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Annette C. Vergunst
David O'Callaghan *Editors*

Host-Bacteria Interactions

Methods and Protocols

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Edited by

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Preface

Bacterial infections are a complex interplay between host and pathogen. Over the last 20 years, there have been great advances in our understanding of the pathogenesis of infectious disease by integrating detailed knowledge of bacterial genetics, cell biology, immunology, and host physiology. This has led to the development of the new field of "cellular microbiology." Early studies began with well-studied organisms such as *Salmonella*, *Shigella*, and *Listeria* and used simple cell culture models such as HeLa cells; however, this rapidly extended to other pathogens and to a wide array of cell types. Cellular microbiology has been instrumental in the identification of bacterial virulence factors required for interaction with the host, their cellular targets, and how the interactions can modulate host cell biology in favor of the pathogen. Recent advances now make it possible to study in great detail the infection in vivo, at the cellular, tissue, and whole animal level.

In this volume we have brought together a set of cutting edge protocols that cover aspects of the investigation of host–bacteria interactions using mammalian and novel non-mammalian infection models, cell biology, OMICS, and bacterial genetics. Our aim is to provide a pathway through the techniques that can be used to investigate different aspects of the physiopathology of bacterial infections, from the whole animal to tissue, cellular, and molecular levels. The pathogens used in the protocols are mainly, but not exclusively facultative and obligate intracellular bacteria, for which we are trying to decipher how the intracellular stages of a pathogen contribute to disease. However, the protocols are generally applicable to most other pathogens. Since the principal goal of the book is to provide researchers with a comprehensive account of the practical steps necessary for carrying out each protocol successfully, the Methods section contains detailed step-by-step descriptions of every protocol. The Notes section complements the Methods with tips based on the authors first-hand experience explaining the "tricks of the trade" and the best ways to deal with any problem or difficulty that might arise.

From the earliest times, infection models have been instrumental in understanding infectious disease. Over recent years, there has been a move away from classical models using mammals and mammalian cells to nonvertebrate systems. In this volume, chapters in Part I will describe how to use *Galleria* (wax moth) larvae (Chapter 1) or *Drosophila* as infection models (Chapter 2), whereas the non-animal models using amoeba (Chapter 9) or plants (Chapters 6 and 11) are included in Part II. The zebrafish has recently emerged as a model where we can exploit both the genetic tractability and optical transparency of developing embryos to follow the infection and assess the role of both host and pathogen factors in real time at the cellular and whole animal level (Chapter 3). Advances in live imaging techniques have also allowed the development of mammalian systems where luminescent bacteria or cells can be seen in the body using highly sensitive cameras (Chapter 4). Two photon microscopy now allows the observation of events at the cellular level in living tissue (Chapter 5).

To fully understand bacterial virulence, it is essential to investigate the host–pathogen interaction at the cellular and molecular level (Part II). Using plant or yeast cells as a surrogate model, we can identify and characterize the bacterial proteins, or effectors,

translocated into host cells through bacterial secretion systems to understand how the bacterium tries to manipulate host defense mechanisms to create its own niche (Chapter 6). We present a protocol that can be used to identify the host targets of a bacterial virulence factor, either when exposed on the pathogen surface or injected into the cell (Chapter 7). We also present protocols to show how pathogens modulate key host cell processes including protein degradation through the proteasome (Chapter 8), phosphoinositide dynamics (Chapter 9), and apoptosis (Chapter 10). Using bimolecular fluorescence complementation, in vivo interactions between host and bacterial proteins can be identified (Chapter 11). There is also a protocol to examine how bacterial pathogens can modulate innate immune signaling through the TLR pathway (Chapter 12).

Technological advances have led to an explosion in the quantity and complexity of OMICS data that can be generated (Part III). A *Drosophila* cell line can be used for siRNA screens (Chapter 13) to identify host factors required for the infection. Two chapters describe the purification of bacteria for proteomic or RNAseq from infected cells (Chapter 14), and the isolation of host phagocytes for RNAseq from zebra fish embryos using FACS sorting (Chapter 15). We also include a protocol for rapid sample production for high-throughput proteomic analysis and data extraction (Chapter 16). Exploiting the masses of data generated in these studies requires powerful bioinformatics support. Chapter 17 describes PATRIC, an NIH-funded database dedicated to OMICS data from pathogens.

Genetic manipulation of the bacterial pathogen is crucial to elucidate the molecular basis of bacteria–host interactions (Part IV). We include three protocols (Chapters 18–20) describing techniques to manipulate bacteria that are either highly recalcitrant or obligate intracellular.

We would like to thank all the contributors, who are leading researchers in the field and have either developed, or are expert users of the presented methods, for providing their comprehensive protocols and tips for this volume. We would like to take the opportunity to thank Dr. John Walker, the Editor-in-Chief of the *Methods in Molecular Biology* series, for giving us the opportunity to edit this volume and his constant support.

We hope you enjoy this volume of *Methods in Molecular Biology*.

Nîmes, France ***Annette C. Vergunst***
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Part I

Infection Models to Study Bacterial Virulence

Chapter 1

Galleria mellonella as an Infection Model for Select Agents

Nicolas Sprynski, Eric Valade, and Fabienne Neulat-Ripoll

Abstract

The use of animal models is a key step to better understand bacterial virulence factors and their roles in host/pathogen interactions. To avoid the ethical and cost problems of mammalian models in bacterial virulence research, several insect models have been developed. One of these models, the larvae of the greater wax moth *Galleria mellonella*, has been shown to be relevant for several fungal and bacterial mammalian pathogens. Here, we describe the use *G. mellonella* to study virulence of the highly virulent facultative intracellular bacterial pathogens: *Brucella suis, Brucella melitensis, Francisella tularensis, Burkholderia mallei*, and *Burkholderia pseudomallei*.

Key words Galleria mellonella, Infection model, Insect model, Brucella, Burkholderia, Francisella

Abbreviations

TS	Trypticase soy
BHI	Brain–heart infusion
CFU	Colony-forming units
MOI	Multiplicity of infection
PBS	Phosphate-buffered saline

1 Introduction

Francisella tularensis, *Brucella suis, Brucella melitensis, Burkholderia mallei*, and *Burkholderia pseudomallei* are highly pathogenic bacteria that have been classified A or B bioterrorism agents by the CDC (Center for Disease Control and Prevention). Study of these pathogens is crucial to better understand their virulence and to develop new therapies. In addition to in vitro approaches, infection models are necessary to understand the role of virulence factors at the whole organism level. Mammalian models are the paradigm for infectious disease studies due to the close relationship to the natural host (human or other mammals). Nevertheless, the use of

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mammalian models to study highly pathogenic bacteria is being complicated: it requires specific laboratory structures (Animal Biosafety Level 3 facility); it is time consuming, expensive and particularly raises ethical considerations. For these reasons, new infection models using insects have been developed (*Drosophila melanogaster, Galleria mellonella*, silkworm larva) [1]. The larva of the greater wax moth *Galleria mellonella* is one of the most used insect models to study virulence, due to several advantages. These larvae are cheap, do not need food or water in their last instar larvae, and have a good size to permit precise injection of pathogens or compounds and infection studies do not require an Animal Biosafety laboratory. After infection, they can be kept at a wide range of temperatures (up to 37 °C) which insures optimal expression of pathogen virulence factors and mimics temperature conditions in mammalian hosts. *G. mellonella* can be infected by a wide range of pathogens including fungi (*Aspergillus fumigatus, Cryptococcus neoformans, Candida albicans*) [2–4] and bacteria (including *Legionella pneumophila, Listeria spp., Burkholderia cepacia* complex, *Yersinia pseudotuberculosis*) [5–8]. Even though insects do not have an adaptive immune response like mammals, they have a complex innate immune system with similarities to that of mammals [9]. The *Galleria* innate immune system is composed of hemocytes which can phagocytose and encapsulate pathogens and a humoral response with the secretion of antimicrobial peptides, melanization, and coagulation of the hemolymph. Furthermore, for several pathogens good correlation between virulence in *Galleria* and mammalian models has been shown [6, 10].

In summary, *G. mellonella* is a powerful good alternative to mammalian models to study virulence factors of mammalian pathogens. Here, we present a protocol to analyze virulence of *B. melitensis*, *B. suis*, *F. tularensis*, *B. mallei* and *B. pseudomallei* by injection of bacterial suspension in the hemocoel.

2 Materials

2.1 Bacterial Strains and Insects

1. *Brucella melitensis* 16 M ^{T} ATCC 23456.
2. *Brucella suis* 1330^{T} ATCC 23444.
3. *Francisella tularensis* SCHUS4 (laboratory collection).
4. *Burkholderia mallei* ATCC 23344.
5. *Burkholderia pseudomallei* SID 4718 (clinical isolate).
6. Larvae of the great wax moth *Galleria mellonella* in last-instar larvae (15–20 per strain tested).

2.2 Injection Apparatus and Equipment

1. Infusion pump KDS 100Y (Kd Scientific, Holliston, MA, USA) remotely triggered by a foot switch (*see* **Note 1**).
2. 1 mL tuberculin syringe (Terumo, Tokyo, Japan).

3. Venofix® A 27G (Braun, Melsungen, Germany) (*see* **Note 1**).
4. Disposable sterile fields.
5. Petri dishes.
6. Microfuge.
7. Pliers.
8. Cotton swabs.
9. 70 % (v/v) Ethanol for disinfection.
10. 37 °C incubator.
11. PSM.
12. Fully equipped BLS3 laboratory.
13. Labeling tape.

2.3 Solutions for Bacterial Growth and Preparation

1. Tryptic soy (TS) broth and agar: Prepare 1.6 % TS agar plates.
2. IsoVitalex™ (Becton Dickinson): Reconstitute with supplied diluent as described by the manufacturer. After reconstitution, use immediately, or store at 2–8 °C and use within 2 weeks.
3. Brain–heart infusion broth and agar (1.6 %) containing 1 % IsoVitalex™: IsoVitalex™ should be added after autoclaving in a precooled medium (approximately 50 °C).
4. Phosphate-buffered saline (D-PBS) 1×.

3 Methods

3.1 Bacterial Preparation

1. *Brucella* and *Burkholderia* are streaked from a frozen stock to a TS plate and then grown in a 37 °C incubator for 3 and 2 days, respectively. *Francisella tularensis* is streaked from a frozen stock to a BHI agar plate supplemented with 1 % IsoVitalex™ and grown in a 37 °C incubator for 3 days (*see* **Note 2**).
2. The day before *G. mellonella* infection, a single bacterial colony is inoculated and grown overnight in 5 mL of the appropriate liquid medium culture (TS for *B. melitensis*, *B. suis*, *B. mallei*, and *B. pseudomallei*; BHI supplemented with 1 % IsoVitalex™ for *F. tularensis*) in a 37 °C shaking incubator.
3. Harvest the bacteria by centrifugation (3 min at 5,500 g in a benchtop centrifuge), wash in PBS, and dilute in PBS to 5×10^9 colony-forming units (CFU)/mL for *B. melitensis* and *B. suis*, 1×10^9 CFU/mL for *F. tularensis*, and 1×10^4 CFU/mL for *B. mallei* and *B. pseudomallei* (*see* **Note 3**).
4. Plate several dilutions of bacterial solutions on TS agar plates for *Brucella* and *Burkholderia* on BHI with 1 % IsoVitalex™ plates for *F. tularensis* to verify the CFU of the different solutions.

3.2 Preparation and Infection of G. mellonella

1. The day before infection, remove the cocoons from the *G. mellonella* larvae (*see* **Note 4**) and 15 larvae are distributed in each Petri dish without food. The larvae are stored until infection in the dark at room temperature.
2. On the day of infection, the cocoons of the larvae are removed (*see* **Note 5**).
3. Set up the infusion pump: Syringe type (Terumo 1 mL), volume of injection to 10 μL, flow rate to 3.6 mL/h.
4. Transfer the bacterial suspension into the syringe and put it on the infusion pump. Plug the Venofix® A into the syringe and purge the Venofix® into a sterile tube.
5. Take out one larva and put it on a disposable sterile field. The larva injection area (the hindmost left proleg) is disinfected before inoculation using a cotton swab impregnated with 70 % ethanol (*see* **Note 6**). Wedge the head of the larva with your index, thumb, and middle finger on the bench. In reaction, the larva will relax itself. Inject 10 μL of the bacterial suspension in the hindmost left proleg (*see* **Note 7**) (Fig. 1). Transfer the injected larva in a clean Petri dish. As negative control, inject a group of 15 larvae with PBS solution (*see* **Note 8**; Fig. 1).
6. After injection of the larvae group, close the Petri dish with adhesive tape. Incubate the Petri dish in the dark in a 37 °C incubator.
7. Every 24 h remove the cocoon from the larvae (*see* **Note 9**) and check the mortality. Caterpillars are considered dead when they display no movement in response to touch (*see* **Note 10**).
8. Stop the experiment when all the caterpillars are dead or when caterpillars begin metamorphosis (*see* **Note 11**).

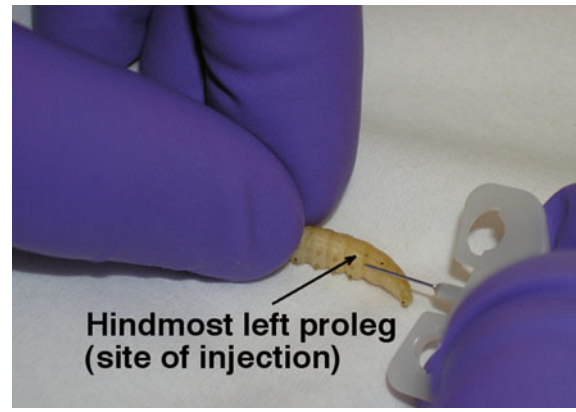

Fig. 1 *G. mellonella* injection: The caterpillar is wedged on the bench with the thumb, index, and middle finger. Prior to injection, disinfect the injection site on the larva with ethanol. Inject 10 μL of the bacterial suspension in the hindmost left proleg (indicated with an *arrow*)

4 Notes

1. For safety, we highly recommend the use of the Venofix® A with an infusion pump remotely triggered by a foot switch. This system allows the manipulator to hold the caterpillar with one hand, insert the needle with the other, and start the injection with their foot. This is more comfortable and safer than other described methods [11].
2. All operations with *B. melitensis*, *B. suis*, *F. tularensis*, *B. mallei* and *B. pseudomallei* must be done in a Biosafety Level 3 laboratory in a microbiological safety cabinet.
3. Correlation of the OD_{600} with the CFU depends on the bacterial strain, the age of the culture, the medium, and the spectrophotometer used. We find that for an OD_{600} of 1, CFUs are 5.10^9/mL for *B. melitensis* and *B. suis*, 1.10^9/mL for *F. tularensis*, and 2.10^9/mL for *B. mallei* and *B. pseudomallei*. This has to be determined empirically for each species and strain used.
4. Food privation has a direct implication in the immune response. Larvae deprived of food have a reduced immune response and thus an increased susceptibility to infection [12]. In order to compare different infection experiments, keep the same time of fasting before infection. Here, we propose 24 h as standard.
5. To remove the cocoon of the caterpillars, use small forceps to carefully tear up the cocoon. Pieces of cocoon are light and volatile. In order to avoid aspiration of pieces of cocoon by the microbiological safety cabinet, put the pieces of cocoon on a tissue soaked in ethanol.
6. Washes, resuspension in PBS of the bacterial culture, and disinfection of the larvae are important steps to avoid humoral response of the caterpillars that are not specific to the pathogen tested. This nonspecific activation of *G. mellonella* humoral response can lead to an important decrease in virulence in the assay.
7. For the injection, insert 2–3 mm of the needle in the hindmost left proleg of the larvae in the direction of the head (*see* Fig. 1). Activate the injection with the foot switch. At the end of the injection, gently remove the needle. Sometimes, a small amount of hemolymph bleeds from the injection point. This phenomenon does not affect the infection assay. If the needle passes through the larva, exclude the caterpillar from the experiment.
8. The group of PBS-injected larvae are essential to show pathogen-specific death of caterpillars. Do not use the data if one of the control-injected larva dies due to injection injury.
9. Sick larvae make fewer or no cocoons.

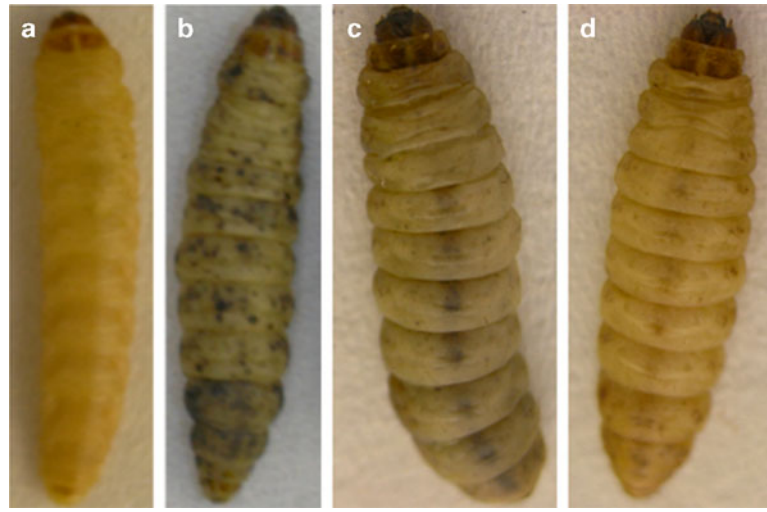

Fig. 2 Phenotype of dead *G. mellonella* with different pathogens. (**a**) Uninfected larva; (**b**) larva infected with *B. mallei* (same phenotype with *B. pseudomallei* and *B. melitensis*); (**c**) larva infected with *F. tularensis*; (**d**) larva infected with *Brucella suis*

10. To verify the death of the larvae touch it gently with the forceps. If the larva does not display any body movement or any proleg movement, the larva is considered dead. Phenotypes of dead caterpillars can change according to the infecting pathogen. For example, *Burkholderia (mallei and pseudomallei)* and *Brucella melitensis* cause high larva melanization. *F. tularensis* and *B. suis* cause middle and low larva melanization, respectively (Fig. 2). "Cannibalism" can occur on rare occasions; do not use data from these experiments.
11. For indication, with the specified MOI (multiplicity of infection), 100 % of the larvae die in 3 days for *B. mallei and B. pseudomallei;* in 7–10 days for *B. melitensis* and *F. tularensis;* and 50 % in 7–10 days for *B. suis*. All dead larvae and larvae undergoing metamorphosis are removed from the experiment and autoclaved. A stooped head easily identifies the larva at the beginning of the metamorphosis.

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Chapter 2

Drosophila as a Model for Intestinal Infections

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Abstract

Drosophila melanogaster is a powerful model to study infections thanks to the power of its genetics and knowledge on its biology accumulated for over a century. While the systemic humoral immune response against invading microbes has been intensively studied in the past two decades, the study of intestinal infections is more recent. Here, we present the methods that are currently in use to probe various aspects of the host-pathogen interactions between *Drosophila* and ingested microbes, with an emphasis on the study of the midgut epithelium, which constitutes the major interface between the organism and the microbe-rich ingested food.

Key words *Drosophila melanogaster*, Innate immunity, Host-pathogen interactions, Resilience/tolerance, Intestinal stem cells, Enterocyte, Phagocytosis, *Serratia marcescens*, *Pseudomonas aeruginosa*, Intestinal homeostasis

1 Introduction

The digestive tract represents a major frontier between our organism and the outside world. The gut must be able to perform several functions such as nutrient and solute absorption while simultaneously preventing the entry of pathogens and preserving the microbiota, which usually fulfills beneficial functions. Thus, it is an ideal organ to study the interplay between the immune response and the preservation of the essential physiological functions of an organ. Much work has been performed in vertebrates, the emphasis being placed currently on studies of the microbiota [1] and also of the continual replacement of enterocytes that form the major population of the gut epithelium, most noticeably with the identification of intestinal stem cells [2]. However, this is a complex system in terms both of mucosal immunity, which includes both innate and adaptive immunity, and of microbiota, which encompasses 400–1,000 species in humans with the microbial count overwhelming

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the number of human cells by a factor of 10. Thus, a simpler model to study all immune and physiological dimensions of intestinal infections is desirable. *Drosophila melanogaster*, with the power of its genetics and the sophistication of its genetic toolbox [3], is a formidable model for such investigations.

Several *Drosophila* models of intestinal infections have been developed in the past decade and revealed the existence of five major arms of defense, four belonging to innate immunity and the fifth one constituting resilience [4, 5]. The first is a physical barrier constituted by the peritrophic matrix that lines the midgut epithelium and allows the passage of nutrients while preventing the direct contact of enterocytes with ingested microbes. The second and third are chemical defenses that are finely regulated. One such defense is the production of reactive oxygen species by the Dual oxidase enzyme [6], which kill incoming microbes. A complementary chemical defense is the production of potent antimicrobial peptides, the expression of which is under the control of the Immune deficiency pathway. Finally, some microbes nevertheless manage to escape from the digestive tract and penetrate the general cavity (hemocoel). The cellular immune response, through hemocytes that phagocytize microbes, deals with these incoming pathogens. Finally, if the infection becomes generalized, the systemic humoral immune response may become activated [7, 8]. However, host defense is not limited to innate immunity and encompasses a second dimension, resilience, the ability to withstand and/or repair damages inflicted either by the pathogen or the host's own immune response. For instance, the homeostasis of the intestinal epithelium is maintained by the proliferation of intestinal stem cells (ISCs), thus leading to the replacement of damaged enterocytes that undergo anoikis [9, 10], a type of programmed cell death involving the detachment of epithelial cells from the surrounding cells and the extracellular matrix.

Drosophila is a widely used model to investigate most major biological questions, from molecular biology to evolutionary genetics. A good source of information on its biology is provided by the "grey" book from Dr. M. Ashburner and associated laboratory manual [11, 12]. A good introduction to *Drosophila* genetics has been written by Dr. R. Greenspan [13]. An essential online resource is to be found at Flybase (http://flybase.org/), which regroups the information on this organism. It is important that the fly stocks used for experiments be free as much as possible from viral, bacterial, or fungal infections. As part of our standard quarantine procedure for stocks received from stock centers or other laboratories, we routinely check by qPCR or qRT-PCR for the presence of several microbes and provide a list of primers used to this end in Table 1. Here, we provide a protocol to perform intestinal infections (*see* Subheading 3.1) and monitor the survival of

Table 1
Primers used to monitor the infectious state of *Drosophila* stocks

Target	Name	Template	Sequence
Wolbachia	Wolb_For Wolb_Rev	gDNA	TTGTAGCCTGCTATGGTATAACT GAATAGGTATGATTTTCATGT
Microsporidia	T.r._For T.r._Rev	gDNA	TCTCACAGTAGTGGCGAATG AACACCGTATTGGAATACAG
DCV	DCV_For DCV_Rev	cDNA	TCATCGGTATGCACATTGCT CGCATAACCATGCTCTTCTG
FHV	FHV_For FHV_Rev	cDNA	TTTAGAGCACATGCGTCCAG CGCTCACTTTCTTCGGGTTA
Nora virus	Nora_For Nora_Rev	cDNA	AACCTCGTAGCAATCCTCTCAAG TTCTTGTCCGGTGTATCCTGTATC

DCV Drosophila C virus, *FHV* Flock house virus, *T.r. Tubulinosema ratisbonensis. Wolbachia*: It is important to monitor the infection status with this intracellular parasite. It has been shown to protect flies from some viral infections. To cure fly stocks of viral or microsporidial infections, it is effective to collect eggs and bleach them before placing them in vials with clean food, making sure that no hatched larvae are present. It may be required to repeat the procedure. To eliminate Wolbachia, a treatment with tetracycline at 200 μg/ml should be sufficient. It is advisable to wait a couple of generations before starting experiments with tetracycline-treated flies

infected flies (*see* Subheading 3.2). While this chapter has been essentially written based on our published experience with two bacterial pathogens, *Serratia marcescens* and *Pseudomonas aeruginosa* [7, 8, 14], most protocols can easily be adapted to fungal infections, although one should avoid conditions in which fungi switch to alcoholic fermentation, ethanol being able to kill flies despite the presence of an alcohol dehydrogenase in the *Drosophila* genome. An essential step is to assess the microbial titer not only in the gut (*see* Subheading 3.3) but also within the intestinal epithelium for microbes that are able to pass the peritrophic matrix and enter enterocytes. Some of them may just be crossing the epithelium and ultimately are found in the hemocoel (*see* Subheading 3.4). The microbial titer may then be measured in this compartment by directly crushing the rest of the body once the gut has been dissected away and plating the extract. An alternative is to collect the hemolymph and to plate it (*see* Subheading 3.5). In our experience with injected *Candida glabrata*, we found that the fungal titer was tenfold lower when comparing the hemolymph versus the whole-body extract [15]. To determine whether microbes may be able to pass the intestinal barrier at a low rate and to determine whether the cellular immune response is important, an easy assay consists in saturating the cellular host defense by the injection of latex beads, which are phagocytized but cannot be degraded, thus efficiently ablating this arm of the host defense. The important part of the

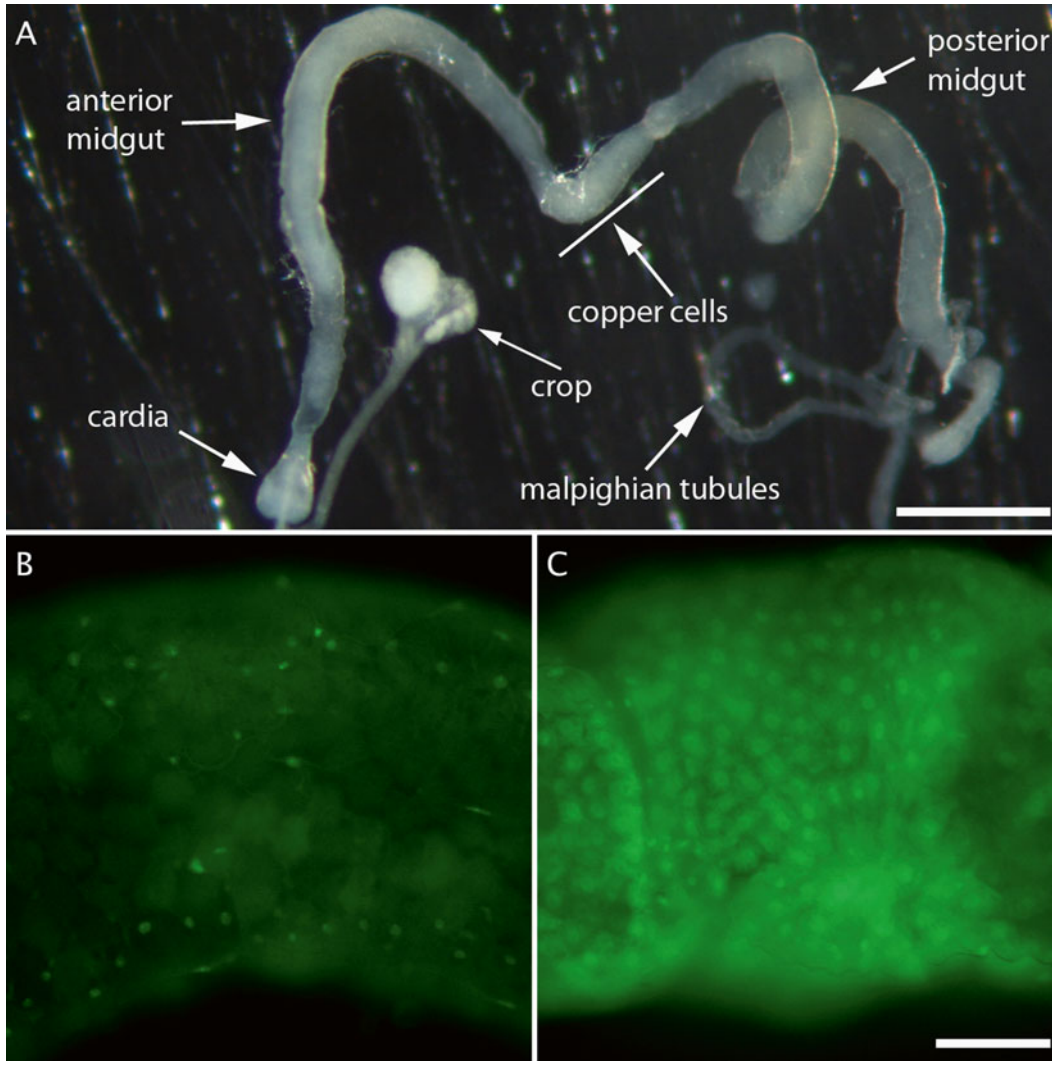

Fig. 1 The dissected *Drosophila* midgut: Monitoring the integrity of epithelial cells with a SYTOX Green assay. (**a**) Dissected *Drosophila* midgut. The frontier between the foregut and the midgut is delimited by a valve-like structure, the proventriculus, which synthetizes the peritrophix matrix that lines the inside of the midgut epithelium. The crop is a diverticulum that branches off the foregut just before the proventriculus. The copper cell region corresponds to the stomach of the fly and is characterized by a low pH, H^+ ions being secreted by specialized cells in this region. The midgut ends at the point of insertion of paired Malpighian tubules, which fulfill the function of kidneys in flies. Like the foregut, the hindgut is covered by cuticle. (**b**–**c**) SYTOX Green staining of control (**b**) and SDS-challenged flies (**c**). SYTOX Green is a nucleic acid stain that cannot permeate live cells. Note the background staining in control flies, which may correspond to the nuclei of longitudinal gut muscles

digestive tract in which solute and nutrient absorption takes place is the midgut, which is divided into several regions (Fig. 1a). The bipartite Gal4-UAS system [16] is commonly used to drive the expression of selected transgenes in distinct regions of the gut

or in the different cell types that form the midgut epithelium (Figs. 2 and 3). Methods for its dissection (*see* Subheading 3.7), fluorescent immunohistochemistry (*see* Subheading 3.8), and mounting are provided (*see* Subheading 3.9). These techniques allow visualizing different cell types (Fig. 3) as well as monitoring ISC proliferation by anti-phosphohistone H3 (PH3) staining. A list of common antibodies is provided in Table 2. When characterizing an infection, it is important to document whether the integrity of epithelial cells or of the digestive tract is affected (SYTOX Green assay (*see* Subheading 3.10; Fig. 1b, c) and Smurf assay (*see* Subheading 3.11)). The SYTOX assay has been used by Ligoxygakis and co-workers to probe the integrity of the larval gut epithelium, namely the integrity of enterocytes, after a *Candida albicans* infection [17]. SYTOX Green is a nucleic acid stain that fails to penetrate life cells. Thus, if it stains nuclei, one may infer that the corresponding cell has been damaged (Fig. 1b, c). The Smurf assay has been developed by Walker and co-workers to monitor the physical integrity and impermeability of the *Drosophila* digestive tract [18]. The principle is quite simple and consists of feeding flies with a dye that remains confined within the gut lumen when the midgut epithelium is intact. The authors showed that the physical integrity of the gut was disrupted in aged flies as the dye could be visualized in the whole body, hence the name of the assay. Quantitation of gene expression by qRT-PCR or transcriptomics requires the preparation of RNA extracts described in Subheading 3.12. We provide in Table 3 the list of primers we use to measure the expression of antimicrobial peptides. Transcriptomics approaches are nowadays often complemented by mass-spectrometry data to identify proteins expressed at various stages of the infectious process. A method to make protein extracts is provided in Subheading 3.12. It has been recently shown that highly pathogenic bacteria such as *Pseudomonas entomophila* are able to block protein synthesis in the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster*, a process that can be visualized using the BONCAT method (*see* Subheading 3.13) [19]. The principle of the method is to monitor *de novo* protein synthesis by feeding flies with L-azidohomoalanine, a methionine analog, which can be revealed using Click-it® chemistry. This method [20] has first been adapted in the *Drosophila* gut by Chakrabarti et al. [19]. Finally, it is likely that intestinal infections impact the physiological functions of the midgut. We provide a simple technique that allows us to assess the function of the stomach region of the midgut (*see* Subheading 3.14; Fig. 4). Of note, we have also published a methods chapter that focuses specifically on *Pseudomonas aeruginosa* infections [21], which complements this chapter.

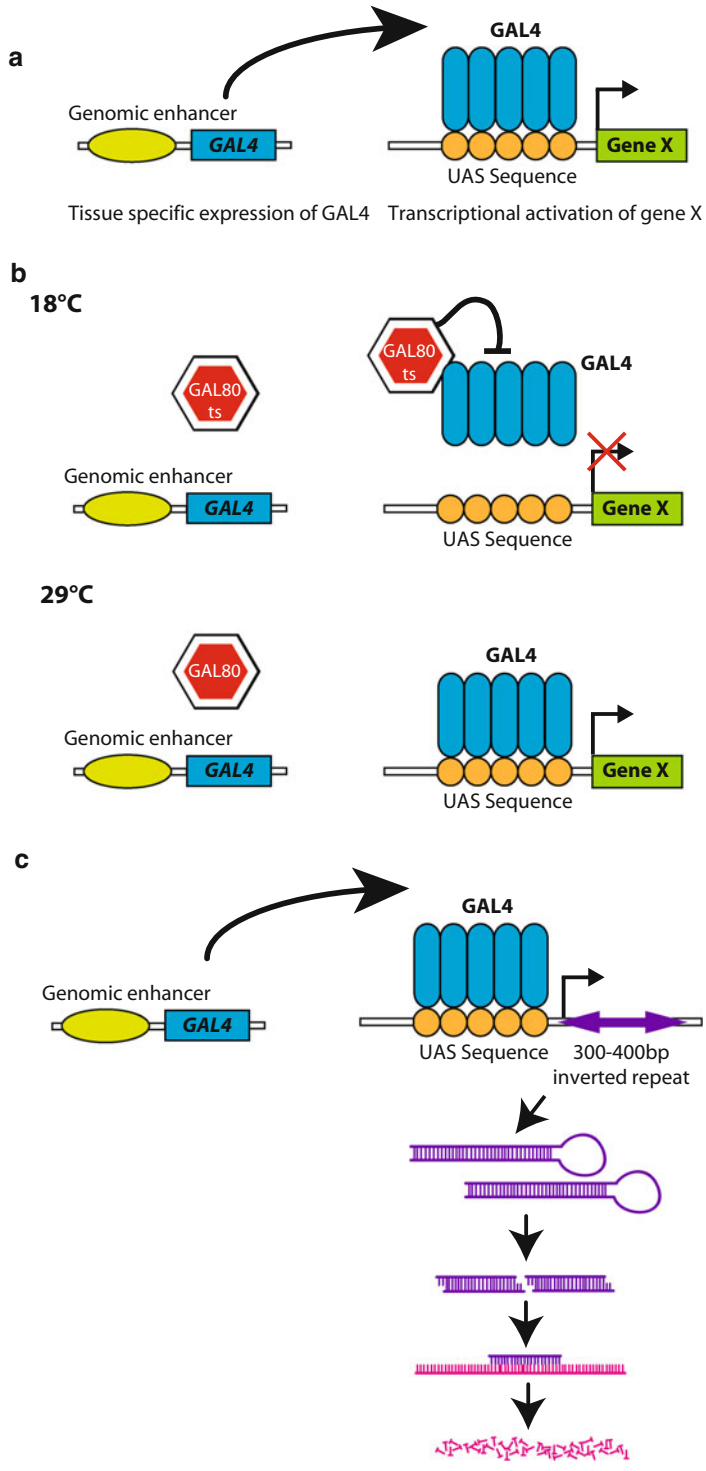

Fig. 2 Adaptation of the yeast Gal4/UAS system to *Drosophila* genetics. The yeast Gal4 transcription factor binds to multiple copies of its binding site upstream of its target genes collectively known as upstream activating sequences (UAS). This system has been adapted to *Drosophila*, the genome of which does not contain UAS sequences, although this statement needs to be modulated. The principle of this bipartite system is to cross transgenic lines, one parental line carrying a transgene in which Gal4 is expressed under the control of a given promoter and

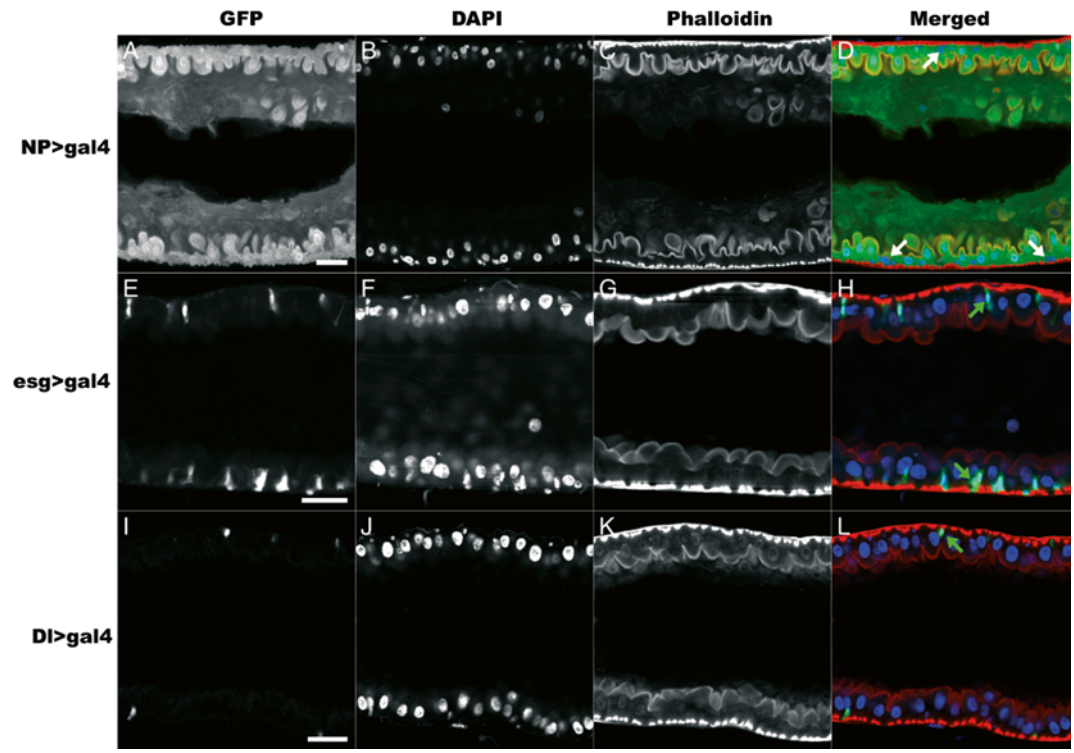

Fig. 3 Common Gal4 drivers used to visualize or express transgenes in specific cell types of the gut epithelium. The dissected flies result from a cross between a specific Gal4 driver line and a UAS-GFP transgene. The NP1 driver results from an enhancer trap in the Myo31DF gene and is expressed only in enterocytes. The *escargot* (*esg*) gene is expressed only in diploid cells, which correspond in the midgut epithelium to intestinal stem cells (ISCs) and enteroblasts that originate from the division of ISCs and differentiate either into enterocytes or enteroendocrine cells, depending on the amount of Delta signal they receive from the progenitor ISC. *Delta* (*Dl*), a gene encoding one *Drosophila* Notch ligand, is expressed specifically only in ISCs, at least in wild-type flies. GFP is displayed in *green*, nuclei in *blue* as revealed by a DAPI stain, and actin is visualized with phalloidin-dsRed. Note the strong staining originating from circular intestinal muscles on the midgut periphery and the weaker signal corresponding to the brush border on the apical side of enterocytes. *Arrows* on the figure point to enterocytes (NP panel), to ISC and/or enteroblast (esg panel), or to an ISC (DL panel) (Color figure online)

Fig. 2 (continued) the other line carrying the UAS sequences coupled to a basal promoter and a gene of interest. In offspring containing both transgenes (**a**), the Gal4 protein will be expressed in a tissue-specific manner and drive the expression of the target gene that has been placed under UAS control. In case the product is "toxic," it is possible to control its temporal expression. This is achieved by introducing another transgene in which a thermosensitive repressor of Gal4 is expressed under the control of a ubiquitous promoter. At the low repressor permissive temperature (18 °C), the Gal80 repressor is active and the gene placed under UAS control is not expressed. At the high restrictive temperature (29 °C), the repressor is no longer able to inhibit Gal4, which drives the expression of its target gene. Note also that 29 °C is the optimal temperature of function of the yeast Gal4 transcription factor. This principle has been adapted to perform RNA interference by expressing a transgene encoding a hairpin designed to hybridize with only one target gene or a family of related genes (**c**). Thus, the Gal4/UAS system is highly versatile

Table 2
Antibodies and reagents used commonly to stain the *Drosophila* midgut

Antibody and reagents	Localization	Species	Dilution	Source
α-Armadillo	Cell junction	Mouse	1/20	DSHB
α-Crumb	Cell junction	Mouse	1/50	DSHB
α-Coracle	Cell junction	Guinea pig	1/2,000	DSHB
α-Disc large	Cell junction	Mouse	1/100	DSHB
α-PH3	Mitotic cells	Rabbit	1/2,000	Millipore
α-Delta	ISC	Mouse	1/100	DSHB
α-GFP	GFP fusion protein	Mouse	1/500	Roche
Texas red phalloidin	Actin cytoskeleton	na	1/100	Invitrogen
FITC phalloidin	Actin cytoskeleton	na	1/50	Sigma-Aldrich

Table 3
Primers used for the quantitation of the expression of *Drosophila* antimicrobial peptide genes

Antimicrobial peptide	Primer	
Ribosomal protein L32 (RP49)	Fw Rv	GACGCTTCAAGGGACAGTATCTG AAACGCGGTTCTGCATGAG
Drosomycin (Drs)	Fw Rv	CGTGAGAACCTTTTCCAATATGATG TCCCAGGACCACCAGCAT
Diptericin (Dipt)	Fw Rv	GGCCCATGCCAATTTATTCA TGGTGGAGTGGGCTTCATG
Attacin A (AttA)	Fw Rv	GGCCCATGCCAATTTATTCA AGCAAAGACCTTGGCATCCA
Defensin (Def)	Fw Rv	GCTCAGCCAGTTTCCGATGT TCCTGGTGGGCATCCTCAT
Cecropin (Cec)	Fw Rv	ACGCGTTGGTCAGCACACT ACATTGGCGGCTTGTTGAG
Metchnikowin (Mtk)	Fw Rv	CGTCACCAGGGACCCATTT CCGGTCTTGGTTGGTTAGGA
Drosocin A (Dro)	Fw Rv	TGAAGTTCACCATCGTTTTCCTG CACCCATGGCAAAAACGC

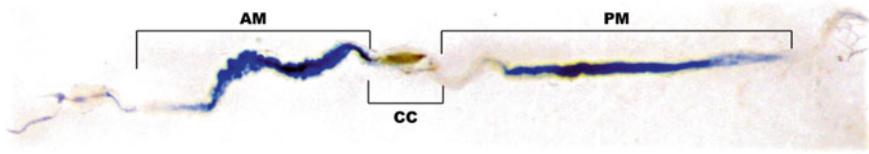

Fig. 4 Visualization of pH in subregions of the midgut. Flies have been fed with a solution containing bromophenol blue. This dye is blue at basal pH, which is found in the anterior (AM) and posterior (PM) midgut. The copper cell region is morphologically distinct from the rest of the midgut and is highly acid. Note that the acidic pH is progressively neutralized in the proximal part of the posterior midgut. This assay allows checking rapidly whether the gut performs one of its physiological functions normally

2 Materials

For all procedures, the use of the highest quality reagents available and ultrapure water is advised.

2.1 *Drosophila* Culture

1. Fly strains (wild type, mutants, driver lines, RNAi lines, …): *See* Subheading 2.2 below.
2. Fly culture incubators or rooms (18, 25, and 29 °C, 60 % humidity).
3. Standard *Drosophila* vials and foam plugs (different sizes: small, medium, large).
4. *Drosophila* standard food (*see* **Note 1**).
5. Fresh baker's yeast suspension: To be prepared by mixing dry or wet live yeast in water; autoclave if axenic conditions are needed.

2.2 *Drosophila* Strains and Handling of Flies

1. Most mutant and transgenic strains can be ordered from the Bloomington Stock Center or other stock centers (e.g., the Kyoto Stock Center) listed in the Resource section of FlyBase (http://www.flybase.org).
2. One important resource to order transgenic RNAi fly lines is the Vienna *Drosophila* RNAi Center (VDRC: http://stockcenter.vdrc.at/control/main), which hosts two collections of transgenic lines that contain transgenes designed to interfere each with a specific predicted gene of the genome. A less extensive collection is available at the Japanese National Institute of Genetics (NIG-Fly: http://www.shigen.nig.ac.jp/fly/nigfly/) and also at the Bloomington Stock Center (Transgenic RNAi Project=TRiP: http://www.flyrnai.org/TRiP-ACC.html) (*see* **Note 2**).
3. Quarantine equipment (quarantine room for storage, with dedicated work space if possible).
4. Standard CO_2 from a gas bottle, or ether for anesthetizing (*see* **Note 3**).

5. Dissection microscope.
6. Fine brushes.
7. Forceps.
8. Morgue with 70 % ethanol for fly disposal.
9. Fly pipette.

2.3 Bacterial Cultures

1. –80 °C freezer.
2. Frozen stocks of bacteria for infection (glycerol stocks kept at –80 °C).
3. Incubators (30 and 37 °C, shaker for liquid culture).
4. Glassware (Erlenmeyer flasks of different sizes).
5. 15 and 50 ml conical polypropylene centrifuge tubes, sterile.
6. Sterile loop.
7. Sterile pipette tips.
8. Pipettes.
9. Petri dishes, disposable.
10. Gloves.
11. Laboratory coat.
12. Safety goggles.
13. Lysogeny broth (LB): 1 g tryptone, 5 g yeast extract, 10 g NaCl. Add to 1 L of deionized water and autoclave.
14. LB agar: As above, additionally add 15 g/L of agar prior to autoclaving.
15. Antibiotics (stock concentration of antibiotics: streptomycin: 50 mg/mL in water; ampicillin: 100 mg/mL in water; kanamycin: 50 mg/mL in water, filter sterilized as above; all antibiotics are sterilized with 0.22 μm filter and kept at –20 °C), as dictated by the bacterial genotype.
16. Class II microbial safety cabinet (*see* **Note 4**).
17. Bunsen burner (*see* **Note 4**).
18. Cuvettes, disposable.
19. UV/visible spectrophotometer.
20. Benchtop centrifuge plus rotors.

2.4 Oral Infection and Survival Assays

1. Laminar flow hood (*see* **Note 5**).
2. Laboratory coat, safety glasses, and gloves.
3. *Drosophila* adult female flies (4–7 days old, 20 flies per infection assay).
4. Suspension of bacteria overnight culture (*see* **Note 6** for media and growth temperatures).

5. Fresh sterile culture media (depending on the bacterium).
6. 50 and 100 mM sucrose solutions (filter sterilized; 0.22 μm filter).
7. 37 mm diameter absorbent pads (e.g., Millipore, ref. AP1003700).
8. 68 ml flat bottom *Drosophila* plastic vials (Greiner Bio-One, 36/82 mm).
9. 38 mm diameter "mite proof" plugs (K-TK, Retzstadt, Germany; www.drosophilacenter.com).
10. 50 ml sterile tubes.
11. Inoculum: Determine the optical density of the overnight culture, harvest by centrifugation with a benchtop centrifuge (4,000 × *g* 10 min), and resuspend the bacterial pellet in the appropriate volume of sterile LB to obtain the desired 10× concentrated bacterial suspension (e.g., OD600 = 1 for final OD600 = 0.1, OD600 = 10 for final OD600 = 1, OD600 = 100 for final OD600 = 10 or OD600 = 1,000 for final OD600 = 100) (*see* **Notes 7** and **8**). To make 50 ml of inoculum (2 ml per infection assay), add 5 ml of the 10× bacterial solution to 45 ml of 50 mM sucrose solution (45 mM sucrose final concentration).
12. Fly culture incubators (25, 27, and/or 29 °C).

2.5 Bacterial Counts in Hemolymph

1. Phosphate-buffered saline (1× PBS).
2. Dissecting microscope.
3. Nanoject II replacement capillaries (Drummond Scientific).
4. Flaming brown micropipette puller (SUTTER Instrument, model P-97) (*see* **Note 9**).
5. Handheld Nanoject II auto nanoliter injector (Drummond Scientific) with indication of the exact injected volume (nanoliter scale).
6. LB agar culture plates with appropriate selective antibiotics.

2.6 Blockade of Phagocytosis

1. "Latex" beads: 0.3 μm carboxylate-modified latex beads (Interfacial Dynamics) washed in PBS and concentrated 4× (5–10 % solids in the final solution).
2. Nanoject II and capillaries (*see* Subheading 2.5).
3. 4 % Trypan blue.
4. FITC-labeled bacteria, e.g., bioparticles (Molecular Probes) prepared according to the instructions of the manufacturer.

2.7 Gut Dissection

1. Adult female *Drosophila* flies (3–7 days old).
2. Standard CO_2 source to anesthetize the flies (*see* **Note 3**).
3. Dissection pad, Petri dish, or depression glass slides.

4. Two fine-tipped dissecting forceps for dissection (Dumont #5).
5. Dissecting microscope and cold light source.
6. Dissecting solution: Phosphate-buffered saline (1× PBS). Store in glass bottles at room temperature (*see* **Note 10**).

2.8 Gut Fixation

1. Phosphate-buffered saline (1× PBS).
2. Gloves.
3. Glass depression slide or small dish (Petri or Syracuse watch glass).
4. 16 % paraformaldehyde (16 % PFA) solution.
5. Fixation solution: 4 % formaldehyde in 1× PBS, obtained by diluting 16 % paraformaldehyde solution in PBS (*see* **Notes 11** and **12**).
6. PBT solution: 0.1 % (v/v) Triton X-100 in 1× PBS. Store at room temperature.
7. Chemical hood.

2.9 Gut Immunostaining

1. 10× PBX: PBS 10×, 3 % Triton X-100.
2. 10 % bovine serum albumin (BSA) in 1× PBS. Aliquot the BSA solution in 1.5 ml Eppendorf tubes. Store at −20 °C.
3. Blocking and permeabilization solution: 1 % BSA in 1× PBX. Store at 4 °C (*see* **Note 13**).
4. Oscillating tube mixer (e.g., ELMI RM-2 L Intelli-Mixer, Alberta Research Scientific Ltd) (*see* **Note 14)**.
5. Aluminum foil.
6. Primary antibodies: A list of the primary antibodies we regularly use can be found in Table 2. A comprehensive list of antibodies used to study the development and biology of the *Drosophila* digestive tract can be found in [22].
7. Secondary antibodies can be purchased from many different companies. We use secondary antibodies of goat anti-mouse, goat anti-rat, goat anti-rabbit, and goat anti-guinea pig IgG conjugated to Alexa Fluor 488 or Alexa Fluor 594 or Texas Red from Invitrogen and Cell Signaling. Store all secondary antibodies in a dark place at 4 °C. We used a 1:1,000–2,000 dilution of all secondary antibodies in 1× PBX with 1 % BSA.
8. PBT (*see* Subheading 2.8, **step 6**).

2.10 Mounting of the Dissected Gut on Microslides, Imaging, and Data Analysis

1. 24 × 60 mm microscope cover slips.
2. PFTE-coated diagnostic 8-well slides.
3. Quick-dry nail polish.
4. Dissecting microscope.
5. Fluorescence stereomicroscope with FITC, DAPI, and dsRed filter.

6. VECTASHIELD Mounting Medium with DAPI (Vector Laboratories H-1200).
7. Waterproof permanent marker to label the slides.
8. Confocal microscope with computer and associated software for image processing.

2.11 SYTOX Green Assay

1. Staged female flies (3–7 days).
2. Overnight culture of bacteria.
3. Dissection materials (described in Subheading 2.7)
4. 50 mM sucrose.
5. PBS.
6. 16 % PFA.
7. 2 % SDS (for positive control experiment).
8. SYTOX Green (1:1,000 dilution of 5 mM stock in DMSO).
9. VECTASHIELD Mounting Medium with DAPI (Vector Laboratories H-1200).
10. Glass slides.

2.12 Smurf Assay

1. Female flies (3–7 days old for infections, positive control can be 45-day-old flies).
2. Food dye (FD&C blue dye #1).
3. 37 mm diameter absorbent pads.
4. 68 ml flat bottom *Drosophila* plastic vials (Greiner Bio-One, 36/82 mm).
5. Overnight culture of bacteria.
6. 50 mM sucrose solution, sterile.
7. 2 % SDS.

2.13 RNA Extraction

1. Minimum of ten dissected midguts per point.
2. TRI Reagent® RT to extract nucleic acids.
3. 4-Bromoanisole (BAN): RNase inhibitor.
4. Micro-pestle (*see* **Note 15**).
5. 1.5 ml Eppendorf tubes.
6. Isopropanol.
7. 70 % Ethanol.
8. RNase-free water.
9. Microcentrifuge.
10. Nanodrop (Thermo Scientific).
11. Bioanalyzer (*see* **Note 16**).

2.14 Protein Extraction

1. 3–7-day-old female flies (x h P.I.), 60 flies/condition.
2. Microbial infection solution.
3. Microcentrifuge.
4. 50 mM sucrose, sterile.
5. PBS/Protease Inhibitor cocktail (Complete Ultra Tablets EDTA-free, Roche) inhibiting serine, cysteine, aspartic proteases, 1 mM PMSF, or equivalent.
6. 1.5 ml Eppendorf tubes.
7. Dry ice.
8. Cell lysis buffer: 10 % glycerol, 1 % NP-40, 20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1× protease inhibitor cocktail.
9. 10 % glycerol.
10. 1 % NP-40.
11. Minipestle fitted for a 1.5 ml Eppendorf microtube.
12. Ice.
13. Bradford protein assay dye reagent.
14. Spectrophotometer (with a plate reader).

2.15 BONCAT Method to Monitor Global Translation

1. 3–7-day-old *Drosophila* female flies.
2. Microbial solution.
3. 50 mM sucrose, filter sterilized
4. 37 mm diameter absorbent pads.
5. 68 ml flat bottom *Drosophila* plastic vials (Greiner Bio-One, 36/82 mm).
6. DMSO (dimethyl sulfoxide).
7. PBS (phosphate-buffered saline).
8. 4 % PFA in PBS.
9. PBT: PBS, 0.1 % Triton X-100.
10. Click-iT® AHA (L-azidohomoalanine) (Invitrogen™): Solubilize Click-iT® AHA with DMSO to make a 50 mM (1,000× stock solution). Aliquot and store any unused reagent at −20 °C. The stock is stable for up to 1 year.
11. Alexa Fluor® 488 alkyne (Invitrogen™).
12. Click-iT® Cell Reaction Buffer Kit (Invitrogen™).

2.16 Monitoring the Acidification of the Copper Cell Region

1. *Drosophila* adult female flies (3–7 days old).
2. Overnight culture of bacteria.
3. 0.25 % (w/w) Bromophenol blue in 50 mM sterile-filtrated sucrose solution.

4. Bromophenol-containing infection and control solutions: Prepare the infection and control solutions as indicated in Subheading 2.4, **step 11**, using the 0.25 % (w/w) bromophenol blue and 50 mM sucrose solution instead of the 50 mM sucrose solution.
5. Gut dissection material as described in Subheading 2.7.

3 Methods

These methods can be conducted either on wild-type or mutant flies. As regards UAS-RNAi strains, we are using Gal4 driver strains also containing a tubulin-Gal80^{ts} expression transgene to avoid any developmental or essential requirement of the targeted gene [23] (Fig. 2, *see* **Note 17**).

3.1 Oral Infection

There are likely as many methods to perform oral infections as there are laboratories [24, 7, 25, 26]. Here, we describe the technique we usually follow for *S. marcescens* Db11 or Db10 infections. Some important parameters to take into consideration when designing the experiment are further discussed in the notes.

1. Prepare the infection solutions as described in Subheading 2.4 (*see* **Note 18**).
2. Infection and control tubes: Place two superposed 37 mM diameter absorbent pads at the bottom of a 68 ml flat-bottom plastic *tub*e. Make sure that the pads cover the entire surface of the bottom of the tube (*see* **Note 19**). For the infection tube, soak the pads with 2 ml of freshly made infection solution. The infection tubes should be used within 1 hour. For the controls, the inoculum is replaced by 2 ml of sterile 50 mM sucrose.
3. Anesthetize the flies with CO2, until flies are no longer moving. Add the flies in the infection and control tubes (twenty 3–7-days-old flies/tube) (*see* **Note 20**).
4. Place the tubes and flies in a fly culture incubator at the desired temperature for the duration of the oral infection.

3.2 Survival Assay

1. Prepare the inocula, tubes, and the control tube as described in Subheading 3.1, **steps 1** and **2**.
2. Anesthetize the flies with CO_2.
3. Transfer the flies to the infection and control tubes (3–7-day-old flies x 20/tube; *see* **Note 21**) and clearly label the tubes with the genotype of flies used and the condition of the experiment (pathogen, concentration, control); *see* **Note 22**.
4. Place the tubes and flies in the fly culture incubator at the desired temperature for the duration of the assay.

5. To ensure a constant and sufficient carbon source for the flies and the bacteria, add, on a daily basis, 200 µl of sterile 100 mM sucrose solution (*see* **Note 23**).
6. Survey the evolution of the survival assay by regularly counting the remaining living flies (those that are still moving after gently tapping the tube) in the infection and control tubes.
7. Analyze the data using appropriate statistics (*see* **Note 24**).

3.3 Bacterial Counts in Whole Flies

1. Infect the flies orally as described above in Subheading 3.1.
2. After the desired time of infection (e.g., for *Serratia marcescens* Db11, typically hours or days [7]), crush the flies in an Eppendorf tube in 0.5 ml of sterile LB medium using a micro-pestle.
3. Perform serial dilutions (starting from undiluted to 1/10; 1/100; 1/1,000 dilutions) of the homogenate in LB medium.
4. Plate these dilutions on LB agar plate with the appropriate antibiotics (*see* **Note 25**).
5. Determine the number of colony-forming units (CFU) through growth overnight at the appropriate temperature.

3.4 Bacterial Counts in the Hemolymph

1. Mount an empty capillary on a Nanoject II (*see* **Note 26**).
2. Prick the fly with the capillary at the thoracic smooth cuticle level and wait for the hemolymph to fill the needle by capillarity (typically 100 nL per fly).
3. Pool the hemolymph from batches of ten flies in sterile PBS on ice (1 µL hemolymph in 9 µL PBS).
4. Perform a serial dilution of the collected hemolymph in sterile PBS (*see* **Note 27**).
5. As described above, plate on LB agar plate with the appropriate antibiotics and determine the number of colony-forming units (CFU) after growth at the appropriate temperature.

3.5 Bacterial Counts Within the Gut Epithelium

1. Infect flies orally as described above in Subheading 3.1.
2. Following the desired time post-infection shift the flies in new culture vials containing filters that were soaked with gentamicin (100 µg/ml) solution in PBS (*see* **Note 28**).
3. After 2 h, flip the flies in new tubes containing filters with sterile sucrose (100 mM) solution for 30 min. Repeat this transfer to wash away any remaining antibiotics.
4. Dissect the gut as described in Subheading 3.7.
5. To get rid of any surface contamination of the dissected gut epithelium resulting from the dissection itself or from the adherence of some potential hemolymphatic bacteria, wash the

dissected guts by shortly dipping them in 70 % ethanol and then in distilled sterile water (*see* **Note 29**).

6. Crush the dissected gut in PBS, make a dilution series of this solution, and plate it on the proper antibiotic selective culture plate (*see* **Note 30**).

3.6 Blockade of Phagocytosis

To assess the importance of phagocytosis in controlling pathogenic bacteria that have crossed the intestinal barrier, one easy technique is to block the cellular arm of the immune response by saturating the phagocytic apparatus of hemocytes with nondigestible "latex" beads (actually often polystyrene beads) [27–29]. This is achieved by injecting these beads in the hemocoel by inserting the capillary in the lateral part of the thorax, under the wing hinge.

1. Prepare an aliquot of 10 μl of sonicated suspension of sterile latex beads and load the capillary (*see* **Note 31**).
2. With the capillary, penetrate the hemocoel by piercing the thorax smooth cuticle.
3. With the Nanoject II, inject 69 nl of fourfold concentrated Surfactant-Free Red CML Latex beads.
4. Wait for 24 h for the injected flies to recover from the procedure and then proceed with your experiment. A control may be performed at this step to check that phagocytosis is indeed blocked (*see* **Note 32**).

3.7 Gut Dissection (see Note 33)

1. Add 20–100 μl of 4 % PFA at the bottom of the glass cupules and cover the solution.
2. Anesthetize the flies with CO_2.
3. Use a clean dissection pad and put it under the dissecting microscope.
4. Place a few drops of sterile 1× PBS solution on the dissection pad.
5. Take the flies with fine forceps and place flies on the dissection pad in a drop of PBS solution.
6. With one pair of forceps, decapitate the flies making sure to cut the esophagus.
7. With one pair of forceps, hold the abdomen, and with the other pair of forceps pull out the external genitalia and rectum. Carefully pull out the gut and remove the ovary, Malpighian tubules, hindgut and foregut, and crop. After an oral infection, the crop can be full of bacteria and inflated causing its volume to represent an obstacle to the dissection of the midgut (*see* **Note 34**).
8. Transfer directly the dissected midguts to the Syracuse watch glass containing the 4 % PFA fixative solution and label the watch glass for the specific genotype and condition.

Alternatively, fixation can be performed in an Eppendorf tube containing 200–400 µl 4 % PFA fixative solution.

9. Cover the watch glass and fix the guts by incubation and at room temperature for 20–40 min (*see* **Note 35**).
10. After fixation remove the fixative solution and rinse the guts two times for 5 min in PBT without agitation.
11. Using a cut 200 µl tip carefully aspirate and transfer the guts with PBT in a clean 1.5 ml Eppendorf tube (*see* **Note 36**).
12. Allow the guts to settle down in the tube and take out the remaining PBT.

3.8 Blocking and Immunostaining of Guts

1. Add 200–500 µl blocking solution to fixed guts and incubate the guts overnight at 4 °C or 1 h at room temperature (*see* **Note 37**).
2. Aspirate the blocking solution.
3. Dilute the primary antibody in 1 % BSA and 1× PBX to the desired concentration and vortex. Centrifuge briefly and add 50–100 µl of diluted primary antibody to the guts.
4. Incubate the gut tissues with the primary antibody overnight at 4 °C or 2–3 h at room temperature (*see* **Note 38**).
5. After incubation collect the primary antibodies and save them at 4 °C for reuse (*see* **Note 39**).
6. Rinse the gut three times for 5 min with the 1 % BSA and 1× PBX solution.
7. Then wash the gut on shaker at room temperature for 15 min (three times) in 1 × PBX.
8. Prepare the secondary antibodies to the desired concentration in 1 % BSA and 1 × PBX (*see* **Note 40**).
9. Add 100–200 µl of diluted secondary antibodies to the gut tissues after a brief centrifugation step.
10. Wrap the tube with aluminum foil to avoid the exposure to light.
11. Incubate the gut with secondary antibodies at room temperature for 2 h or overnight at 4 °C with gentle agitation.
12. Remove the secondary antibody from the tube.
13. Rinse the gut three times for 15 min with PBT.
14. After final wash, rinse in 1× PBS and prepare for mounting.

3.9 Mounting of the Guts

1. Put a small drop of DAPI containing VECTASHIELD Mounting Medium in each well of the diagnostic slide.
2. With the help of a cut 200 µl tip carefully aspirate and transfer the guts with PBS in a clean glass cupule.

3. Under the dissecting microscope, with one pair of fine forceps dispose and arrange 1–3 guts in each well and make sure that they are covered with the mounting medium.
4. Carefully place a cover slip to the slide containing the guts.
5. Seal the edges of the cover slip with nail polish.
6. Use a permanent marker to label the slide for the specific genotype and experimental condition.
7. Put the prepared slides in a slide box and store the slides in the dark at 4 °C until observation with an epifluorescence or a confocal microscope (*see* **Note 41**).

3.10 SYTOX Green Method to Probe the Integrity of the Epithelium

1. Infect or mock infect (no bacteria) flies, as described in Subheading 3.1, and dissect the guts as described in Subheading 3.7, up to **step 8**.
2. Add 60 µl of 1:1,000 SYTOX Green directly to 20 µl 16 % PFA. Fix for 20 min.
3. Wash 3 × 5 min in PBS. SYTOX Green penetrates cells with compromised plasma membranes. As a positive control, feeding flies with a 2 % SDS-sucrose solution incubation will lead to the entry of SYTOX Green in damaged cells.
4. Mount in Vectashield and DAPI on a glass microslide.
5. Spread guts under dissecting microscope.
6. Place cover slip on top of the microslide.
7. Seal the edges of the cover slip with nail polish.
8. Label the slide with marker.
9. Examine slide by fluorescence microscopy (*see* **Note 42**).

3.11 Smurf Assay to Probe the Integrity of the Digestive Tract

1. Collect female flies (3–7 days old).
2. Set up infection tubes as described previously in Subheading 3.1.
3. Add blue food dye (FD&C blue dye #1) to the infection mix. As a positive control, add 2 % SDS, or use staged old flies (>45 days).
4. Incubate flies in infection vials with blue dye at 29 °C.
5. Score the "smurf" flies, i.e., flies with extended blue coloration that is not limited to the proboscis and crop.

3.12 RNA Extraction from Adult Midguts

As RNA extraction requires RNase-free working, please proceed with the gut dissection as fast as possible until the intestines are placed in TRI Reagent®RT.

1. Dissect the midguts as described in Subheading 3.7 (minimum ten per point).
2. Transfer them directly in a 1.5 ml tube containing 150 µl of TRI Reagent®RT and keep the tubes on ice.

3. Homogenize the midgut tissues by crushing them with micro-pestle and complete to 350 µl with 200 µl of TRI Reagent®RT.
4. Add 17.5 µl of BAN (5 % v/v).
5. Vortex and incubate for 5 min at room temperature.
6. Centrifuge for 15 min at 15,000 g at 4 °C.
7. Collect the upper aqueous phase and transfer to a new RNase-free 1.5 ml Eppendorf tube.
8. Add 300 µl of isopropanol.
9. Vortex and incubate for 10 min at room temperature.
10. Centrifuge for 15 min at 15,000 g at 4 °C
11. Discard the supernatant.
12. Wash the pellet with 500 µl of 70 % ethanol and centrifuge for 10 min at 15,000 g at 4 °C.
13. Repeat the washing step and centrifuge.
14. Take out the supernatant and let the RNA pellet to dry.
15. Resuspend the pellet by adding 30 µl of RNase-free water.
16. Quantify the RNA preparation with the Nanodrop (*see also* **Note 16** *for the use of a Bioanalyzer*).

3.13 Protein Extracts of Dissected Guts

1. Collect flies.
2. Set up a control and infection as described in Subheading 3.1. Incubate at the desired temperature and for a period as long as needed by the experiment.
3. Dissect guts as described in Subheading 3.7 (up to **step 8**) in PBS/proteinase inhibitor, transfer to 1.5 ml Eppendorf tubes cooled on dry ice, and store at −80 °C.
4. Add 80 µl cell lysis buffer; use minipestle to homogenize.
5. Incubate on ice for 1 h.
6. Spin for 10 min at 14,000 rpm at 4 °C.
7. Collect around 70 µl supernatant, and store at −80 °C.
8. Measure the protein concentration using a Bradford protein assay.

3.14 Monitoring the Synthesis of Proteins by the BONCAT Method

1. Add Click-iT® AHA/50 mM sucrose or Click-iT® AHA/bacteria/50 mM sucrose solution directly to two filters placed in medium tube. Add the flies and incubate at 29 °C until needed.
2. Dissect and fix the guts as described in Subheading 3.7 (up to **step 10**: fixation time: 20 min at room temperature).
3. Wash three times for 10 min in PBT.
4. Wash in PBT/BSA.

5. To detect the Click-iT reaction, prepare freshly Click-iT® Cell Reaction Buffer cocktail in 1.5 ml Eppendorf tube (440 μl 1× Click-iT cell reaction buffer, 10 μl CuSO4, 50 μl Click-iT cell buffer additive, add to 1 μM AHA final concentration; total volume is 500 μl and serves well for four conditions to test) (*see* **Note 43**).
6. Remove PBT/BSA from the guts, but leave a minute amount of liquid so that the guts do not dry out. Add 100 μl cell reaction buffer cocktail to guts in glass wells, and cover with aluminum foil to protect from light. Incubate for 30 min at room temperature.
7. Wash three times in PBS.
8. Mount the guts on glass slides with Vectashield/DAPI.
9. Continue directly to image acquisition, since the samples and the click reaction fade (*see* **Note 44**).
10. Measure the intensity of reaction on micrographs with Image J.

3.15 Monitoring the Physiological Function of the Acid-Secreting Region of the Drosophila Midgut

1. Transfer the flies on bromophenol blue-containing sucrose solution at least 2 h before infection (*see* **Notes 45** and **46**).
2. Transfer the flies on bromophenol blue-containing bacterial suspensions and control solutions for the desired duration of the infection as described in Subheading 3.1.
3. Dissect the fly midguts in 1× PBS as described in Subheading 3.7 without the fixation step.
4. Mount the dissected midguts on a glass slide in a drop of 1× PBS and directly proceed to image acquisition (*see* **Note 47**).
5. The dissected gut of control wild-type flies fed on sterile solution appears blue in the anterior and posterior midgut regions (pH > 4) and yellow in the copper cell region (pH < 2.35) (Fig. 4).

4 Notes

1. The quality of the food is an important parameter that influences the outcome of intestinal infection experiments. In flies raised on protein-poor medium intestinal stem cells will not proliferate after hatching which leads to shorter guts [30]. The major source of protein in the food is provided by yeast. The quality of the yeast can be critical. It is better to use live yeast as some yeast extracts fail to provide essential nutrients. It is not unheard of some resellers changing the quality of their products without warning their customers. Another issue to take into consideration is the microbiota [31, 32]. It has been shown that some strains of *Acetobacter pomorum* or *Lactobacillus*

plantarum are able to compensate for the dietary effects of a protein-poor food [33, 34]. For 1 l of medium, we use 6 g agar, 15 g dry yeast, 60 g sugar, 80 g maize flour, and 5 g of sodium hydroxybenzoate as a preservative. We prepare batches of about 30 l using a professional cooker for collectivities. Smaller amounts can be prepared in a cooking pot.

2. There are different designs for the RNAi transgenes. One consideration is the location of insertion of transgenes. Earlier libraries relied on classical transformation with random insertions [35]. Depending on the site of insertion and thus the chromatin context, the same transgene can be expressed at variable levels. Newer generations use an integrase-based system in which the insertion site has already been selected, thus allowing the generation of transgenic lines with a higher efficiency and consistent expression from one construction to the next. The second consideration is the type of hairpins. Many libraries rely on transgenes that lead to the production of long RNA hairpins, with different programs being used for designing the hairpins. A more recent design relies on short hairpins that deliver shRNAs that affect their target transcripts through the endogenous miRNA pathway [36].
3. For fly anesthesia, a CO_2 setup requires a more "heavy" investment (bottle, a pressure regulator, and anesthesia pad). Ether is an alternative that however requires practice. When flies are exposed to a too heavy dose, they may never recover. The common mistake is to use not enough ether, which leads to a premature awakening of the flies and the temptation to expose the flies again to ether for a longer period that becomes lethal. A good idea is to use a first batch of flies and measure the recovery time after exposure to determine how long the flies will remain asleep. The time it takes for flies to be anesthetized gives a clue as to how long the flies will remain asleep. One should aim for 30–60 s.
4. For harmless bacteria, a Bunsen burner on a bench may be sufficient to prevent contaminations, although we prefer to use a class II microbiological safety cabinet. Its use is imperative for more pathogenic class II microbes for safety reasons. In any case, the best protection of the worker is his/her own immune system and it is not advisable to perform this type of work when immunosuppressed (HIV patient, patient undergoing chemotherapy, …).
5. The preparation of the sucrose solution, the infection solutions, as well as the infection tubes should be performed under sterile conditions. For this we advise to work as much as possible under a clean laminar flow hood.
6. Depending on the nature of the bacterium used, adapt the temperature and the media used for the overnight culture

(e.g., *Serratia marcescens*: LB broth media and 37 °C under agitation, Ecc15: LB broth media and 30 °C under agitation, *Lactobacillus plantarum*: MRS media, 30 °C without agitation).

7. Depending on the nature of the assay (immunostaining or survival) different bacterial concentrations can be used. A high concentration (OD600 = 10 or 100) ensures a rapid and more systematic ingestion of bacteria by the treated flies, allowing analysis of early modifications of the gut shape or physiology. For longer experiments as survival assays, the use of lower concentrations (OD600 = 0.1 or 1) can reduce the pathogenicity of the bacterium which can help for the observation of subtle differences in survival between different genotypes. Of note, these concentration changes may not be neutral. For instance, higher bacterial concentrations trigger a stronger host ROS response [24].
8. In the original publications on intestinal infections of *Drosophila* with *S. marcescens*, the protocol that was followed was slightly different in that only OD0.1 was used and that the overnight bacterial culture (usually around OD1.7) was not centrifuged but diluted to OD1 by adding fresh sterile LB and then brought to OD0.1 by dilution with a 50 mM sucrose solution [7].
9. Any needle puller can be used. The tip must be sharp and broken under a dissecting microscope with a pair of tweezers.
10. *Drosophila* Ringer solution can also be used.
11. The fixative should ideally be prepared fresh every time; alternatively, it can be stored up to a week at 4 °C or longer at −20 °C. Some investigators, e.g., [24], use PBTw (PBS with 0.1 % Tween20) instead of PBS, a change that may enhance fixation by permeabilizing the sample.
12. Formaldehyde is toxic by inhalation, ingestion, and direct cutaneous contact. It has also been classified as a carcinogenic substance. To prepare the solution, it is best to work under a chemical fume hood. Wear gloves at all times and change them if they come into contact with the solution. Because it is difficult to perform the dissection under a fume hood, the exposure can be limited by placing a cover on the fixation dish. Discard according to local regulations; do not throw away in the sink.
13. BSA concentration may have to be adjusted from 0.5 to 2 % to decrease background staining, depending on the primary antibody used.
14. It is important to use an oscillating platform rather than a rotating platform as the intestines may otherwise become intertwined.

15. The pestle should be cleaned (50 % bleach or 10 % hydrogen peroxide treatment for at least 30 min) and autoclaved.
16. This equipment is not essential. However, it is advised to check the quality of samples prior to transcriptomics analysis on such a machine. It is also useful for troubleshooting when encountering problems with qRT-PCR.
17. To limit the activity of the Gal4 transcription factor during development, all the crosses and development of the F1 progeny are conducted at 18 °C until the desired developmental stage is reached, usually imaginal stages. Prior to starting the oral infection assay, the F1 flies are transferred for 3 days at 29 °C to release the repression of the thermosensitive $Gal80^{ts}$ and promote the Gal4 activation of the UAS-RNAi transgene since Gal4 functions better at 29 °C. Of note, the efficiency of RNA interference depends on the transgene, but also on the stability of the gene product. Thus, optimal conditions may need to be worked out depending on the experiment.
18. In the protocol we describe here, the flies are continuously exposed to the pathogen as they are feeding on it. One reason is that *S. marcescens* does not readily colonize the digestive tract. The sucrose present in the solution makes it palatable to the fly and also is used as a carbon source by both bacteria and fly. For some highly pathogenic strains such as *Pseudomonas entomophila*, a single round of feeding on highly concentrated bacterial solution is sufficient to cause the demise of the fly [37]. Other nonpathogenic strains such as *Erwinia carotovora carotovora 15* are able to colonize the gut and require at least one specific "colonization" factor [38]. When fed at high concentrations, this bacterial strain indirectly causes important damages to the gut with the loss of up to 50 % of the enterocytes that are mainly killed by the strong host ROS reaction [24]. The host is nevertheless able to compensate these losses within 48 h through the proliferation of ISCs. In these paradigms of infections, the flies may be placed back onto normal fly food medium after ingestion. It is also possible to deposit the bacterial solution onto regular food vials. Some investigators place the concentrated bacterial solution in the cap of an Eppendorf tube in an otherwise empty vial. Finally, the presence of bacterial growth medium can strongly alter the virulence of the bacteria. For instance, *Pseudomonas aeruginosa* kills wild-type flies when incubated with its growth medium but fails to do so when incubated only with sucrose solution [8, 39]. The actual design of the experiment depends on the pathogen under investigation and the question being asked.
19. To ensure that the filters are flat on the bottom of the tube, especially bumps on the circumference that allow flies to go

underneath the filters, we use a slightly smaller tube (for instance, an unscrewed inverted 50 ml Falcon tube) that we use as a plunger to flatten the surface.

20. When investigating early time points of the infection, it might be desirable to synchronize fly feeding by prior short-term starvation. This is achieved by placing flies in an empty vial for a couple of hours or a vial containing just water for longer periods (on water, the starvation has to last for longer than 18 h to induce significantly higher feeding behavior [40]). We have found that most flies feed within 5 min of being put on the sucrose-containing medium. This strategy is used by many investigators. However, one should keep in mind that food may be diverted preferentially to the crop after starvation, thus changing the conditions of the assay.
21. It is important to add always the same number of flies to improve reproducibility. We found that 20 flies is a good number for the vials we use and the amount of bacterial solution. In addition, it represents high enough a number of flies to detect the effects. Usually, we take only females. Should one mix males and females, care should then be taken to ensure that the same ratio is used in all tubes of the experiments.
22. Controls usually consist of flies feeding on sugar solution. However, one can add also *Escherichia coli*. When investigating specific virulence processes, the best control is a microbial mutant affecting the virulence factor under consideration. Of note, sometimes fungal infections can develop in the control vials as there is no microbial competition for the use of sucrose.
23. In the case of *Staphylococcus xylosus*, we have determined that the bacteria and the flies rapidly consume the sucrose initially present on the filters. Just adding water to preserve the humidity is usually not sufficient, although the combination of starvation and pathogenesis may yield valuable information, as determined in our original *S. marcescens* infection model [7, 14]. An alternative is to place flies on new vials and fresh bacterial solution every day to every two days. This is much more cumbersome. With *S. marcescens*, we have not observed major differences between the two techniques (add sucrose daily or change vials). This suggests that the deposition of feces on the sides of the vials does not pose a specific problem.
24. The most important point is to reproduce the data in at least three independent experiments performed at different times. When there is a large effect, the conclusion is generally obvious, as unlike experiments for mice, a large number of flies is used in these experiments. For effects of lower magnitude, more flies might be needed to reach statistical significance. As a rule of thumb, if when comparing two conditions, the survival curve for

A is always delayed as compared to that of condition B in several independent experiments, then there is likely a significant difference. The appropriate statistics to two survival curves in one set of experiments is the log-rank test, for instance as implemented in the Prism package. Of note, the data can be displayed either using Kaplan-Meyer plots or using Excel scatterplots. The latter type of representation makes the comparison between two curves easier than with Kaplan-Meyer plots. If the survival curves have the same shape, an alternative is to measure the LT50 (time it takes for 50 % of the flies to die) in several independent experiments and then compare the LT50 data [8].

25. The microbiota is usually sensitive to most antibiotics. In young flies, the quantity of microbiota is low as compared to the number of ingested bacteria.
26. For this experiment in which fluid is drawn out from the injected fly by capillarity, it is essential NOT to preload the capillary with oil prior to mounting on the Nanoject II apparatus.
27. The expected number of CFUs varies depending on the bacterium, the time of the infection, and whether the cellular immune defense is functional [7, 8].
28. This step is to clear bacteria that are present in the gut lumen as gentamicin does not cross the eukaryotic cytoplasmic membrane. If you just need to check the total titer of ingested microbes, omit the gentamicin treatment steps.
29. The presence of microbes sticking to the gut from the hemolymph compartment is a concern with ingested bacteria that cause a systemic infection after escaping from the digestive tract (e.g., *P. aeruginosa*) or when the cellular immune response is impaired [7, 8].
30. The titer depends on the concentration of ingested bacteria and is usually high as compared to that measured in the hemolymph (10^2 to 10^5).
31. The capillary must first be backfilled with oil before mounting on the Nanoject. This is essential to ensure a somewhat accurate delivery of definite quantities. When we checked the accuracy of volumes actually injected by delivering a radioactive solution, we found an error rate of 100 % with the smallest dose delivered with a Nanoject I (4.2 nl). Latex beads tend to aggregate. To prevent this from happening, an optional step is to place the aliquot containing the beads in an ultrasound water bath for 30 min. To prevent the clogging of the capillary, it is best to fill it slowly so that one is able to dislodge the aggregate as soon as it enters the needle. As regards the injection itself, one should proceed rapidly for the injections from one fly to the next. A foot pedal plugged in the Nanoject is helpful to increase the speed.

32. To check that phagocytosis is indeed blocked, inject FITC-labeled bacteria into the pretreated flies and PBS-injected controls. After half an hour, inject 300–400 μl of trypan blue to quench the fluorescence emitted by noningested bacteria. Check under a dissecting microscope equipped with epifluorescence or under a fluorescence microscope. You should observe fluorescent dots, especially close to the dorsal vessel, in the PBS-preinjected flies, and hardly any fluorescence in the latex-bead-injected flies. This control is worth doing whenever preparing a new batch of beads or for especially important experiments. In any case, it is advisable to check the results obtained by this technique with a genetic approach, that is, by testing flies deficient for phagocytic receptors, e.g., *eater* mutant flies.
33. The following sections describe the procedure followed for making immunofluorescent stainings. Note that with some transgenic fluorescent reporter lines and fluorescent bacteria (GFP, dsRED, mCherry, …), it is possible to directly observe the samples after dissection and even to follow processes by live microscopy. One way to enhance the longevity of the preparations is to dissect the guts in Schneider medium. Another is to ease the gut outside of the body cavity without severing the gut. In this case, peristaltic contractions may pose a problem. This may be alleviated by sticking the dissected gut on polylysine-coated slides.
34. Unless an important aspect of the infection takes place in the crop, e.g., the formation of a biofilm [41], it is recommended to discard the crop as it may be filled with bacteria that may survive fixation and degrade the final preparation (*see also* **Note 39**).
35. The fixation time has to be empirically determined for each antigen. Too long a fixation step may lead to antigen cross-linking or masking of epitopes. In practice, periods of between 20 and 40 mn are advisable.
36. To facilitate the transfer, rinse the tip in PBT solution prior to collecting the sample.
37. Permeabilization is ensured by the Triton detergent present in the solution whereas nonspecific binding sites for the antibody are saturated by BSA, thus decreasing background staining. The blocking step at 4 °C can be extended over longer periods (e.g., weekend) without a noticeable loss of quality.
38. Too extended an incubation period may lead to a higher background.
39. The primary antibody may be reused many times. The quality of the staining may actually increase as nonspecific antibodies cross-react and become depleted in the antibody solution.

However, a risk is that the solution becomes contaminated by microbes, for instance those that are present in the gut and not killed with 100 % efficiency by fixation (*see* **Note 34**).

40. The secondary antibody should be protected from light so as to preserve the fluorophores. The tubes are usually kept wrapped in aluminum foil.
41. It is better not to wait too long before observing guts infected with bacteria. We have found that not all bacteria are killed by a 4 % formaldehyde fixation. 16 % may help but on the other hand may prevent access to the epitopes.
42. The nuclei of longitudinal intestinal muscle cells appear to incorporate SYTOX Green even in the absence of experimental damage (Fig. 1).
43. Use the Click-iT reaction cocktail within 15 min of preparation. It is important to minimize the exposure to light when handling azidohomoalanine.
44. The intensity of the staining decreases strongly by 24 h.
45. To visualize the alkaline regions of the intestine the experiment can be conducted with the pH-sensitive dye Phenol-red (Sigma) instead of bromophenol blue [42]. Both dyes work by incubating the flies for 2 h in order to allow the pH-sensitive dye to fill the intestine and to change color depending on the acidity of the different regions of the fly midgut.
46. According to Shanbhag and Tripathi, an alternative pH estimation of the midgut region can also be achieved without the use of pH-sensitive dye. The dissected guts are directly laid on a piece of pH indicator paper, and carefully punctured with a thin needle to allow the gut content to diffuse on the pH paper and react at its contact [43, 44]. This technique presents the advantage of direct detection of both the acidic and the alkaline zone.
47. Dissected guts may no longer secrete acid in the copper cell level and the remaining peristaltic movement of the intestine might blur the regions by mixing of the gut contents of adjacent areas if one waits too long before imaging.

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Chapter 3

Zebrafish Embryos as a Model to Study Bacterial Virulence

Jennifer Mesureur and Annette C. Vergunst

Abstract

In recent years the zebrafish has gained enormous attention in infection biology, and many protocols have been developed to study interaction of both human and fish pathogens, including viruses, fungi, and bacteria, with the host. Especially the extraordinary possibilities for live imaging of disease processes in the transparent embryos using fluorescent bacteria and cell-specific reporter fish combined with gene knockdown, transcriptome, and genetic studies have dramatically advanced our understanding of disease mechanisms. The zebrafish embryo is amenable to study virulence of both extracellular and facultative intracellular pathogens introduced through the technique of microinjection. Several protocols have been published that address the different sites of injection, antisense strategies, imaging, and production of transgenic fish in detail. Here we describe a protocol to study the virulence profiles, ranging from acute fatal to persistent, of bacteria belonging to the *Burkholderia cepacia* complex. This standard operating protocol combines simple survival assays, analysis of bacterial kinetics, analysis of the early innate immune response with qRT-PCR, and the use of transgenic reporter fish to study interactions with host phagocytes, and is also applicable to other pathogens.

Key words Zebrafish, *Burkholderia cepacia complex*, *Burkholderia cenocepacia*, Bacterial virulence, Intracellular bacteria, Infection profiles

1 Introduction

Opportunistic microbial infections are a major cause of respiratory failure in cystic fibrosis (CF). Bacteria of the *Burkholderia cepacia* complex (Bcc), specifically *B. cenocepacia*, are particularly harmful for CF patients, and infection leads to increased morbidity and mortality [1]. Although infection with Bcc can be asymptomatic, it can unpredictably result in chronic progressive worsening of lung function and sometimes acute fatal necrotizing pneumonia and sepsis, termed cepacia syndrome. Several highly transmissible strains, including *B. cenocepacia* J2315 [2] and K56-2 [3], have caused a lot of havoc amongst patients, and since these bacteria have a high intrinsic resistance to antibiotics there is no effective treatment. The development of cell culture, non-vertebrate (*Galleria mellonella*, nematodes, *Drosophila*), vertebrate (zebrafish), and mammalian infection models [4–12] has contributed to a better understanding of the behavior of this pathogen,

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and bacterial virulence factors including lipopolysaccharide (LPS), flagella, secretion systems, and catalases have been identified [13]; however, the precise mechanisms underlying the disease and causing these sudden exacerbations and the induction of an excessive pro-inflammatory response are still not clearly understood.

Here we describe in great detail the protocol that we developed to study virulence of the Bcc using zebrafish embryos, although the method is generally applicable to other bacterial pathogens. The zebrafish has now been firmly established as an infection model in the study of human infectious disease [14], including for a number of facultative intracellular pathogens, such as *Mycobacterium, Salmonella, Listeria*, and *Burkholderia* [11, 15–17]. Especially the unprecedented possibilities to follow the infection of fluorescently labeled bacteria in real time in the transparent embryos allows the analysis of the role of host phagocytes during infection of cell-specific reporter fish, for instance those expressing GFP in neutrophils [14, 18] or mCherry in macrophages [19]. Importantly, the embryos have an innate immune system that is very similar to that of humans [20–23] and this allows studying the role of the innate immune response during infection in great detail, whereas an adaptive system is not functionally mature until at least 2 to 3 weeks postfertilization. The research community's efforts to develop this animal as a valuable additional and important tool for disease studies and drug screens [24] have produced many useful techniques (e.g., antisense RNA gene knockdown using morpholinos (MO) [25, 26] and transgenesis [27, 28]) and materials (e.g., transgenic zebrafish lines) that will help the researcher to address relevant biological questions from both the host and the bacterial side using bacterial mutants. Recent global transcriptome studies have also greatly extended our understanding of the host response to infection of adult zebrafish or embryos in response to pathogens using microarrays or RNAseq [29–32], and dual RNAseq combined with proteomics will soon allow the simultaneous identification of host and bacterial factors essential during specific disease stages in the whole animal, or specific cells (*see* Chapter 15).

Earlier we have shown that different clinical Bcc isolates, introduced by microinjection directly in the blood circulation of 30 h postfertilization (hpf) embryos, are rapidly phagocytosed by macrophages and can cause infection in zebrafish embryos that ranges from symptomless persistent, with bacteria (e.g., *B. stabilis* LMG14294) surviving in macrophages but unable to disseminate, to acute pro-inflammatory infection (e.g., *B. cenocepacia* K56-2), with bacteria surviving and multiplying in macrophages, followed by a highly pro-inflammatory infection that becomes rapidly fatal [11]. This model therefore offers great possibilities for detailed analysis of the early innate immune response, interaction with host phagocytes, and the importance of intracellular stages in disease development during both persistent and acute infection. In this chapter, we describe a protocol that allows analyzing virulence potential of environmental and clinical

isolates, ranging from simple CFU counts to determine bacterial load in the embryos and survival assays to intravital imaging and qRT-PCR analysis of important host immune response genes. Several zebrafish infection protocols have recently been published, including very elegant videos [33] and other interesting publications that describe techniques including transfection and morpholino injections in fine detail [34, 35]. Our aim is to present a protocol for newcomers in the field, and provide useful suggestions that might help to quickly adopt the protocol. The method will also be applicable to other bacterial pathogens, although some details (for instance growth conditions, counting strategies) may not be valid for other bacterial pathogens.

1.1 Microinjection

1. Microinjector, e.g., Femto Jet (Eppendorf).
2. Mechanical xyz micromanipulator arm, e.g., M-152 (Narishige).
3. Stereo microscope.
4. Microloader pipette tips.
5. Borosilicate glass capillaries, e.g., with filament O.D.:1 mm, I.D.: 0.78 mm, 10 cm length (*see* **Note 12**).
6. Agarose plates (1—1.5 % agarose in E3 medium) for injection containing slots of 1 by 1 mm (*see* **Note 13**).
7. Tricaine (MS222) 20×, 400 mg in 100 mL of sterile water, adjust pH 7.0 with Tris–HCl 1 M pH 9 (around 2.1 mL). Aliquot in 15 mL tubes and freeze at −20 °C. After use, keep at 4 °C.
8. Pasteur pipettes and latex bulbs.
9. 24- and 48-well tissue culture plates.

2 Materials

2.1 Obtaining Eggs and Preparation for Infection

1. Zebrafish facilities (*see* **Notes 1** and **2**).
2. Adult wild-type AB, Golden (*see* **Notes 3** and **4**) and Tg(*mpx::GFP*) transgenic fish [18].
3. Spawning tanks (*see* **Note 5**).
4. Incubator at 29 °C.
5. Embryo water (E3 medium) [36]: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ (from sterilized stock solutions) in sterilized water, supplemented with 1 μl of 10 % methylene blue (MB) per liter (*see* **Note 6**).
6. Stereomicroscope.
7. Petri dishes 90 × 14 mm.
8. Very fine forceps, Tweezers #5 Dumont, Dumoxel/Biology Grade.
9. Tissue culture-quality Petri dishes 60/15 mm (*see* **Note 7**).
10. Plastic Pasteur pipettes (7 mL) (*see* **Note 8**).

2.2 Bacterial Culture and Preparation of Inoculum

1. *Burkholderia cenocepacia* K56-2 and *B. stabilis* LMG14294, expressing a fluorescent reporter. Here we use strains harboring plasmid pIN29, encoding DSRed [11] (*see* **Notes 9** and **10)**.
2. Luria Bertani (LB) broth: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl. Add to 800 mL H_2O. Adjust pH to 7.5 with NaOH. For agar plates add 1.5 % Bacto agar. Adjust volume to 1 L with dH_2O and sterilize by autoclaving (121 °C, 20 min).
3. Chloramphenicol (Cm), 100 mg/mL stock solution in 100 % ethanol (store at −20 °C): For *Burkholderia*, we use Luria Broth (LB) medium with 100 mg/l of chloramphenicol (*see* **Note 11**).
4. Phosphate-buffered saline (PBS) 1×.
5. Phenol red (PR) solution, 0.5 %.
6. Spectrophotometer.
7. Inoculation loops.
8. 18 × 180 mm glass tubes with loose-fitting metal lids for bacterial growth.
9. 37 °C shaking incubator.
10. 37 °C incubator.

2.3 Micro Injection

1. Microinjector, e.g. Femto Jet (Eppendorf).
2. Mechanical xyz micromanipulator arm, e.g., M-152 (Narishige).
3. Stereo microscope.
4. Microloader pipette tips.
5. Borosilicate glass capillaries, e.g. with filament O.D.:1 mm, I.D.: 0.78 mm, 10 cm length (*see* **Note 12**).
6. Agarose plates (1–1.5 % agarose in E3 medium) for injection containing slots of 1 by 1 mm (*see* **Note 13**).
7. Tricaine (MS222) 20×, 400 mg in 100 mL of sterile water, adjust pH 7.0 with Tris–HCl 1M pH9 (around 2.1 mL). Aliquot in 15 mL tubes and freeze at −20 °C. After use, keep at 4 °C.
8. Pasteur pipettes and latex bulbs.
9. 24 and 48 well tissue culture plates.

2.4 Analysis of Bacterial Multiplication

1. LB 1.5 % agar plates (*see* Subheading 2.2, **step 2**) with appropriate antibiotics (*see* **Note 14**).
2. LB 1.5 % agar plates, square dishes 125/15 mm (~50 mL per plate) with appropriate antibiotics (*see* **Note 15)**.
3. Bacterial safety cabinet.
4. Eppendorf tubes.
5. Pipetman and tips.

6. 10× Trypsin/EDTA, diluted 10× in sterile H_2O.
7. Triton X-100 2 % in H_2O.

2.5 Tools for Survival Assays and Intravital Imaging

1. Inverted fluorescence and fluorescence multizoom microscopes with camera and supplied imaging software (*see* **Note 16**).
2. 24- and 48-well tissue culture plate (*see* **Note 17**).
3. 35 mm Glass-bottom dishes.
4. Microscope depression slides.

2.6 Tools for qRT-PCR and RNA-Seq Analysis

1. RNase-free work zone.
2. RNase-eliminating solution such as RNase Away.
3. RNase, DNA free 2 ml.
4. 1.5 and 0.5 ml Eppendorf tubes.
5. RNase, DNA-free water.
6. TRIzol® (Invitrogen).
7. Chloroform.
8. Isopropanol 100 %.
9. Ethanol 70 %.
10. DNase I, RNase free.
11. RNeasy MinElute Cleanup kit (Bio Rad).
12. Photospectrometer to quantify RNA.
13. iScript cDNA Synthesis kit (Bio Rad).
14. qPCR Primers with Tm of 60 °C (*see* **Note 48**).
15. qPCR machine with software: Light Cycler 480 (Roche).
16. 96-well white plates with transparent sealing foils (Roche).
17. SYBR Green mix adapted for your machine: For the LC480: Light Cycler 480 SYBR Green I Master (Roche).

3 Methods

Figure 1 shows a typical work plan of an infection experiment.

3.1 Obtaining Eggs and Preparation of Embryos for Infection

1. To obtain eggs, prepare several tanks containing one or two adult couples of the desired line (WT or Tg(*mpx:GFP*)) (*see* **Note 5**).
2. Carefully rinse eggs with tap water using a fish net with very fine maze, and transfer eggs in a standard Petri dish with E3 medium. Remove the remaining waste (*see* **Note 18**).
3. Put the plates in the incubator at 29 °C. During the day remove bad eggs (abnormal embryo development or infected eggs).
4. The next day, 2 h before microinjection (*see* **Note 19**), remove the protective membrane of the embryos under a dissecting

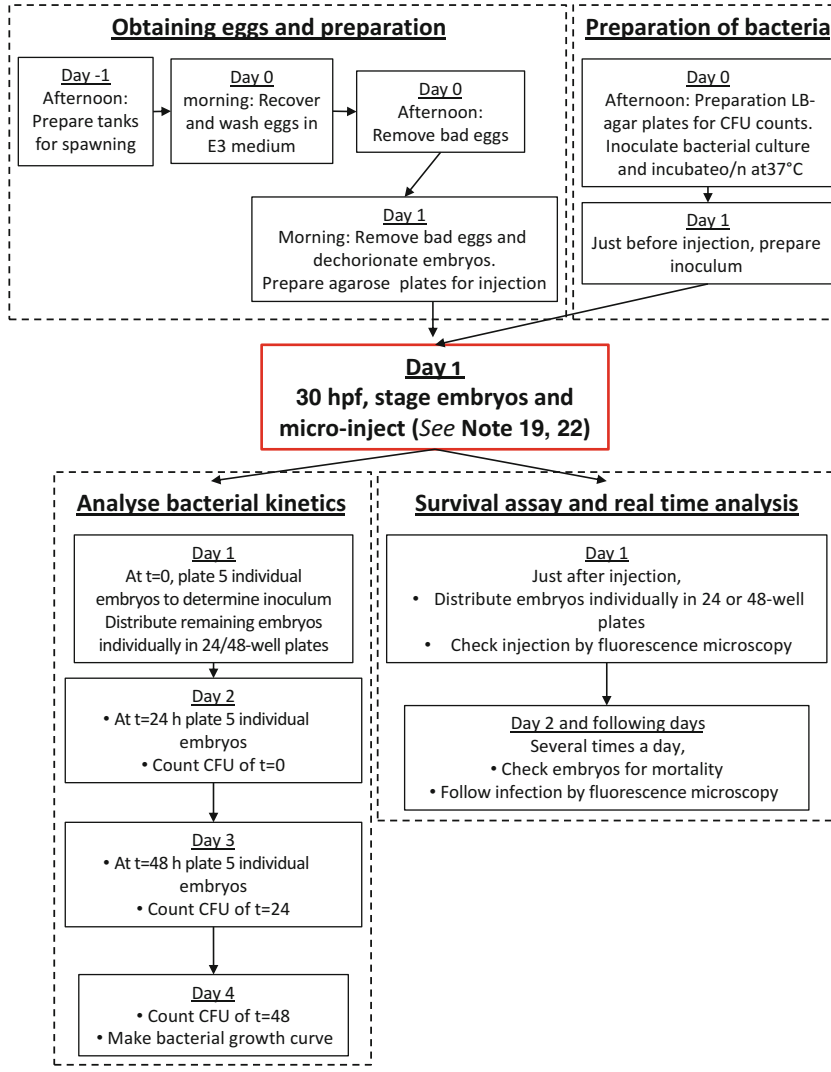

Fig. 1 Typical work plan for an infection experiment. OD: optical density at 600 nm; hpf: hours post-fertilization; hpi: hours post-injection/infection; CFU: colony-forming unit

microscope by delicately opening the chorion with very fine forceps (*see* **Note 20**).

5. After dechorionation, place the embryos in a tissue culture plate 60/15 mm with fresh E3 medium (*see* **Notes 6** and **7**), and maintain at 29 °C until the time of injection.

3.2 Growth of B. cenocepacia for Infection and Preparation of Inoculum

1. One day before the infection experiment, grow *B. cenocepacia* in LB medium from frozen glycerol stock (*see* **Note 11**). To maintain plasmid pIN29, add 100 mg/L Cm (LB-Cm100). Pipette 5 mL LB-Cm100 medium into a sterile glass tube and inoculate with a loop full of bacteria. Incubate in an orbital shaker (200 rpm) for 16–18 h at 37 °C.

2. Just before infection, measure the OD_{600} and prepare a dilution to obtain 50 bacteria per nL (*see* **Notes 21–23**) as follows: Transfer the required volume of bacterial culture to obtain 1 mL of an OD 1 to an Eppendorf tube, and centrifuge at 3,500 × *g* for 2 min. Remove supernatant and add 1 mL PBS to the bacterial pellet. Resuspend by gently pipetting up and down using a pipet man. Add 50 µL of the suspension (OD 1) to 850 µL PBS. To be able to visualize the injection, add 5 µL phenol red 10× to 45 µL of the bacterial dilution.

3.3 Microinjection (See Fig. 2 for Setup)

1. Prepare agarose plates for injection and tricaine 2× in E3.
2. Switch on the microinjector (*see* **Note 24**).
3. Pipette 5 mL of E3 containing 2 % tricaine onto the agarose plate and position 60 30 hpf embryos in the slots (*see* **Notes 25** and **26**).
4. Position the micromanipulator next to the stereomicroscope (Fig. 2a).
5. Load 3–4 µL of the bacterial injection suspension (from Subheading 3.2, **step 2**) into a pulled capillary pipette using a microloader tip, and place on the micromanipulator.
6. With the micromanipulator, place the tip of the needle in the center of the image. Adjust time and/or pressure to obtain an injection volume of around 1 nL (*see* **Notes 27** and **28**).
7. Place the agarose plate with embryos on the microscope, and place the needle above the caudal vein or the blood island region. Pierce the skin with the capillary needle (Fig. 2b), by descending the z-axis of the micromanipulator. When the capillary needle is at the correct location and in the blood circulation, inject the bacteria with a single pulse. The phenol red allows visualization of correct injection directly in the blood circulation (*see* **Note 29**) (Fig. 2c, d). Remove any embryos that are not properly injected.
8. Place five embryos in 10–20 mL sterile E3 medium to rinse embryos (*see* **Notes 30** and **31**) to determine inoculum, T = 0.
9. Transfer the embryos individually to a sterile 1.5 mL tube with a Pasteur pipette, and remove any E3 with a micropipette. Add 100 µL trypsin, and immediately disrupt the embryo by pipetting up and down (30–40 times) with a micropipette.
10. Incubate for 20 min at room temperature and pipette up and down (30–40 times) to completely disrupt the tissue.
11. Plate the total lysate from each embryo on LB-Cm100 plate and count colonies after an overnight incubation at 37 °C (*see* **Notes 32** and **33**).
12. Proceed with the remaining injected embryos to Subheading 3.4 (determination of survival rates), Subheading 3.5 (determination of bacterial multiplication), Subheading 3.6 (real-time analysis), and/or Subheading 3.7 (analysis of host gene expression). Using a fluorescence microscope (with 10× objective) discard any embryos that do not have any fluorescent bacteria.

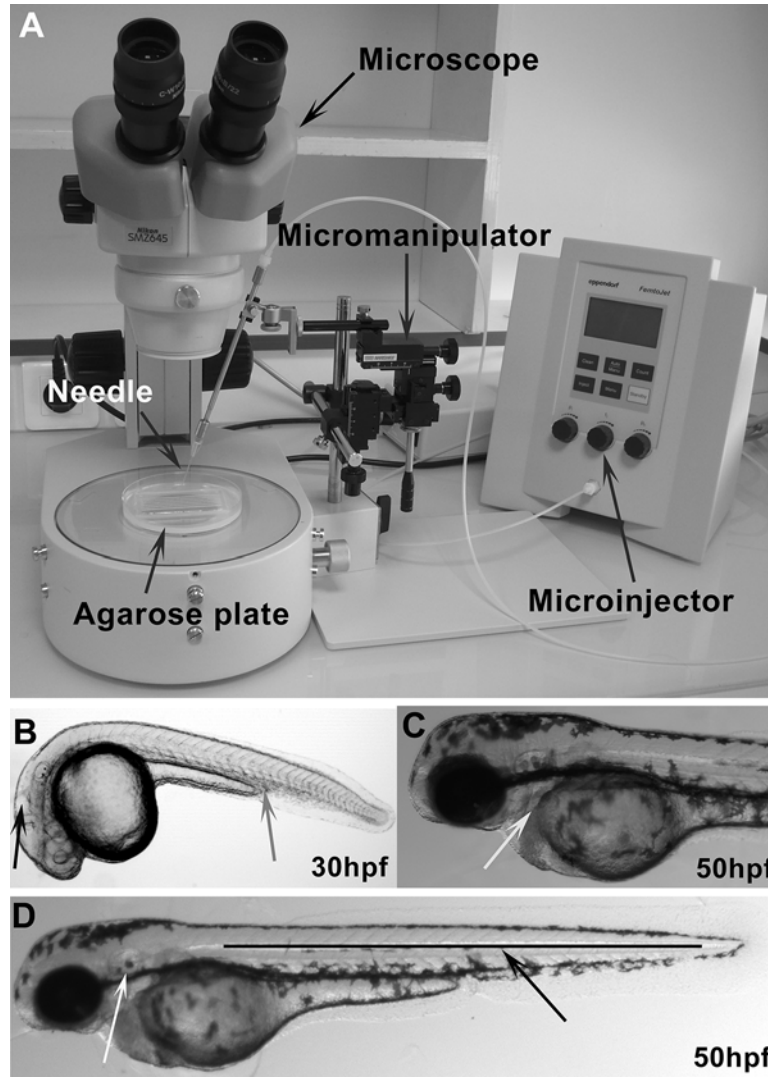

Fig. 2 Injection setup and injection sites. (**a**) Typical injection setup. (**b**–**d**) Injection sites for systemic (**b** *grey arrow*, **c**) or local (**b** *black needle*, **d**) bacterial infection in the zebrafish embryo [35]. (**b**) At 30 hpf, two preferred sites are used for microinjection; the blood island (*grey needle*) or the hindbrain (*black needle*). (**c**) Duct of Cuvier injection site at 50 hpf. (**d**) In 50 hpf embryos, the otic vesicle (*white arrow*), subcutaneously (*black needle*, [44]), and the notochord (*black line*)

3.4 Embryo Survival Studies

1. Just prior to injection prepare 24- or 48-well tissue culture plates with 1 mL E3 medium per well.
2. Transfer a minimum of 20 infected embryos individually into wells and put in the incubator at 29 °C.
3. Observe the embryos at least once a day and every 2–3 h during the critical phase of infection under a stereomicroscope and record mortality. An embryo is considered dead when the blood circulation has stopped and the heart no longer beats (*see* **Note 34**).

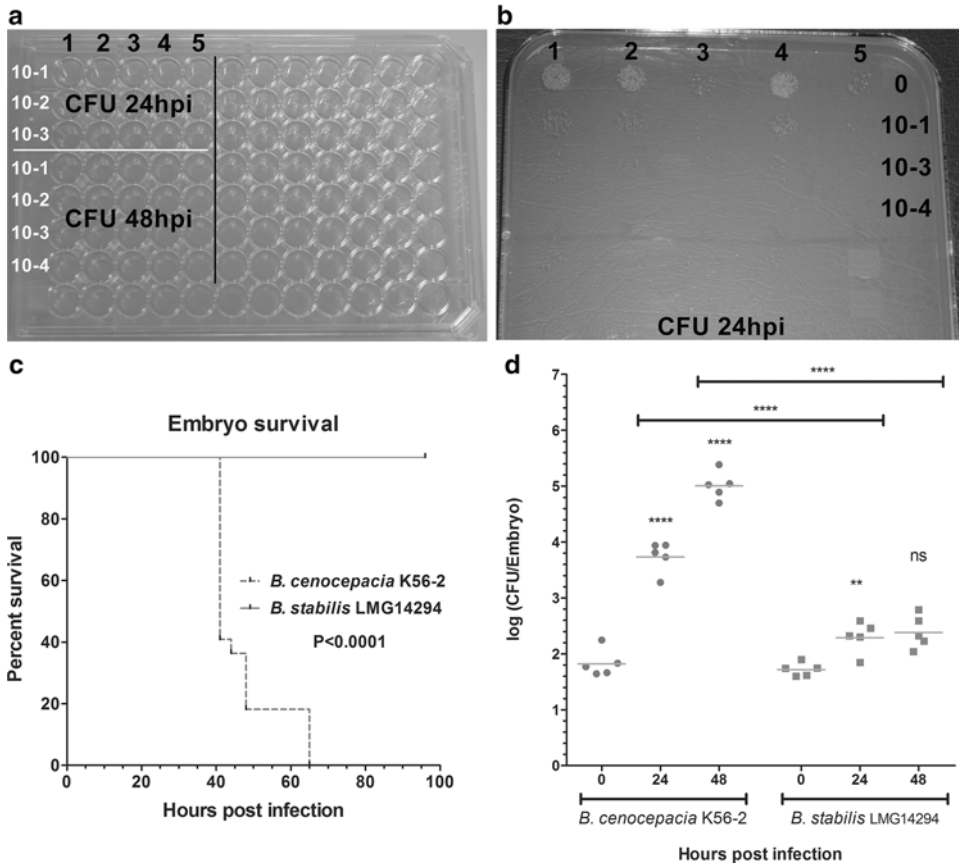

Fig. 3 Determination of bacterial multiplication and embryo survival. (**a**) A 96-well plate can be used to prepare serial dilutions for CFU counts at 24 and 48 hpi. (**b**) Plating method to determine bacterial CFU in tenfold serial dilutions. (**c**) Embryo survival following infection with *B. cenocepacia* K56-2 and *B. stabilis* LMG 14294 ($n = 20$ for each strain) with Kaplan-Meier representation. Significance is determined with a log-rank (Mantel–Cox) test. (**d**) Bacterial multiplication of *B. cenocepacia* K56-2 and *B. stabilis* LMG 14294 during infection. Five embryos per time point per experiment, with grouped column scatter representation. Each dot represents CFU per embryo; geometric means are indicated by bars. Comparison between 0 and 24 hpi, 24 and 48 hpi for the same strain and the difference between two different strains at 24 and 48 hpi with unpaired student *T*-test $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$

4. Represent mortality/survival rates using Kaplan-Meier graphic representations and analyze data with a log-rank or other appropriate statistical tests (Fig. 3c).

3.5 Determination of Bacterial Multiplication as Readout for Virulence

1. Prepare LB-agar-Cm100 plates.
2. After microinjection (subheading 3.3, **step 7**), transfer five infected embryos per time point per strain to be analyzed individually into wells of a 24- or a 48-well culture plate, and place in the incubator at 29 °C.
3. Wash five embryos at the desired time point by transferring the embryos to 10–20 mL E3 (*see* **Note 35**).

4. Transfer the washed embryos individually to sterile 1.5 mL tubes with a Pasteur pipette, and withdraw the E3 with a micropipette. In contrast to T = 0, add 45 μL trypsin, disrupt the embryo by pipetting up and down with a yellow tip (30–40 times), add 50 μL 2 % Triton X-100, mix by flicking the tube, and incubate for 30 min at room temperature. Repeat disruption by pipetting up and down 30–40 times (*see* **Note 36**).
5. Plate the total lysate on LB Cm100 agar plates (10 cm), or plate serial dilutions as follows (**steps 6–10**; *see* **Note 37**).
6. Prepare a sterile 96-well plate with 90 μL of PBS in each well.
7. For each embryo lysate (Fig. 3a) add 10 μL to a well in row A containing 90 μL of PBS, and pipette 20 times up and down to mix evenly.
8. Transfer 10 μL from well A to well B, and mix by pipetting up and down 20 times.
9. Repeat **step 8** until the desired number of dilutions is obtained.
10. Change pipette tip and spot 10 μL of each dilution and 10 μL of embryo lysate (non-diluted) on LB-Cm 100 cm^2 square plates as in Fig. 3b. Let the drops dry into the plate without spreading (normally takes 30 min), and incubate the plate overnight at 37 °C (*see* **Note 38)**.
11. Count colonies of each dilution (*see* **Note 39)** and determine total CFU per embryo.
12. Use an appropriate software program for analysis of bacterial multiplication rates and plot data using a semilogarithmic scale (*see* **Note 40**) (Fig. 3d).

3.6 Real-Time Observation During Infection

1. For global observations, embryos can be visualized directly in the 24-well plate with a 10× objective. If the embryos move, add tricaine (1× final concentration in E3).
2. For observation at higher magnification embryos can be placed in a microscope depression slide or in a glass-bottom dish in a small drop of 1× tricaine in E3 (make sure that the liquid does not evaporate during imaging).
3. Use 40×, 63×, or 100× oil objectives and bring embryo in focus using bright field.
4. Take representative images with bright field, Nomarski, and/or the different filter sets to visualize GFP and DSRed or mCherry (*see* **Note 16**, Fig. 4).
5. After observation and imaging, pipette the embryo back into E3 medium in the 24-well plate and return to the 29 °C incubator.

3.7 Extraction of RNA for qRT-PCR and RNA-Seq Analysis

1. Follow the steps for preparation of the embryos, preparation of inoculum, and microinjection until Subheading 3.3, **step 7** (*see* **Note 41**).

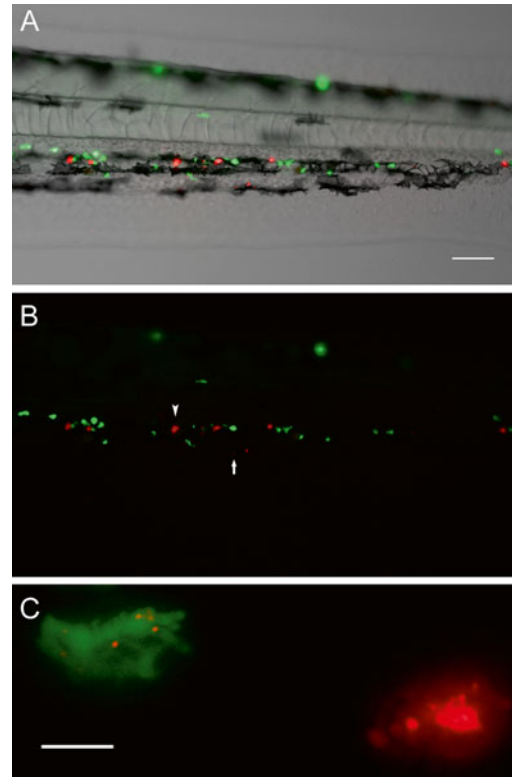

Fig. 4 Real-time imaging using fluorescence microscopy. (**a**, **b**) Images 2 hpi of the tail region of a Tg(*mpx-GFP*) embryo, with neutrophils expressing GFP, injected at 60 hpf with K56-2, expressing DSRed. (**a**) Representative bright-field and fluorescence (*green* and *red channel)* overlay image. *Arrowhead*: K56-2 bacteria (*red*) inside a phagocytic cell that is not a neutrophil. *Arrow*: Individual bacterium. *Scale bar* 50 μm. (**b**) Image as in A taken with *red* and *green channel* only. (**c**) Fluorescence overlay image (*red* and *green filters*) 18 hpi representing an individual GFP-expressing neutrophil that contains several red fluorescently labeled K56-2 bacteria. On the right, a GFP^{minus} cell containing many bacteria. *Scale bar* 10 μm. [11]

2. After injection, for each time point required for analysis at each of the conditions (for instance: strain K56-2, T = 3 hpi (=target sample A), PBS, T = 3 hpi (=control sample)), transfer a pool of 10–25 embryos to 500 μl of TRIzol. Vortex well until all tissue has dissolved. Transfer the tubes immediately to −80 °C.
3. Extract and purify the RNA from each pool using the RNeasy MinElute Cleanup kit according to the manufacturer's instructions or as previously described [35]. The final volume of purified RNA is 13 μl (*see* **Note 42**).
4. Analyze the quantity and the quality of RNA using 1 μl of the sample (*see* **Note 43**).

3.8 qRT-PCR Analysis (See Note 44)

1. Reverse transcribe each sample (500 ng total RNA is enough) with a cDNA synthesis kit following the manufacturer's instructions (*see* **Note 45**). The final volume is 25 µL.
2. Dilute each cDNA sample ten times to obtain the final volume required to set up the qRT-PCR reaction (*see* **Note 46**).
3. Prepare the SYBR Green mix for each condition (target and control samples at each of the different time points). For each condition, both the target gene and the reference genes should be analyzed (*see* **Note 47**). Volume per well: Pipette 5 µl master mix (2×), 1 µl primer mix (5 µM Fw and Rev primers, *see* **Note 48**), and 0.5 µl PCR water. Prepare the SYBR Green mix in an Eppendorf tube for the number of wells that you need plus two volumes to be sure to have enough.
4. Put the 7.5 µl of SYBR Green mix in each well and then add 2.5 µl of cDNA (*see* **Note 49**).
5. Use the following qPCR program (Table 1).
6. Analyze the qRT-PCR results (*see* **Note 50**).

4 Notes

1. Although more extensive zebrafish facilities are needed when keeping multiple transgenic fish lines (we acquired a Zebtec stand-alone system from Tecniplast), if only wild-type fish are needed for egg production a few aquaria (30 L) with adults are sufficient.
2. Authorization to keep and handle animals should be acquired according to your local and national regulations. In addition, permits should be obtained if you wish to produce transgenic animals or perform infection experiments (GMO regulations, ethical committees). Infection experiments using embryos and larvae that have not yet reached the free feeding stage are not considered as animal experiments (directive 2010/63/EU).
3. Pigment formation in the young larvae can interfere with imaging (fluorescence, light microscopy, histology). We suggest using Golden adults [37], which have a mutation in the gene *slc24a5* that prevents pigment formation. Another possibility is to use Casper fish. We avoid using phenylthiourea (PTU) to prevent melanization as it has an effect on fish biology [38] and immune signalling.
4. Zebrafish can be obtained from several stock centers, including the ZIRC (http://zebrafish.org/zirc/home/guide.php). We normally compare virulence of at least one bacterial strain in the different genetic backgrounds to exclude differences in virulence.
5. Male and females are put together in the evening in spawning tanks, which contain an inner tank with holes. The next morning, usually half an hour (sometimes longer) after the light in

Table 1
qPCR program used

Pre-incubation

Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:10:00	4.4	

Amplification

Cycles	45	Analysis Mode	Quantification	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:15	4.4	
60	Single	00:00:40	2.2	

Melting Curve

Cycles	1	Analysis Mode	Melting Curves	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:05	4.4	
65	None	00:01:040	2.2	
97	Continuous		0.11	5

Cooling

Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
40	None	00:00:10	1.5	

the room is turned on automatically (fish are maintained with a regime of 14-h light, 10-h dark), the females will lay eggs which fall through the holes. This avoids the parents from eating the eggs. If no spawning tanks are available, glass containers filled with glass beads or marbles can be placed in the aquarium the night before. We noticed that the fish prefer dark underground, and it may help also to include (plastic) plants underneath the inner container. Although we have had good spawning with females that had been separated from males for longer time periods, as a general rule do not keep females separate for longer than 1 or 2 weeks as they may become egg bound. A female can lay between 50 and 200 eggs. We usually set up several couples to be sure to have enough eggs. If it is difficult to obtain eggs it might help to separate males and females in the same tank a few days before.

6. Methylene blue (MB) is an antiseptic used in aquaria mainly against fungal growth, but with low antimicrobial activity. It is

therefore necessary to wash the eggs well before putting them in the E3 medium. We use a very fine fish net to rinse the eggs under running tap water, and then further separate the eggs from faecal materials under a dissecting microscope, before placing them in E3 medium. When you plan to work with fixed embryos (e.g., for in situ hybridization, immune staining), or image fluorescent phagocytes in live animals for further analysis with software tools (e.g., Image J) [39], avoid using MB since it can accumulate in the yolk and cause autofluorescence. For qRT-PCR analysis of host gene expression, it is better to use E3 without MB.

7. Once dechorionated, the embryo tail can stick to the plastic of Petri dishes damaging the tail. This may increase variation in infection outcome, and can induce wound-induced immune responses. We suggest using cell culture-quality dishes, such as Greiner Cellstar 60–15 mm, after the embryos have been dechorionated to prevent this. An ambient room temperature of around 23–25 °C is optimal for handling embryos.
8. We use 7 mL plastic Pasteur pipettes to transfer eggs. At later stages, after dechorionating the embryos, glass Pasteur pipettes should be used instead to prevent sticking of the embryo to the plastic pipette. Take great care taking up the embryos with the pipette and try to avoid wounding the embryos. Pipetting the embryos "head-first" works well.
9. Following the infection in real time requires the use of fluorescently labeled bacteria. For this we have constructed a series of plasmids with the genes encoding a selection of fluorescent proteins (GFP, DsRed or mCherry, CFP, YFP, mTurquoise, E2-Crimson) expressed from a strong constitutive *tac* promoter sequence. In the examples described here we use very bright, red fluorescent K56-2 that were obtained by transforming bacteria with the pIN29 (DSRed) vector by electroporation [11]. For colocalization studies, the actual fluorescent strain used will depend on the fluorescent proteins expressed in host phagocytes, or other immune-labeled host proteins. Avoid the use of CFP or mTurquoise for imaging of live embryos, since the high-energy light with the blue filter set affects the embryos.
10. Bacteria can be obtained from culture collections such as the BCCM/LMG bacterial culture collection.
11. Prepare overnight bacterial cultures directly from glycerol stocks kept at −80 °C, or from fresh agar plates started from −80 °C stocks. Do not keep Bcc strains on LB agar plates by repeatedly re-streaking the bacteria, as mutations resulting in attenuated virulence are likely to accumulate. Do not use bacteria that have been kept on agar plates or in liquid medium for longer times than needed, and do not store Bcc at 4 °C. For some Bcc strains (like *B. stabilis* LMG14294, or *B. cenocepacia*

J415), liquid cultures inoculated directly from −80 °C glycerol stocks often do not grow, and we suggest in that case to first streak out bacteria from frozen glycerol stocks onto LB plates containing appropriate antibiotics 2 days before an infection experiment and incubate at 37 °C for 1 day. In the evening prior to infection inoculate a loop full of bacteria in liquid LB medium and incubate on a rotary shaker with good aeration at 37 °C overnight. Avoid using airtight plastic tubes.

12. To prepare pulled microcapillary pipettes (borosilicate glass capillaries, e.g., with filament O.D.:1 mm, I.D.: 0.78 mm, 10 cm length) we use a micropipette puller (Sutter Instruments Inc., Flaming/Brown p-97) with settings to get a relatively short needle tip. We use air pressure 550, heat 990, pull 40, velocity 80, and time 200. This should be tried experimentally though for personal preference. In addition, the settings depend on the type/size of heating filament. The tip of the needle is closed after pulling, and should be opened by gently touching the fine point with a fine forceps under a stereomicroscope.
13. To prepare agarose plates with slots of 1 by 1 mm (6 cm in length) that hold the embryos in place for microinjection we use a house-made aluminum mold (kindly provided by Nicolas Cubedo). We have recently seen similar glass molds available commercially (MidSci).
14. We do not bleach the eggs for each experiment. To enumerate CFU, it is important to use LB agar plates containing an antibiotic to allow growth of Bcc only, and avoid growth of bacteria from the natural microflora that might obscure the counts. Usually the antibiotic resistance marker present on the reporter plasmid is used for this purpose. Due to the high intrinsic resistance to antibiotics of Bcc, you can also use polymyxin B or gentamicin when the bacteria do not contain a reporter plasmid. Of course it should first be analyzed whether the strains or the mutants used are resistant to these antibiotics. We have never encountered changes in virulence due to the presence of the reporter plasmid. We have found that the pBBR series of plasmids can be stably maintained in the Bcc during infection of zebrafish embryos in the absence of antibiotic pressure for over 5 days. For the Bcc, we suggest to test this once in the beginning by for instance comparing CFUs in individual embryos plated on LB with polymyxin B and LB with antibiotics used for selection of the plasmid, and then analyzing the colonies for expression of the reporter plasmid under a fluorescence microscope. All Bcc colonies should express the fluorescent reporter, indicating that the plasmid has been maintained in the absence of selection pressure.
15. We use square plates (140 mm) to apply 10 μl drops of 10× dilution series per individual embryo (*see* Fig. 3b). This method is highly reproducible and avoids having to plate multiple dilutions

per individual embryo on single Petri dishes. It is very important that the square plates are level when the agar solidifies and when applying the bacterial drops, to avoid the drops running into each other once applied to the plate. It is equally important to properly dry the plates. We dry the plates without lid in a microbiological safety cabinet for exactly 30 min, not longer. Bcc do not grow well on plates that are too dry. For Bcc, use freshly prepared agar plates (do not use plates older than 1–2 days), and store the plates at room temperature to avoid condensation when stored at 4 °C.

16. An inverted fluorescence microscope with possibilities for large magnification is required for detailed analysis of bacteria-cell interactions. Our microscope is equipped with 5, 10, 40, 63, and 100× objectives. The possibility to use Nomarski optics will greatly enhance the quality of the imaging of host cells and tissue. The microscope should also be equipped with the different filter sets needed to visualize the different fluorescent markers, such as GFP and DSRed. For our Leica microscope, we use filter sets L5 (band pass (BP) 480/40; beam splitter (BS) 505; emission BP527/30) and N2.1 (515–560; BS 580; emission long pass (LP) 590), respectively. For imaging we used a Coolsnap fx (Roper Scientifique) and MetaVue software, and images are further processed using Adobe Photoshop. In addition, a stereomicroscope equipped for fluorescence (the Nikon AZ100, or alike) which allows rapid analysis of embryos and imaging of complete embryos (1×, 2×), but also allows more detailed imaging, is very useful. Placement of embryos on agarose plates in E3 medium with tricaine 1× is also a good option for imaging [33]. In this case, to mobilize the embryos on the plate use a loading tip (that can be shortened if needed).
17. Six-, 12-, 24-, or 48-well tissue culture plates can be used to culture embryos after injection. If possible, avoid using 96-well plates because the wells are too small for embryos meaning that they will not develop correctly if kept for more than 2 days in the wells.
18. Do not keep more than 100 eggs per Petri dish. Use multiple plates or larger 150 mm Petri dishes to reduce the risk of contamination and allow normal embryo development. At this stage remove non-fertilized and empty eggs under a dissecting microscope as they may be a source of infection. Prevent transgenic eggs from being washed away through the sink and dispose of them properly.
19. The best time point after fertilization to inject the embryos depends on the site of injection, the pathogen used, and the questions to be addressed. We usually microinject embryos in

the blood circulation at the site of the blood island or caudal vein (*see* Fig. 2) between 28 and 32 h post-fertilization (hpf). At this stage immature macrophages and neutrophils are capable of phagocytosing and killing nonpathogenic bacteria, and expression of genes involved in innate immune response, resembling those in human infections, can be detected by qRT-PCR. For the Bcc we can easily distinguish between strains that induce a strong inflammatory response, and kill embryos within 48 h (e.g., *B. cenocepacia* K56-2), and less virulent strains that do not kill embryos within the duration of the experiment but remain persistent in macrophages (e.g., *B. stabilis*) [11]. *Mycobacterium marinum* infections will require several days for the development of granuloma-like structures [15]. Microinjection in the inner ear or hindbrain (to study recruitment of phagocytes) and in the notochord is typically performed in older (>50 hpf) embryos. When studying the role of certain virulence factors by microinjecting bacterial mutants, it may be advisable to compare virulence with the WT strain by injecting at different time points after fertilization. A type III secretion mutant of *Pseudomonas* was for example attenuated when injected 50 hpf, but not at 30 hpf [40].

20. Two fine tweezers can be used, each "gripping" the chorion, placing them close next to each other. By gently moving the tweezers apart the chorion will open and liberate the embryo. Avoid touching the yolk, as it will "disintegrate" upon touching. Alternatively, one pair of tweezers can be used; gently touch the chorion with the closed points of the tweezers, and then immediately but gently open the tweezers to disrupt the membrane. We do not use proteolytic enzymes such as pronase to remove the chorion, as it can damage the embryos if left too long, and may have an effect on immune responses.

21. The calculations are based on a total number of 1×10^9 bacteria per mL for an OD =1. A 20-fold dilution will result in 50 bacteria per nL. We prepare 50 μL total volume of inoculum (this can be reduced to 10 μL). The number of bacteria in a culture at OD_{600} 1 should be determined experimentally for each bacterial strain by plating different dilutions. The number of colony-forming units (CFU) can differ between strains under different growth conditions. For *B. cenocepacia* K56-2 the culture density can sometimes reach an OD_{600} of 8 with almost all bacteria in the culture viable (contributing to CFU), whereas with other strains bacteria may start to die at this high density resulting in a discrepancy in the relationship between the OD and the number of CFU. For injections in other sites, such as the otic vesicle, higher bacterial inoculum will be required, since pressure and time of injection have to be reduced (s*ee* **Note 27**).

22. For several *B. cenocepacia* [11] and *Salmonella* strains [16], a low infectious dose (<10 CFU) is sufficient to cause a lethal infection. We generally inject around 50 CFU to determine virulence of isolates or mutants. For other pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* [41, 42], much higher inocula (>1,200 and 1,500 CFU, respectively) are needed to induce virulent infections. When initiating experiments with new bacterial species, we suggest finding the optimal inoculum, as well as different bacterial growth stages, as differences in virulence outcome may be apparent after infection of log-phase compared to stationary-phase-grown bacteria.
23. Although we do not have this problem with Bcc, some bacterial species tend to form aggregates in culture. This could result in rapid clogging of the injection needle. In this case, pass the bacteria through a gauge needle several times to break up the aggregates.
24. Switch on the microinjector with the injection cable unplugged several minutes before injection to allow it to reach the correct pressure.
25. One could transfer the embryos prior to injection to a Petri dish containing E3 with 1 % tricaine, and subsequently position the embryos on the agarose plate for microinjection. However, we transfer embryos directly from E3 onto the agarose plate containing 5 mL of 2 % tricaine in E3 using a Pasteur pipette. When doing this try to avoid adding too large a volume of E3 with the embryos. The embryos will "sink" to the bottom of the tip by holding it briefly in a vertical position, allowing positioning the embryos very rapidly in a small volume. We have found that incubation of embryos in tricaine (without injection) reduces expression of some innate immune response genes (IL-8 and IL-1b) during the first hour after incubation compared to non-treated control embryos (unpublished). Therefore it is essential when performing qRT-PCR to always treat PBS control embryos in an identical manner, including the time of incubation in tricaine. As soon as embryos start to twitch when touched with the injection needle, you should replace the tricaine solution. A 20× solution can be stored at 4 °C for a maximum of 2 weeks. Do not reuse or store 2× dilutions, as the activity will go down.
26. Stage the embryos at 30 hpf: They should have a consistent blood circulation and a straight tail [43]. To analyze virulence potential, we typically inject 60 embryos per bacterial strain. Five embryos are sacrificed at different time points to determine CFU (T = 0) (*see* **Note 30**); 24 and 48 hpi (72, and later if desired), at least 24 embryos are used for mortality

assays (*see* Subheading 3.4), and around 10–20 embryos are kept for intravital observations of the infection. Each experiment should be repeated at least three times. If host gene expression is to be analyzed by qRT-PCR the number of infected embryos should be adjusted as required (*see* Subheading 3.7).

27. There are several options to determine the correct injection volume. Due to the fact that pulled capillaries are used there will be significant variation in the bore of each needle. With the help of a scale bar on a microscope slide or in the ocular, adjust the pressure and or pulse time to obtain the desired diameter of a drop [34]. Alternatively, a test injection can be done into a drop of glycerol on a glass slide, and fluorescent bacteria can be counted using a fluorescence microscope. But through experience, the trained eye will be able to rapidly determine the injection volume after performing a test injection in liquid E3 in a Petri dish. Typically, we use a pressure of 400 hPa and an injection time of 0.6 s. As noted below (*see* **Note 28**) different settings will be required when injecting in different anatomical sites (such as the otic vesicle), normally reducing both time and pressure, thus injecting a smaller volume to avoid rupture of the vesicle. The bacterial density in the inoculum should be adjusted to give the correct infectious dose.
28. In order to reduce problems with clogging of needles and to ensure injection of reproducible numbers of bacteria, apply a low output pressure (<10 hPa) for a continuous flow.
29. The injection site can influence the observed virulence outcome, and this should be taken into account when analyzing virulence potential by analyzing embryo survival only. Neutrophils for instance were shown to need a surface to efficiently sweep up bacteria, including *Bacillus* [44], whereas in the blood circulation macrophages were the major phagocytosing cells. Bacteria that are normally killed by neutrophils may therefore be avirulent when injected subcutaneously in zebrafish embryos, but survive when injected in the blood and phagocytosed primarily by macrophages. This injection site-dependent phagocyte behavior permits the study of the interaction of pathogens with different host cells in more detail, and identification of different host cell-specific mechanisms in virulence. Intravenous injection permits to study macrophage behavior and subsequent immune responses, including recruitment of neutrophils during later stages, and subcutaneous injection in the embryo tail permits to study virulence in the context of neutrophil-dominated phagocytosis. There are other sites for injection, each offering different experimental possibilities (Fig. 2b–d, *see* **Note 19**) [35]. Injection into the yolk sac circulation valley, where bacteria will pass through the heart before entering the circulation, is an attractive

alternative to injection into the blood island to introduce bacteria into the circulation. *M. marinum* can be injected into the yolk sac, and an automated injection system was developed for yolk injection [24]. We do not find the yolk to be a good site of injection to study virulence and inflammatory response for the Bcc, as this may be a site of relative immune privilege in the zebrafish embryo, as described for instance for *S. aureus* [41]. The otic vesicle is a closed cavity that is used to study innate immune cell migration. The hindbrain ventricle is a closed cavity which contains very few macrophages (0 to 2 at 30 hpf). After injection into the hindbrain ventricle it is possible to follow the migration of macrophages and neutrophils to the infection site. Recently, the notochord has been described as another compartment for infection. Macrophages are unable to enter the notochord because they cannot cross the collagen sheath [45].

30. To determine the precise inoculum (T = 0), five embryos are "plated" individually immediately after microinjection for each strain. We found this more accurate than microinjection directly in a drop of LB or PBS on an LB agar plate. Collect embryos at different times during an injection series (for instance when injecting 50 embryos with one needle, take an embryo every other 10 embryos directly for determination of the inoculum). This will allow showing whether the inoculum is constant throughout the injection series. Disrupt the embryo immediately after injection, as non-virulent bacteria may be phagocytosed and killed rapidly, and this would result in an underestimation of the inoculum size.
31. Prior to disrupting each embryo in trypsin/Triton, transfer the embryo to a Petri dish with 10 mL fresh E3. Taking embryos directly from the injection plate and transferring to an Eppendorf tube carries the risk of carrying over bacteria present on the injection plate.
32. For some Bcc strains longer incubations are needed. Bacteria can display different morphotypes. Sometimes ghostlike or egg-like colonies can be observed at different frequencies after passing through the animals. These are Bcc, and should be included in the CFU counts (and could be verified after longer growth with a fluorescence microscope for the presence of the reporter plasmid). Such morphotype variations may give interesting information about the bacterial strains after interaction with host cells.
33. If bacterial numbers greater than 250 are expected (inoculum, or during infection by increasing bacterial numbers) bacteria are plated in serial dilutions, as described in Subheading 3.5.

34. Infection experiments using zebrafish embryos are not considered animal experimentation until they reach the free feeding stage. Experiments should therefore be terminated before this stage, unless the experiment requires longer infection times and authorization has been obtained (*see* **Note 2**).
35. For determination of bacterial multiplication rates, CFU in five individually treated embryos per time point per strain should be analyzed. Pooling of the embryos before plating will not allow statistical analysis of variation in virulence. We generally determine CFUs for Bcc at 24 and 48 hpi. For each additional time point, for example to analyze the onset of bacterial replication in more detail (*B. cenocepacia* K56 is taken up by macrophages and survives and starts to replicate intracellularly around 6 to hpi [11]) five extra embryos are required. Large differences in bacterial counts can be expected between different Bcc strains. For Bcc, it is not useful to determine intracellular vs. extracellular bacteria, as for instance done for *Salmonella* [16] by using gentamicin or another antibiotic treatment, as Bcc are resistant to most antibiotics.
36. Bcc are generally resistant to prolonged treatment with trypsin and Triton-X-100 at the indicated concentration. We advise however to check whether bacterial survival of your strain or mutant is affected by this treatment by incubating bacterial dilutions from an overnight culture in E3 with and without trypsin/Triton for 10, 20, and 30 min, prior to plating on LB agar plates.
37. Depending on the virulence of the strains, the bacteria can multiply to high numbers during infection, and it may be necessary to dilute the samples for counting CFU. Whether a strain is multiplying and to which extent can be visualized by fluorescence microscopy. For K56-2 at 24 hpi 10 μl drops of 10^{-1}, 10^{-2}, and 10^{-3} dilutions and at 48 hpi 10 μl drops of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of infected embryos are typically plated (*see* Fig. 3). For *B. stabilis*-infected embryos 10^{-1} dilutions and the undiluted remaining 90 μl are plated.
38. It is important to know the growth rate of the bacterial strain on LB agar. When incubated too long, the colonies will start overgrowing each other very rapidly. The small colonies should be counted under a stereomicroscope when visible and not yet touching each other. For some bacterial species it may be required to incubate the plates at 30 °C overnight (such as *Salmonella*).
39. It is important to count each (countable) dilution for each embryo; this allows seeing whether dilutions were performed

correctly. Spots with more than 150 colonies should not be considered due to the risk of overlapping colonies and underestimating CFUs. Use the dilution with a value of between 20 and 150 CFU to calculate the total CFU per embryo. Do not forget to multiply by 10 because only 10 μL from the 100 μL embryo lysate was used to prepare the dilutions.

40. For statistical analysis we use the GraphPad Prism software, which we find very useful to represent both bacterial multiplication data and Kaplan-Meier survival assays, including statistical analysis.
41. To study the global host immune response at the transcriptional level during infection, qRT-PCR (quantitative reverse transcription-PCR) and RNA-seq experiments can be performed. Extreme care should be taken in handling and manipulating the embryos because any wounding during embryo development can induce innate immune responses, and will add to experimental variation. Inject each embryo only once and very carefully. We found that the anesthetic has an immunosuppressive effect on embryos and we can see this effect on cytokine gene expression at least up to 1 h after injection. Therefore, do not leave embryos too long in the anesthetic solution, and treat all controls (PBS injection) in exactly the same manner. Try to inject the different pools without too much time difference, since also the development of the embryos during this stage is very fast. We use a pool of at least 10 embryos (up to 25 works well) per condition for each time point. For qRT-PCR or RNA-seq analysis, a PBS-injected control group is essential. For Bcc, we generally analyze embryos 3–4, 7–8, and 24 hpi.
42. It is important to work in RNase- and DNA-free environment. It is possible to treat the bench and objects with RNase inactivator such as RNase Away. After extraction, RNA can be stored for a long time at −80 °C.
43. For ten embryos we obtain around 200–300 ng/μl of RNA. Especially for RNA-seq experiments in which highly pure and non-degraded RNA is required it is essential to analyze the quality of the RNA, for instance with a Bioanalyzer (Agilent).
44. Before the experiment, determine the number of genes you wish to analyze. Always include a housekeeping gene. It is imperative that the expression level (CP value) of the housekeeping gene does not vary from one condition to the other (infected, PBS, etc.). For zebrafish qRT-PCR, several housekeeping genes such as *PPIAL* or *EF1α* genes have been used. We have good results with *PPIAL*. Results with *EF1α* were less reproducible during Bcc infection.

45. It is important to use exactly the same quantity of RNA in all samples; otherwise the different levels of gene expression cannot be compared. cDNA can be conserved for several months at −20 °C.
46. A volume of 2.5 μl cDNA is used per reaction, and each condition should be set up in triplicate. Be careful, diluted cDNA is less stable and it is advised to store only one night at 4 °C.
47. We use a Light Cycler 480 from Roche for qPCR analysis, with the SYBR Green kit from Roche. Using another light cycler, other kits may be optimal.
48. The Tm of the primers must be around 60 °C and the PCR product should be between 100 and 150 nucleotides (nt) in order to reach maximum efficiency during the PCR reaction. The size of the primers should be between 20 and 25 nt. The Tm for oligonucleotides can be calculated as follows: $[(A+T)\times 2+(G+C)\times 4]\times[1+(N-20)/20]=Tm$ in °C, where N = number of nucleotides. This formula works for an oligonucleotide with more than 20 nt. Programs can be used to calculate the Tm.
49. Avoid bubbles; if there are bubbles in the wells perform a short centrifugation step: 2 min at 700 g.
50. Before starting the analysis, check whether the housekeeping gene gives reproducible results under the different conditions and whether all replicates have a reproducible CP (small variations of ~0.5 cycle are allowed). Then, perform a melting curve analysis; there must be only one peak per gene for all reactions. If there are several peaks this indicates that there are several PCR products; either the sample is not pure or the primers are not specific. Next, analyze the data with the second derivative or ΔΔCt method [46]. As a (simplified) example: PBS, T = 3 hpi (=control sample), strain K56-2, T = 3 hpi (=target sample). Reference gene = PPIAL, and target gene = IL-8.

    For each sample (control and target), calculate 1st ΔCT: c = b−a, where

    a = average of replicates of reference gene of sample A (e.g., PBS 3hpi/PPIAL).

    b = average of replicates of target gene of sample A (e.g., PBS 3 hpi/IL-8).

    The c-value of the control sample (in our case PBS, 3 hpi) becomes the second ΔCT = C.

    The normalized value for target gene expression is then $2^{-\Delta\Delta CT}$ where ΔΔCT = c − C.

    In the example (Table 2) below the IL-8 expression is enhanced 3.58 times during K56-2 infection at 3 hpi.

Table 2
Example of qRT PCR comparing target and reference gene expression under one experimental condition (K56-2 infection at 3 hpi)

	CP n°1	CP n°2	CP n°3	AVG reference gene	
PBS 3 hpi	21.31	21.36	21.37	21.35	
K56-2 3 hpi	21.19	21.23	21.20	21.21	
	CP n°1	**CP n°2**	**CP n°3**	**AVG target gene**	
PBS 3 hpi	24.61	24.67	24.65	24.64333333	
K56-2 3 hpi	22.69	22.6	22.7	22.66333333	
	1st Δct	2nd Δct	ΔΔct	$2^{-\Delta\Delta CT}$	Normalized values
PBS 3 hpi	3.30	3.30	0.00	1	1
K56-2 3 hpi	1.46	3.30	−1.84	3.580100284	3.580100284

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Chapter 4

Studying Host-Pathogen Interaction Events in Living Mice Visualized in Real Time Using Biophotonic Imaging

Gary Splitter, Jerome Harms, Erik Petersen, Diogo Magnani, Marina Durward, Gireesh Rajashekara, and Girish Radhakrishnan

Abstract

Despite progress in mouse models of bacterial pathogens, studies are often limited by evaluating infections in an individual organ or tissue or at a given time. Here we present a technique to engineer the pathogen, e.g., *Brucella melitensis,* with a bioluminescent marker permitting analysis of living bacteria in real time during the infectious process from acute to chronic infection. Using this bioluminescent approach, tissue preference, differences between virulent and mutant bacteria, as well as the response of the bacteria to host metabolites can provide extraordinary data enhancing our understanding of host-pathogen interactions.

Key words *Brucella*, *Brucella melitensis*, Biophotonic imaging, Lux operon, Mice, Host-pathogen, Animal models

1 Introduction

1.1 Visualizing a Bioluminescent Pathogen

Identifying how a pathogen interacts, persists, or is cleared from an animal is often viewed as a single time point event. However, introducing a bioluminescent operon in the pathogen and then visualizing the bioluminescent pathogen in living mice using a highly sensitive charge-coupled device camera permit following infection in real time. The bacterial operon encodes the necessary enzymes required for substrate biosynthesis resulting in bioluminescence not visible to the human eye. However, an ultra-sensitive CCD camera can image the bioluminescence even within an animal.

Brucellosis caused by *Brucella melitensis* has a high incidence in developing countries, and the World Health Organization considers brucellosis as one of the seven neglected zoonoses, a group of diseases that contribute to the perpetuation of poverty [1]. Despite progress in mouse models of brucellosis, much remains unknown regarding dissemination and tissue localization of the zoonotic intracellular pathogen *Brucella melitensis.* Visualizing infectious bacteria in real time in a living animal provides the

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opportunity to address questions regarding host-pathogen interactions not possible with classical experimental approaches. Here, we report the application of bioluminescent *B. melitensis* to study host-pathogen interaction events in living mice in real time using biophotonic imaging.

Questions that biophotonic imaging can address are the following: (1) What is the dissemination pattern of virulent brucellae and how does the dissemination pattern differ with non-virulent or mutant bacteria? (2) Where do brucellae remain in a chronically infected animal? (3) What is the level of persistence, duration, and location of live attenuated *Brucella* vaccines in an animal? (4) Why greater than 5 % of infected individuals treated with antibiotics relapse with infection? (5) Can antibiotic treatment be monitored to distinguish recovery or relapse? Addressing these questions using bioluminescent *Brucella* and the mouse model will provide insight into mechanisms of *Brucella* pathogenesis and improve our understanding of vaccine development against this serious intracellular pathogen.

1.2 Acute Virulent Brucella Infection in a Mouse Model

Brucella infection of humans and most domestic animals occurs via ingestion and inhalation. Brucellosis of domesticated animals (cows, goats, and pigs with *B. abortus, B. melitensis,* and *B. suis,* respectively) is also characterized by a chronic infection resulting in orchitis in infected males and spontaneous abortions in infected females [2]. However, except for these changes in reproductive performance, few clinical signs are reported. In contrast, acute human brucellosis is characterized by infection with multiple presentations including undulating fever, malaise, sweats, arthralgia, lower back pain, splenomegaly, osteoarticular involvement, cervical lymphadenitis, hepatomegaly, genitourinary involvement, and cholecystitis [3, 4]. These acute clinical symptoms in humans are reported when seeking medical assistance.

Engineering bioluminescent virulent *B. melitensis* permits the evaluation of acute brucellosis in a mouse model. Bioluminescent virulent *B. melitensis* during the acute phase of infection in the mouse localizes to sites similar to those reported in humans (Fig. 1). Findings of bioluminescent bacteria in the spleen, liver, and osteoarticular regions are particularly prominent. In addition, early localization of bacteria in the salivary gland of the oral cavity as well as the testes in male mice is observed (Fig. 2). Testicular infection of domestic animals and humans is often reported [5–7]. Since oral ingestion is a frequent route of exposure for domestic animals and humans, the presence of bioluminescent *Brucella* in the oral cavity (Fig. 3), of the mouse following intranasal infection, oral infection, or even intraperitoneal injection is an interesting observation. Localization of brucellae to the oral region perhaps due to specific host factor(s) may signal entry to a newly infected animal and trigger bacterial gene expression important for early survival in the new host.

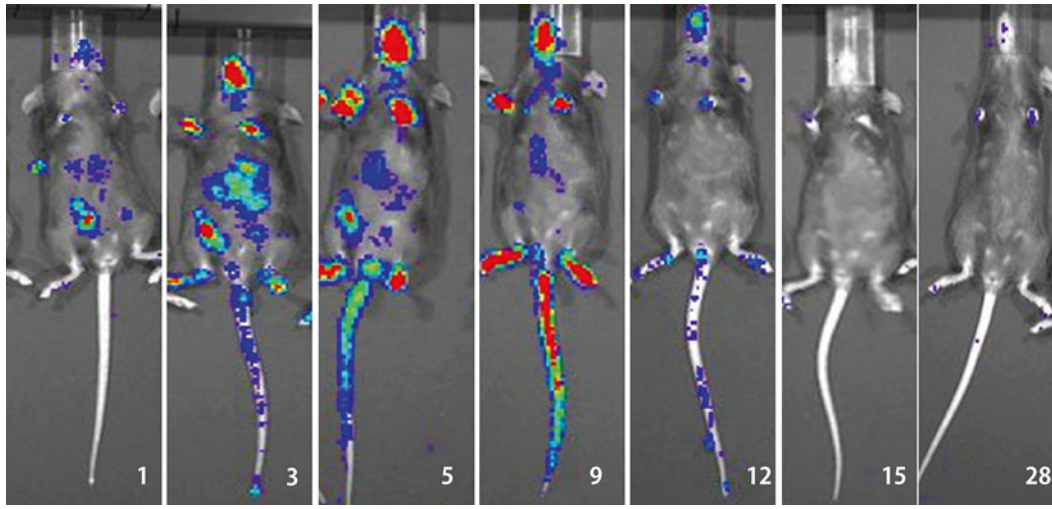

Fig. 1 Infection of C57BL/6 mice with bioluminescent virulent *B. melitensis* strain GR023. Mice were infected with *B. melitensis* strain GR023 (1×10^7 CFU) in the lower peritoneal cavity and infection was monitored for 28 days. A large number of bacteria are present in the oral cavity at days 3–14 after infection. *Number in the lower right* of each panel is the day of post-infection. The rainbow scale represents approximate photon counts

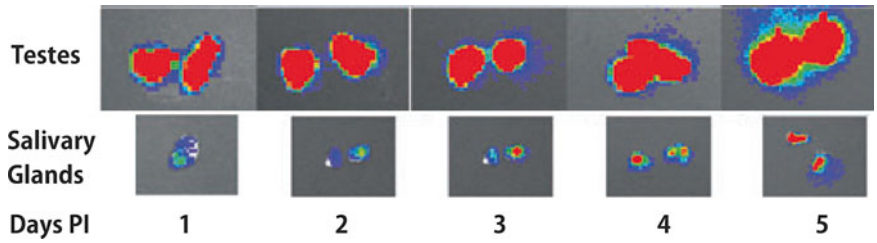

Fig. 2 Ex vivo imaging of testes and salivary glands from IRF-$1^{-/-}$ mice at days 1, 2, 3, 4, and 5 following infection with *B. melitensis* GR023 (1×10^7 CFU) (figure in part from Fig. 4a of [10])

1.3 Chronic Virulent Brucella Infection in a Mouse Model

If humans remain untreated, chronic brucellosis is a frequent outcome [8]. Chronic brucellosis in man has serious protean clinical manifestations, such as orchitis, osteoarthritis, spondylitis, endocarditis, and several neurological disorders [3, 4]. Again, the mouse model infected with bioluminescent *B. melitensis* provides a picture of chronic brucellosis with osteoarthritis, and the variable nature of *Brucella* among mice (Fig. 4). For example, infection of the heart (Fig. 5), and frequently infection of the urogenital clitoral gland in female mice has been observed [9], whereas bioluminescence of the liver and spleen is not observed in chronic murine brucellosis which often correlates with the absence of detectable bacteria from these organs.

1.4 Mutant Brucella Infections in a Mouse Model

Virulence factors of *Brucella* were identified while screening a transposon library for loss of virulence and reduced lethality using immune-deficient IRF-$1^{-/-}$ mice [10, 11]. Further, differences in

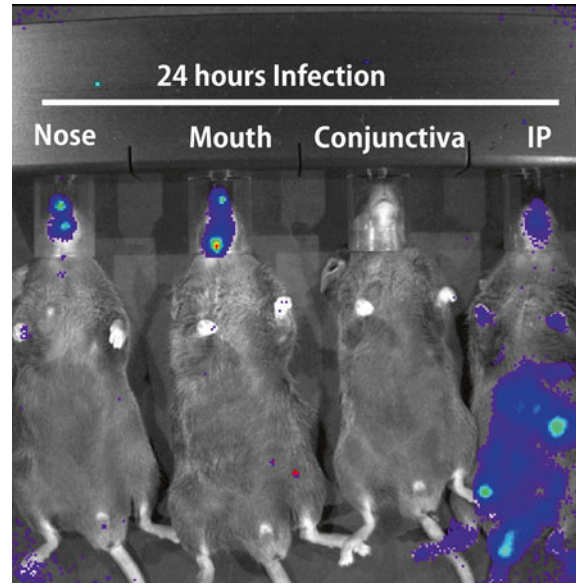

Fig. 3 Infection at 24 h of IRF-1$^{-/-}$ mice with *B. melitensis* GR023 (1×10^7 CFU) given in the nose, mouth, conjunctiva, or intraperitoneal cavity. The rainbow scale represents approximate photon counts

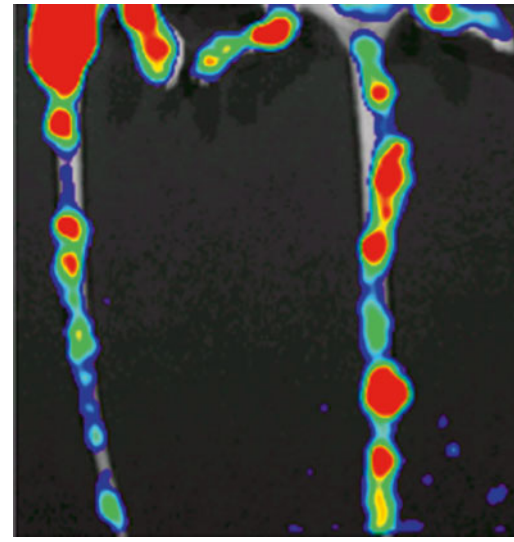

Fig. 4 Infection of BALB/c mice with bioluminescent virulent *B. melitensis* strain GR023. Mice were infected with *B. melitensis* strain GR023 (1×10^6 CFU) in the lower peritoneal cavity and infection monitored on day 14. Notice the individual segments of coccygeal vertebrae containing bioluminescent bacteria as well as the bacteria in joints of the feet. The rainbow scale represents approximate photon counts

Fig. 5 Infection of a BALB/c mouse with bioluminescent virulent *B. melitensis* strain GR023. Mouse was infected with *B. melitensis* strain GR023 (1×10^7 CFU) in the lower peritoneal cavity and infection monitored on day 270. A large focal infection was present in the region of the heart. The rainbow scale represents approximate photon counts

bacterial growth and dissemination among mutant strains were observed in mice. VirB is a type 4 secretion system required for brucellae survival within macrophages as well as to establish and maintain persistent infection in mice [12–16]. However, a mutant lacking *virB* expression can still disseminate and persist in the host for days prior to immune elimination as observed by biophotonic imaging of a *B. melitensis virB* mutant [10]. More recently this approach of examining bioluminescent *Brucella* in vivo has been used to study the role of a TIR domain-containing protein, TcpB (also termed Btp1), of *B. melitensis* during infection in mice [17]. This protein targets the Toll-like receptor adaptor protein TIRAP also known as MAL (MyD88-adapter-like) to inhibit TLR2 and TLR4 activation. Furthermore, using biophotonic imaging, TcpB is required for the initial growth and spread of *B. melitensis* in vivo [17]. Bioluminescent *B. melitensis* containing directed gene deletions now permits identifying in real time the influence of particular bacterial genes on dissemination, persistence, and localization among mutants compared to wild-type virulent *B. melitensis*.

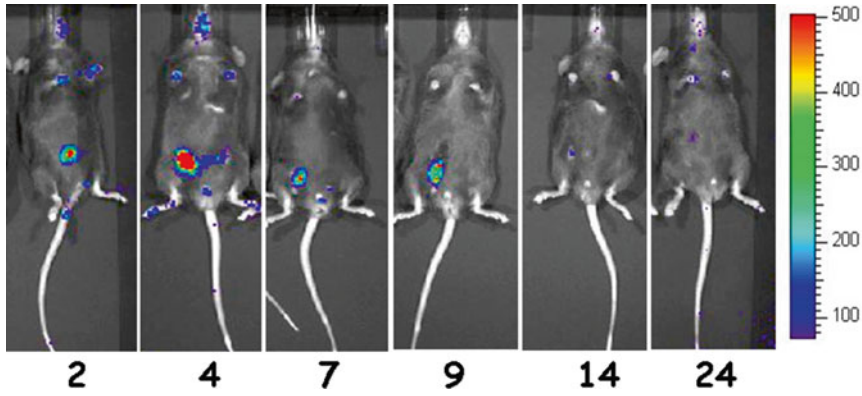

Fig. 6 IRF-1$^{-/-}$ mice vaccinated and challenged. IRF-1$^{-/-}$ mice are highly susceptible and die by 14 days with virulent *B. melitensis* (1×10^6 CFU) infection. Mice were immunized with an attenuated non-luminescent *virB* mutant strain of *B. melitensis* followed 6 weeks later by challenge with bioluminescent virulent *B. melitensis* strain GR023. Note minimal dissemination of virulent bacteria following challenge. Days of infection after challenge are under each image. The rainbow scale represents approximate photon counts

Evaluation of bioluminescent brucellae has the following advantages. First, in vivo infection can be monitored in the entire animal providing a more complete picture of dissemination, persistence, and localization than determined strictly from colony-forming units of bacteria from the spleen or the liver as typically performed in mutant bacterial studies. In non-bioluminescent studies, kinetic changes cannot be followed in the same mouse which is important in infections with inconsistent clinical symptoms and localization. Second, bioluminescent bacteria coupled with biophotonic imaging can reduce the number of mice required for a study because an individual mouse can be followed for the duration of infection, rather than using a separate mouse for each time point. Third, monitoring infection in the whole body of individual mice provides the opportunity to identify the protean nature of *Brucella*, i.e., osteoarticular, cardiac, or testicular involvement or involvement of many organs. Fourth, using bioluminescent bacteria permits monitoring dissemination of bacteria from one animal to naïve animals, an important consideration for vaccine development and understanding mechanisms of animal-to-animal transmission.

1.5 Evaluation of Vaccine Candidates

Certain bioluminescent mutant *Brucella* strains, e.g., *virB4*, *galE*, and *BMEI1090-BMEI1091*, are attenuated in mice permitting evaluation of their potential as vaccine candidates [11]. Further, constructions of bioluminescent current vaccines, i.e., *B. melitensis* Rev.1, and *B. abortus* RB51 and strain 19, would provide useful information regarding how current vaccines persist and disseminate in the mouse as benchmarks for alternative vaccine development. Bioluminescent *Brucella* mutants can be monitored in real time for limited in vivo dissemination and faster clearance compared to bioluminescent virulent *B. melitensis*. Notice in Fig. 6 that prior

vaccination of highly sensitive IRF-1$^{-/-}$ mice with an attenuated *B. melitensis virB* mutant provided sufficient immunity to limit dissemination of the bioluminescent virulent GR023 strain and ensure survival of these mice that usually die from virulent *Brucella* infection. Certain mutants, for example, *virB4*, are attenuated in macrophage cultures, while other mutants, *galE* or *BMEI1090-BMEI1091*, are not attenuated in macrophages but highly limited in dissemination in animals that is readily distinguished with biophotonic imaging [11]. These findings provide the opportunity to explore host-pathogen interaction of different mutants in real time in vivo. Further, in vaccine development bioluminescent attenuated candidates can be evaluated for the requirement of vaccine dissemination and persistence to engender differing levels of immunity, a question that has been poorly addressed in *Brucella* vaccine development. Specifically, killed *Brucella* vaccines that are rapidly cleared fail to provide protection, while *Brucella* vaccines that are more virulent and persist in the host appear to provide higher levels of protection.

The optimal design for a safe human brucellosis vaccine requires a nonliving vaccine; nonetheless killed bacterial preparations generally do not confer sufficient protection [18, 19]. We have created a novel vaccine vector, a replication incompetent but metabolically active *Brucella* [20] that possesses *de novo* protein synthesis required for the bioluminescence activity (Fig. 7). Notice in Fig. 7 that *Brucella* irradiated with lower doses and possessing metabolic activity (IR350K, met+) have increased dispersion and persistence in mice compared to mice receiving bacteria treated with higher irradiation doses (IR1000K, met–) and possessing minimal metabolic activity. Therefore, bioluminescent monitoring provides a unique and rapid readout of the activity, dispersal, and persistence of non-replicating vectors, a clear advantage for alternative vaccine approaches.

1.6 Role of the Unique Host Sugar, Erythritol, and Brucella Infection

Erythritol is a four-carbon sugar preferentially utilized by a number of *Brucella* spp., and this unique sugar is produced in the reproductive tract by several domestic ruminant animals [21]. A key site of this *Brucella*-host interface is the infected placenta, and the presence of erythritol especially in the third trimester of pregnancy has been used to explain the localization of *Brucella* as high as 10^{13} bacteria/g of tissue [22] to the placenta leading to abortion [23]. *B. melitensis* will also localize to an artificial site of erythritol within a mouse, providing a potential model system to study the role of erythritol in *Brucella* pathogenesis.

Previously, the presence of erythritol was suggested as the primary cause for localization of *Brucella* spp. in the placenta [24, 25]. To determine if this effect could be replicated in mice with *B. melitensis*, a localized site of erythritol imbedded in a Matrigel plug was introduced into mice. Using bioluminescent virulent *B. melitensis* strain GR023, *B. melitensis* preferentially localized to the site

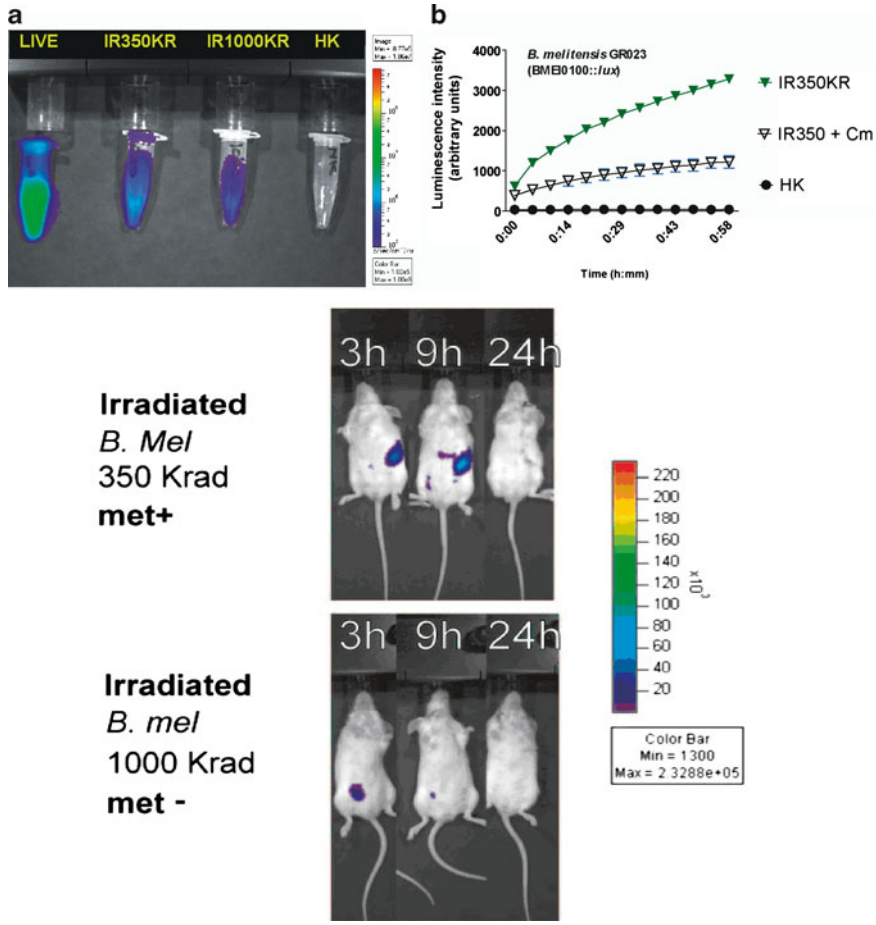

Fig. 7 Non-replicating, irradiated *B. melitensis* localization and persistence in vivo. Mice were immunized with irradiated (350Krad met+; 1000Krad met–) *Brucella* (1×10^9 bacteria/mouse). Infection was monitored by biophotonic imaging at indicated times post-infection. met+, metabolically active; met–, metabolically inactive (figure in part from Fig. 2 of [20])

of erythritol and not glucose (Fig. 8). Whether this is due to a preferentially active process of the bacterium (i.e., a chemotactic system to erythritol), placental neovascularization or an increased level of replication upon reaching the site of erythritol is currently unknown. However, *B. melitensis* growth in vitro is enhanced in erythritol compared to glucose.

Here we describe a protocol to perform biophotonic imaging of brucellae in living animals as a model. This protocol is useful to study bacterial dissemination in acute and chronic infection, virulence, vaccine candidates, and host factors that can influence bacterial localization.

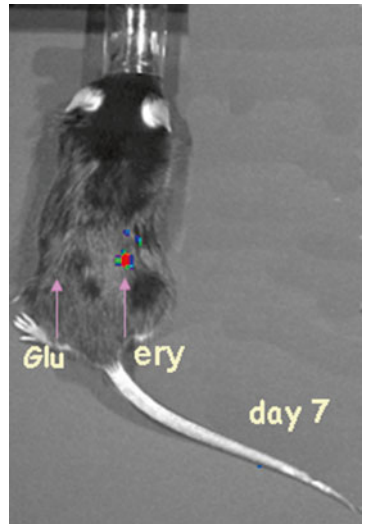

Fig. 8 Preference of bioluminescent virulent *B. melitensis* strain GR023 to localize in Matrigel containing erythritol and not glucose in an IRF-1$^{-/-}$ mouse. A 10 % solution of glucose or erythritol was mixed with the high-concentration Matrigel substrate. Glucose gel (0.5 mL) was injected into the lower left back and the erythritol gel (0.5 mL) was injected into the lower right back of an IRF-1$^{-/-}$ mouse. The mouse was then infected i.p. with 1×10^6 CFU of the virulent, bioluminescent *B. melitensis* strain GR023 in PBS. The mouse was imaged 7 days after infection. Note that biophotonic imaging of the dorsal view revealed bacteria in the gels but not in the deeper organs which are evident when imaging the ventral view

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Store all solutions, buffers, and media at room temperature unless indicated.

2.1 *Brucella melitensis*

1. Wild-type *B. melitensis* strain 16 M (ATCC 23456) (*see* **Note 1**).
2. Mutant strains containing the *lux* operon (*see* **Note 2**) are derived and identified as VirB, GalE, and BMEI1090-91 [9–11].
3. A constitutive bioluminescent *B. melitensis* (GR023) contains the insertion of the Lux operon in *BMEI0101* disrupting this gene whose function is unknown [10] (*see* **Note 3**). Insertion of the Lux operon into this chromosomal location does not alter virulence of *B. melitensis* in macrophage cultures or in BALB/c, C57BL/6, or IRF$^{-/-}$ mice [9–11] (*see* **Note 4**).
4. Luria Bertani (LB) broth: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl. Add to 800 mL H_2O. Adjust pH to 7.5

with NaOH. Adjust volume to 1 L with dH_2O and sterilize by autoclaving.

5. Brucella broth (Difco): Kanamycin (50 μg/mL), carbenicillin (50 μg/mL).
6. Brucella agar (Difco): Kanamycin (50 μg/mL), carbenicillin (50 μg/mL).
7. Phosphate-buffered saline (PBS).
8. Charge coupled device camera (PerkinElmer, IVIS) (*see* **Note 5**).

2.2 Antibiotics

1. Kanamycin (50 μg/mL final concentration): Dissolve 0.5 g of kanamycin into 10 mL of ddH_2O. Filter through a 0.22 μm filter to sterilize. Aliquot and store at −20 °C. Use at 1:1,000 dilution in LB broth or LB-agar (50 μg/mL).
2. Carbenicillin (effective concentration: 50–100 μg/mL): Dissolve 1 g of carbenicillin (Sigma) into 10 mL of ddH_2O. Filter through a 0.22 μm filter to sterilize. Aliquot and store at −20 °C. Use at 1:1,000 dilution in LB broth or LB-agar.
3. Chloramphenicol (final concentration in media is 15 μg/mL): Dissolve 2 g of chloramphenicol in 200 mL of 100 or 95 % ethanol. Sterilize by filtration through a 0.2 μm filter. Store at room temperature, in a screw-top bottle wrapped in foil.

2.3 Plasmid and Preparation of Transposon

1. Plasmid pXen13 (Caliper Life Sciences) that contains Lux genes *luxCDABE* from *Photorhabdus luminescens* (*see* **Note 1**) was used as the source of marker of bioluminescence for the transposon vector.
2. A promoterless bioluminescent plasmid EZ::TN pMod-3 < R6Kγori/MCS > transposon vector (TSM08KR, Epicentre, http://www.epibio.com/pdftechlit/155pl0611.pdf) containing Tn5 mobile elements, kan^R.
3. Gel purification kit.
4. TAE buffer (50× solution)**:** 2.0 M Tris-HCl and 0.05 M EDTA in dH_2O. Adjust to pH 8.3. Solution is 0.2 μm filtered and dispensed into storage bottles. Dilute to 1× with dH_2O for a solution consisting of 40 mM Tris–acetate, pH 8.3 and 1.0 mM EDTA.
5. Microcentrifuge.

2.4 Equipment for Mouse Euthanasia

1. CO_2 cylinder.
2. Enclosed plexiglass chamber.
3. Gas regulator with flow meter (0–1,000 mL/min).
4. Connection tubing and valves.
5. Method of scavenging spent gas (*see* **Note 9**).

2.5 Equipment, Supplies, and Media for Electroporation of Brucella

1. dH_2O.
2. Genepulser II electroporator (Bio-Rad).
3. Disposable 0.2 cm electroporator cuvettes
4. Pipette tips.
5. Petri dishes.
6. Centrifuge tubes, 10 mL.
7. CO_2 incubator.
8. Super Optimal broth with glucose (per liter): 20 g tryptone, 5 g yeast extract, 8.56 mM NaCl or 10 mM NaCl, 2.5 mM KCl. Add ddH_2O to 1,000 mL. 10 mM $MgCl_2$ or 20 mM $MgSO_4$. 20 mM glucose. Adjust pH to 7.0 with sodium hydroxide and sterilize by filtration (0.22 μm filter).

2.6 Mice Models and Infections

1. IRF-1$^{-/-}$, C57BL/6, and BALB/c mice (Jackson Laboratories) (*see* **Note 6**).
2. 27 gauge x ½″ long needles (Terumo SS05M2713).
3. 1 mL syringes.
4. PBS.

2.7 CCD Imaging

1. An intensified charged coupling device by Perkin Elmer (Caliper model 100) or a similar device is used to image bioluminescent bacteria. The minimal detectable photons are specified as less than 100 photons/s/cm^2/sr for a 5-min exposure with a binning of 10 [26] (*see* **Note 7**).
2. Caliper Living Image Software v3.2 (*see* **Note 8**).
3. Isoflurane anesthetic.
4. Isoflurane vaporizer.
5. Oxygen gas.
6. Oxygen gas regulator with flow meter (0–1,000 mL/min).
7. Induction chamber.
8. Connection tubing and valves.
9. Method of scavenging spent gas (*see* **Note 9**).
10. Enclosed plexiglass gas anesthesia chamber for mice.

2.8 Erythritol Trafficking Experiments

1. Non-immunogenic matrix substrate Matrigel.
2. Glucose 10 % solution: 1 volume of glucose in 9 volumes of sterile dH_2O. Sterilize using a 0.22 μm filter. Store in 200 mL aliquots at 4 °C.
3. Erythritol 10 % solution: 1 volume of erythritol in 9 volumes of sterile dH_2O. Sterilize using a 0.22 μm filter. Store in 200 mL aliquots at 4 °C.

3 Methods

This section comprises information about the construction of bioluminescent *Brucella* strains using electrocompetent bacteria, as well as monitoring the bioluminescent bacterial infection in mice and in ex vivo tissues.

3.1 Construction of Bioluminescent Brucella Strains

1. The promoterless Lux operon (*see* **Note 1**) from pXen13 is inserted into the transposon vector EZ::TN pMod-3 < R6Kγori/MCS> that has 19 bp transposon recognition sequences and is modified to contain a Kan^R marker [10]. This construction produces a 10 kb plasmid, termed pUWGR4.
2. Transposon complexes are stored at −20 °C.
3. *B. melitensis* are made electrocompetent by inoculating brucella broth (10 mL) with a single colony from an agar plate streaked with *B. melitensis,* stored at −80° C. Grow to an OD_{600} 0.5–0.7 at 37 °C with vigorous shaking (200 rpm), and chill on ice for 10 min.
4. Bacteria are harvested by centrifugation (5 min at 5,000 g), washed twice with dH_20, and the cell pellet resuspended in dH_2O to 1/50th of the original volume.
5. For transformation, 50 μl of bacteria are mixed with 4 μl of the transposon complex, in an electroporation cuvette prechilled on ice and electroporated using settings of 2.5 kV, 25 uF, and 200 Ω.
6. Following electroporation, cells are suspended in 950 μl of super optimal broth containing glucose, transferred to a 10 mL tube, and shaken for 7 h.
7. Following electroporation the bacterial suspensions (100 μl) are plated on petri dishes containing brucella agar with kanamycin (50 μg/mL), and the dishes incubated at 37 °C with 5 % CO_2 for 5–7 days.
8. Resulting kan^R colonies are screened for bioluminescence using the CCD camera. The *lux* operon allows isolation of phenotypic mutants that constitutively express strong bioluminescence, Fig. 9 (*see* **Note 10**). Bioluminescent colonies are streak purified.
9. To quantify the amount of bioluminescence, bacterial strains are grown in brucella broth containing kanamycin with aeration at 37 °C with 5 % CO_2 for 60 h. Adjust cell density to $OD_{600} = 1.5$, and transfer 100 μl to a 96-well microtiter plate. Quantify bioluminescence by IVIS by defining the ROI and select the highest bioluminescing strain for mice infection (*see* **Notes 11** and **12**).

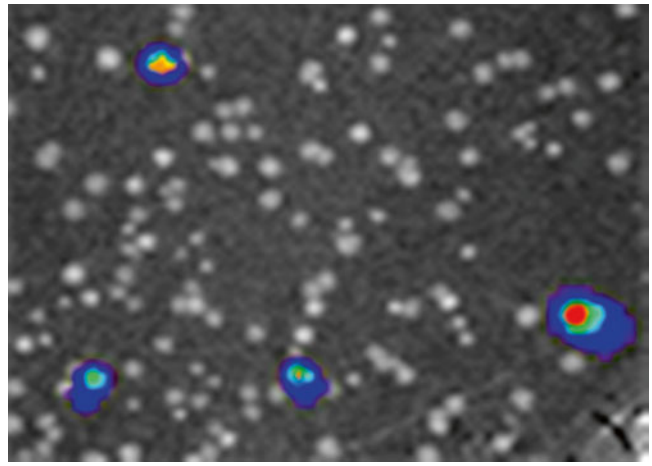

Fig. 9 A heterogenous mixture of bioluminescent and non-bioluminescent *B. melitensis* colonies. *B. melitensis* was electroporated with the EZ:TN transposon containing the promoterless *lux* operon. Depending on the site of insertion in the *Brucella* genome of a given bacterial colony, a *Brucella* promoter activates the promoterless *lux* operon which is detected by biophotonic imaging (figure in part from Fig. 2 of [32])

3.2 Infection of Mice (See Note 13)

1. Bioluminescent *B. melitensis* is grown to logarithmic phase OD_{600} 0.5–0.7 in 10 mL brucella broth.
2. Centrifuge bacteria for 5 min at 5,000 g in a microcentrifuge.
3. Resuspend in PBS to 1–5 x 10^{6-7} CFU of bacteria depending on the experiment [10] (*see* **Note 14**).
4. Inject 200 μl bacterial suspension into the peritoneal cavity using a 27 gauge needle and 1 cc syringe (*see* **Notes 15** and **16**).

3.3 Bioluminescent Imaging of Mice to Monitor Infection

Following imaging, mice are kept warm when they are returned to their cage (*see* **Note 9** regarding scavenging gases).

1. At completion of the procedure, turn vaporizer off and allow animal to breathe supplied gas until it awakens.
2. Place animal in recovery area with thermal support until fully recovered.
3. To monitor infection kinetics, mice are typically imaged at 2–3-day intervals until the experiment is terminated (*see* **Note 18**).

3.4 Ex Vivo Imaging of Tissues

1. Animals are euthanized using a 10 % CO_2 chamber (*see* **Note 19**).
2. Tissues of interest are removed and placed in a sterile petri dish.
3. Tissues in the petri dish are imaged with the CCD camera for 5–10 min with a pixel binning of 10.
4. Bioluminescence from each tissue was quantified using Living Image software after defining the ROI.

3.5 Erythritol Trafficking Experiments

1. Thaw Matrigel aliquot on ice at 4 °C. Chill any pipettes, syringes, or containers that will come in contact with Matrigel (*see* **Note 20**).
2. Mix 0.5 mL of chilled Matrigel with 0.5 mL of chilled 10 % glucose or 10 % erythritol and load mixture into chilled syringe containing an 18 gauge needle. Final concentration of glucose or erythritol is ~5 %.
3. Maintain syringe on ice until material is injected (0.5 mL) into mice where the Matrigel will solidify at 37 °C. Avoid air bubbles in the syringe.
4. Inject 0.5 mL of glucose gel into the lower left back of BALB/c mice (*see* Subheading 3.5, **step 2**, and **Note 21**).
5. Inject 0.5 mL erythritol gel into the lower right back of the mouse.
6. Immediately inject the mouse intraperitoneally with 1×10^6 CFU of the virulent, bioluminescent *B. melitensis* strain GR023 in PBS (*see* Subheading 3.2, **step 4**).
7. Image the mice 3 days after infection using the in vivo imaging system and Living Image software (*see* Subheading 3.4, **step 3**).

To determine localization of *B. melitensis* to sites of erythritol in mice biophotonic imaging is performed on mice containing a non-immunogenic matrix substrate Matrigel. A 10 % solution of glucose or erythritol is mixed with the high-concentration Matrigel substrate (*see* **Note 19**).

4 Notes

1. Because *B. melitensis* is a select agent, CDC inspection and approval of laboratories are required, and laboratory personnel must receive federal approval to handle the agent.
2. The Lux operon comprises five genes (*luxCDABE*) involved in the emission of visible light. *luxA* and *luxB* code for the components of luciferase, and *luxCDE* code for a fatty acid reductase complex that produces fatty acids necessary for the luciferase action to generate light [27]. *luxC* codes for the enzyme acyl-reductase, *luxD* codes for acyl-transferase, and *luxE* encodes the protein required for enzyme acyl-protein synthetase. Luciferase produces blue/green light through the oxidation of reduced flavin mononucleotide and a long-chain aldehyde by oxygen. Importantly, since both ATP and oxygen are necessary for the reactions to occur, only living bacteria produce light.
3. This study describes the random insertion of the promoterless lux operon into the *Brucella* genome. Therefore, activation

of the lux operon is driven by the bacterial promoter that lies upstream of the operon. Depending on the nature of the bacterial promoter, the bacterial promoter could be weak or strong, constitutive or inducible. Construction using a promoterless lux operon requires defining the insertion site in the bacterial genome as well as characterizing phenotypic expression.

4. Bacterial physiology should always be checked following the insertion of external genetic material into the bacterial genome and compared to the wild-type organism.
5. There are several companies that sell imaging systems for detecting biophotonic signals. Select an imaging system that is specifically for use with laboratory animals, is highly sensitive to detect signals in deep tissues, has flexibility to focus on particular regions of interest for signal detection, and can correlate biophotonic signal to anatomical tissue location.
6. BALB/c and C57BL/6 mice are the most common mouse strains used to evaluate brucellosis infection. BALB/c mice are considered the most susceptible, while C57BL/6 mice are more resistant to infection and these two mouse strains produce differing levels of IFN-γ [28]. IRF-$1^{-/-}$ mice are immune-compromised mice producing low levels of several cytokines including IFN-γ and IL-12, as well as low CD8 and Th1 CD4 T cells [29]. When infected with various strains of *Brucella* spp., the life-span of IRF-$1^{-/-}$ mice is dependent on the virulence of a given *Brucella* strain [29]. IRF-$1^{-/-}$ mice are a sensitive model of *Brucella* virulence, and attenuated *Brucella* strains can protect these mice against lethal challenge [29, 30] indicating that adaptive immunity is important in clearance of brucellosis. C57BL/6 and BALB/c mice are immune competent; however, C57BL/6 mice are considered more resistant than BALB/c mice to *Brucella* infection [31].
7. Bioluminescence imaging to determine the number of bacteria in a tissue can be as sensitive as colony-forming counts. Bacterial numbers determined by counting colony-forming units and photon counts determined by biophotonic imaging correlated strongly in at least three mouse strains studied, IRF-$1^{-/-}$ ($r = 0.98$), C57BL/6 ($r = 0.91$), and BALB/c mice ($r = 0.99$). Bioluminescence imaging for CFU determination has the advantage of time and labor which requires about 5 min compared to 3–5 days for CFU determination by culture.
8. Regional area of interest (ROI) for quantifying bioluminescence is determined using the Living Image software and computer cursor to define the boundaries of the ROI. The software computes the photons/s/cm^2/sr in the ROI.

9. Since inhalation anesthetics are in gaseous form, there is significant potential for human exposure to these agents. This is particularly true with systems designed for rodents because of both the necessity to use an induction chamber and the standard use of a nosecone instead of intubation. Both of these components have the potential to leak significant amounts of isoflurane into the environment. An active scavenging system uses a house vacuum or a fume hood to draw the waste gas out of the workspace. Alternatively, a passive system using a pass-through charcoal canister filter in the exhaust gas line can be used.
10. Bioluminescent brucellae containing the lux operon constitutively expressing bioluminescence are identified by imaging culture plates containing individual colonies using the CCD camera of the IVIS system.
11. Because the Lux operon is inserted in the *Brucella* genome downstream of constitutive *Brucella* promoters, bioluminescence is continuous. The constructed bioluminescent strains have been maintained for greater than 7 years without loss of the Lux operon or bioluminescence.
12. Alternatively, it may be desirable to select lux-containing bacteria only when bioluminescent following intracellular infection of host cells. This would most likely evaluate bacterial promoters specifically activated following intracellular infection.
13. Animal handling should be performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. The use of laboratory animals in experiments requires the approval of an institutional animal use committee.
14. CFU determination by serial dilutions, plating, and counting of live bacteria are performed to determine the number of bacteria in a given population. Using 96-well plates tenfold dilutions are made on brucella agar. Make serial dilutions of a solution containing an unknown number of bacteria, plate these bacteria, and determine the total number of bacteria in the original solution by counting the number of colonies and comparing them to the dilution factor. Each colony-forming unit represents a bacterium that was present in the diluted sample. The numbers of colony-forming units (CFUs) are divided by the product of the dilution factor and the volume of the plated diluted suspension to determine the number of bacteria per mL that were present in the original solution.
15. The natural route of *Brucella* spp. exposure is via oral ingestion; however, injection of bacteria into the peritoneal cavity permits greater assurance of known numbers of infecting bacteria in experiments.

16. Both ATP and oxygen are required for bioluminescent *Brucella*. Therefore, killed bioluminescent brucellae will not generate a detectable signal, unlike green fluorescent (GFP)-labeled bacteria where the GFP protein can generate a detectable signal for extended periods even following death of the organism. Also, the bacteria require available oxygen.
17. Biophotonic imaging of mice in dorsal or ventral recumbency may not detect bioluminescent bacteria in all body locations and identifying bacteria may depend on the number of bacteria and the depth of bioluminescent bacteria in the animal. Therefore, imaging should be performed to maximize detection of the desired tissue. Detection limits are approximately 100 bacteria near surface locations and 1,000 bacteria in deeper organs.
18. Mice with severe bacterial diseases may succumb when frequently anesthetized with isoflurane. For example, this is observed when severe liver damage is present.
19. The euthanasia chamber is made of plexiglass and is kept clean to minimize odors that might distress animals subsequently euthanized. Compressed gas should be delivered in a predictable and controllable fashion, at a low flow rate. CO_2 first renders the animal anesthetized and then, with adequate exposure time, will result in death by CO_2 narcosis.
20. Matrigel is a non-immunogenic material useful for monitoring host cell and bacterial migration. Various factors can be readily mixed with Matrigel to study their influence on the host or the pathogen. Here, selected sugars are evaluated for their influence on migration of brucellae in the host. Matrigel is liquid at 4 °C but solidifies at 37 °C.
21. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins to include laminin (a major component), collagen IV, heparan sulfate proteoglycans, and entactin/nidogen. Matrigel also contains TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator, and other growth factors that occur naturally in the EHS tumor. It is considered to be non-immunogenic in mice. To avoid premature solidification of Matrigel, it is important to maintain all pipettes, syringes, and containers at 4 °C.

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Chapter 5

Intravital Two-Photon Imaging to Understand Bacterial Infections of the Mammalian Host

Ferdinand X. Choong and Agneta Richter-Dahlfors

Abstract

Intravital two-photon microscopy (2PM) is an advanced fluorescence based imaging technique that allows for a cinematic study of physiological events occurring within tissues of the live animal. Based on this real-time imaging platform, the pathophysiology of bacterial infections can be studied in the most relevant of model systems—the live host. Whereas traditional animal models of host–pathogen interaction studies rely on end stage analysis of dissected tissues, noninvasive intravital imaging allows for real-time monitoring of infection during shorter or extended time frames. Here we describe the use of advanced surgical techniques for initiation of spatially and temporally well-controlled kidney infections in rats, and how the bacterial whereabouts can be studied while at the same time monitoring the host's altered tissue homeostasis based on real-time deep tissue imaging on the 2PM platform. Whereas this chapter focuses on pyelonephritis induced by uropathogenic *Escherichia coli* (UPEC) in rats, the major concepts can easily be translated to numerous infections in a variety of organs.

Key words Intravital, Two-photon microscopy, Uropathgenic *Escherichia coli*, Pyelonephritis, Microinjection

Abbreviations

2PM	Two-photon microscopy
UPEC	Uropathogenic *Escherichia coli*
GFP	Green Fluorescent Protein
CFU	Colony Forming Unit
PO_2	Tissue oxygen tension

1 Introduction

The establishment of the field "cellular microbiology" during the 1990s has been pivotal in our current understanding of how pathogens interact with their hosts during infection. The generated wealth of knowledge, to a large part based on molecular in vitro

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studies, has essentially molded our understanding of infection biology and guided the development of antimicrobial therapies. However, the true state of an infection within the living host is fundamentally complex and changes over time as infection progresses, sometimes reaching deeper tissues or disseminating into the circulation of the animal, causing sepsis. To fully understand the infection process thus calls for non-reductionistic approaches applicable to the live animal.

The organs are highly complex, comprising a number of different tissues acting in concert to achieve the physiological function of the organ. The kidney is represented by a heterogeneous organization of nephrons, blood vessels, and connective tissues, comprising various cell types of epithelial, endothelial, and immune cell origin. This translates to a range of unique microenvironments in close proximity to each other, where infecting bacteria may reside during the different phases of infection. Depending on the tissue's histology, anatomy, and physiology, the infected organ may communicate with proximal and/or distal organs when mounting a proper response to a local infection. Intravital imaging has revealed the nature of several physiological events that are closely associated with UPEC infection of the proximal tubules in the renal cortex, as exemplified by gradual cessation of renal filtration leading to nephron obstruction and glomerular shutdown [1]. Localized ischemia ensues with tissue oxygen tension dropping rapidly, thus establishing an anaerobic bacterial microenvironment already a few hours into the infection. The ischemic response is in part dependent on activation of the clotting cascade in peri-tubular capillaries, and this was shown to serve as a protective innate mechanism. By containing bacteria at the site of infection, spread to deeper tissues and eventually the systemic circulation is avoided during the neutrophil recruitment phase. To maintain their foot hold inside the tissue in spite of the dynamic alterations in tissue histology and homeostasis, it is very likely that bacteria adapt their metabolomic and proteomic profiles throughout the infection process.

The complexity of infection-associated tissue alterations thus drives the need for novel model systems, which takes the relevant host and microbe complexities into account. As studies based on intravital imaging of infection within organs of live animals forms the basis of the emerging area "tissue microbiology," this field has the potential to deepen our understanding of the integrated pathophysiology of infections [2–4].

Fluorescence-based microscopy of host–pathogen interactions in vivo was for long hampered by several factors, a major being the limited depth penetration of light as it is absorbed and/or diffracted by tissues. Moreover, some of the most commonly used routes of infecting an animal (intravenous, intraperitoneal, intragastric, bladder catheterization) results in poor spatial and temporal predictability of the progression of infections. This is exemplified by the ascending model of pyelonephritis: following infusion of

bacteria into the urinary bladder, it is impossible to foresee which population of the millions of nephrons bacteria will colonize at any point in time. The creative use of advanced surgical techniques for time-controlled bacterial delivery has thus been critical in allowing for high-quality visualization of infection processes within precise locations in the organ of a host animal [5–8].

2PM was originally developed by biophysicists [9] and later adapted to the live studies of dynamic processes in organs by biologists [10]. In essence, reduced phototoxicity and deeper tissue penetration of the excitation light is achieved by the use of pulsed beams of photons possessing half the energy required to excite a fluorophore. A detailed review of the technical aspects of 2PM can be found in ref. 11. 2PM has been applied to multiple medical fields to study processes in a wide array of intact tissues. Non-exclusively, these include the study of calcium fluctuations in individual synapses [12, 13], the role of astrocytes in the brain [13], tumor vascularization [14], embryonic development [15], kidney physiology [16], immune cell homing in inflammation [17, 18], and bacterial infections of the kidney [8, 11, 19, 20].

The applicability of 2PM in studies of bacterial infections is in this chapter exemplified by detailing the procedure for real-time intravital imaging of uropathogenic *Escherichia coli* (UPEC) infection of initially one nephron as a model for pyelonephritis. Many considerations of the procedures are highly generic, and our aim is that this chapter may serve as a guideline for details to be considered by scientists planning to initiate an intravital imaging model for a bacterial infection. The following sections cover detailed descriptions of the setup of intravital 2PM imaging of the kidney, surgical procedures for initiation of infection, as well as generation of the fluorescent probes used to visualize important components at the site of infection.

To visualize bacteria by 2PM imaging, bacteria should express a fluorescent protein, such as GFP+. Preferentially, the protein should be encoded from a stable, single insertion of the gene segment on the chromosome rather than a plasmid to avoid the need of antibiotics in the animal during the time-course of infection [8]. The choice of promoter is also relevant. To circumvent altering levels of bacterial fluorescence due to changes in the local tissue environment, a constitutively active promoter can be used, such as the tetracycline promoter PLtetO-1 [8].

To obtain the spatial and temporal precision required for the dynamic 2PM imaging, surgical procedures can be applied when initiating infection. In the pyelonephritis model, access to the left kidney of a Sprague Dawley rat was achieved by gentle, surgical exposure. Using a fine glass capillary needle, bacteria can be slowly infused into the tissue, with care taken to avoid introducing unnecessary damage to the tissue. In the pyelonephritis model, 10^5 CFU of the UPEC strain CFT073 is slowly infused into the lumen of a

single nephron with an infusion rate and pressure corresponding to the native rate of glomerular filtration. A distant site injected with phosphate buffered saline according to the same principle acted as a sham-operated control site within the same kidney of the anesthesized animal.

Within a live host, the local infection may traverse into or induce responses in several tissue compartments. Tissue autofluorescence originating from a specific subset of cells, e.g., proximal tubular cells, can be used as an advantage to help in the tissue orientation. To highlight the vasculature, fluorophore-conjugated dextrans can be systemically applied [6, 7, 16]. Dextrans above the size-exclusion limit (i.e., 50 kDa dextran) cannot be filtered by the glomerulus and thus remain in circulation. In contrast, small molecular weight dextran (10 kDa) injected into the systemic circulation is rapidly filtered, and can accordingly be used to visualize glomerular filtration and determine filtrate flow rate in the tubular segments of the nephron. If bacteria are introduced into compartments exposed to significant shear stress, such as the renal filtrate in the tubules of the nephron, the bulk of infused bacteria will be flushed away, whereas only few remain in the tissue from where the infection is initiated. To aid in identification of the infected nephron, a fluorophore-conjugated small molecular weight dextran (10 kDa) can be co-infused with the bacterial inoculum. Due to endocytic activities of proximal tubule cells, the fluorophore-conjugated dextran will serve to provide a distinct outline of the apical side of the injected epithelium [8].

2 Materials

2.1 Bacterial and Culture Requirements

1. UPEC strain LT004 (cobS::**ϕ(PtetO-1)** *gfp*+Cmr) [8] (*see* **Note 1**).
2. Luria–Bertani (LB) agar and LB broth.
3. 37 °C shaking incubator for liquid culture growth.
4. 37 °C incubator for agar plates.
5. Inoculating loops.
6. 100-mL conical flask or 15-mL Falcon tube.
7. Centrifuge.
8. 1.5-mL Eppendorf tubes.

2.2 Fluorophore Conjugated Dextrans

1. Sterile 0.9 % saline solution.
2. 10,000 MWCO membrane.
3. Surgical syringe.
4. 10 kDa fluorophore-conjugated dextran in 0.9 % sterile saline (20 mg/ml), store wrapped in foil ≤ 1 month at 4 °C.

5. 500 kDa rhodamine-conjugated dextran in 0.9 % sterile saline (8 mg/ml), store wrapped in foil ≤ 1 month at 4 °C. Dialyze probe solution (5–10 ml) before use against 0.9 % (w/v) sterile saline (5 L) overnight at room temperature using a 10,000 MWCO membrane (*see* **Note 2**).

2.3 Intratubular Microinjection Induced Infection

1. Standard Sprague–Dawley (264 ± 16 g) and Munich-Wistar (255 ± 22 g) male rats. Animal experimentation should follow the local ethical and legal national regulations and be performed by trained individuals accredited by the relevant regulatory bodies.
2. Anesthesia induction chamber.
3. Isoflurane–oxygen mixtures (5 % (v/v) and 2 % (v/v)).
4. Halothane–oxygen anesthesia.
5. Pentobarbital (optional).
6. Buprenorphine.
7. 50 mm dish and 40 mm coverslip.
8. Autoclave tape.
9. Appropriate animal temperature control devices (e.g., circulating water blanket attached to a temperature-controlled circulating water bath).
10. ReptiTherm pads.
11. Homeothermic table.
12. Rectal probe for temperature recording.
13. Vascular catheters (PE-60 tubing for rats and PE-50 tubing for mice).
14. Electric clippers.
15. Germicidal soap.
16. Surgical scissors.
17. A pair of tooth forceps.
18. A pair of hemostats.
19. Stereoscopic microscope.
20. Sharpened micropipettes (5–10 μm inner diameter). Pulled using a micropipette puller and sharpened on a wet spinning grindstone at a 20° angle.
21. Leitz micromanipulator.
22. Micropump.
23. Kidney cup.
24. Sterile 0.01 M phosphate buffered saline prewarmed to 37 °C.
25. Heavy mineral oil.
26. Sudan Black-stained castor oil.

27. WillCo-dish coverslip bottom dishes; 50 mm/40 mm coverslip (Electron microscopy sciences).
28. Appropriate fluorescent probes.
29. Imaging Platform.

3 Methods

3.1 Presurgery Preparation and Maintenance of Bacterial Cultures

Prior to the experiment, bacterial cultures are maintained on LB agar plates (*see* **Note 3**).

1. A day prior to the experiment, prepare a fresh overnight culture of bacteria by inoculating one defined colony into 4 ml of LB broth in a 15 ml Falcon tube, incubate overnight shaken at 230 rpm at 37 °C.
2. On the day of the experiment, prepare a fresh culture by pipetting 40 μl of the overnight culture into 4 ml of fresh LB broth in a 15 ml Falcon tube.
3. Cultivate this culture at 230 rpm, 37 °C until the culture density reaches $OD_{600} = 0.6$.
4. Harvest the bacteria by centrifugation at 5,000 g for 5 min.
5. Discard the supernatant and wash the pellet twice with 0.9 % sterile normal saline. Concentrate the culture to an approximate density of 10^9 CFU/ml (*see* **Note 4**).
6. Store this suspension on ice for use within 2 h.

3.2 Surgical Preparation of Animals

1. Apply anesthesia to the animal by placing it in an anesthesia induction chamber infused with 5 % isoflurane–oxygen.
2. After sufficient effect, transfer the animal to a clean heated surgical area (e.g., a homeothermic table).
3. Supply a 2 % isoflurane–oxygen mixture to maintain anesthesia. Titrate when necessary for effect.
4. Perform a subcutaneous injection of 0.05 g/kg buprenorphine.
5. Prepare the areas for incision by shaving the fur using a pair of electric clippers. Areas include left flank area (kidney exposure), neck (jugular vein and artery access), and inner thigh (femoral vein).
6. Disinfect the respective areas with germicidal soap and water.
7. Dry the areas with a paper towel and thoroughly clear away any remaining cut hairs (*see* **Note 5**).
8. Constantly monitor the temperature of the animal by inserting a rectal probe while awaiting the next step of the experiment (*see* **Note 6**).

3.3 Rat Surgery and Bacterial Infusion

1. Prepare the inoculum for microinjection. At least two micropipettes need to be prepared, the first is loaded with the bacteria culture, and a second filled with 0.9 % NaCl which functions as the sham (*see* **Notes 7–9**).
2. Prepare an optional third micropipette by aspirating Sudan-Black castor oil. Castor oil remains in the nephron when injected. When introduced to nephrons neighboring the infection site, this becomes a visual marking for the easy location of the infection site (*see* **Note 10**).
3. Begin preparing the animal for infection by first inserting a venous access line by making a small incision above the femoral vein.
4. Make a small cut in the vessel, insert a PE-60 tube and secure with sutures.
5. Once all preparative steps have been completed, locate the kidney and estimate its size by palpating the left flank of the animal.
6. Grasp the skin of the animal with a pair of tooth forceps.
7. On the intended path of incision, pinch the skin to crush the tissue with a pair of hemostats to prevent bleeding.
8. With a pair of surgical scissors, perform 0.5–1 cm incisions through the tissue. Repeat **steps 6–8** for the outer muscle layer to expose the inner muscle layer.
9. At this point, re-palpate the tissue to locate the kidney.
10. Repeat the **steps 6–8** again to gain access to the peritoneal cavity. Initial incisions should be shorter than the length of the kidney. The incision can be increased subsequently if needed (*see* **Note 11**).
11. Remove the adipose tissues encapsulating the kidney by careful manual searing with a pair of forceps (*see* **Note 12**).
12. Transfer the animal to a clean heated surgical area on the stereoscopic microscope stage.
13. Gently raise the kidney out of the peritoneal cavity by grasping the hilar fat pad with forceps.
14. Place the kidney in a kidney cup and stabilize the setup.
15. Supply normal saline through the femoral access, and add drops over the exposed kidney to maintain hydration. This should be performed throughout all subsequent steps.
16. Switch the anesthesia supplied to the animal from the original isoflurane–oxygen mixture to halothane–oxygen anesthesia. This allows for fine adjustments of anesthesia depth to be made, as well as recovery.
17. Shift the animal onto the stereomicroscope stage.
18. Illuminate the intended site of infection with a mercury levelling bulb. Focus at this site and increase the magnification to 96× (Fig. 1). Adjust the lighting where necessary (*see* **Note 13**).

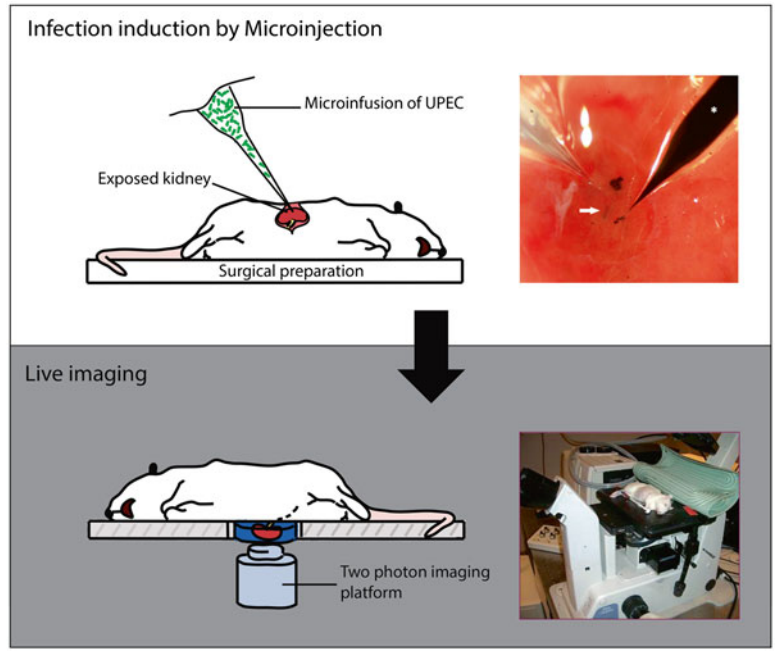

Fig. 1 Schematic representation of intravital infection and imaging. UPEC is injected into a single nephron on the surface of the exposed kidney (*Top panel*). In parallel, a Sudan Black dye (*asterisk*) is injected into a nearby nephron to mark the infection foci (*white arrow*) for subsequent analysis. After injection, the animal is transferred to the 2PM stage for fluorescence microscopic analysis (*Bottom panel*). Adapted from Choong et al. 2012a and Choong et al. 2012b) [2, 22]

19. Focus the field of view slightly about the kidney surface such that the kidney is now blurred.
20. Mount the bacterial suspension-filled micropipette onto the Leitz micromanipulator.
21. Bring the tip of the micropipette into the field of view just above the kidney (*see* **Note 14**).
22. Refocus onto the kidney surface.
23. Position the needle tip above a target nephron. The orientation of the needle should be aligned with the tubular walls. Approximately 90 % of surface-localized tubules are proximal convoluted tubules.
24. Advance the needle slowly into the proximal convoluted tubule until the needle tip breaks the tissue barrier and enters the luminal space. Upon contact with the tissue, the kidney capsule will offer a sizable degree of resistance (*see* **Notes 15** and **16**).
25. Infuse the bacterial suspension at a rate of 50 nl/min for 10 min, giving a total injected amount of approximately 5×10^5 CFU of bacteria.

26. Withdraw the needle from the tissue (*See* **Note 17**).
27. Replace the current micropipette with the Sudan-Black stained Castor oil filled micropipette (*see* **Note 18**).
28. Repeat **steps 16–20** to make two marks on either side of the injection site for subsequent orientation on the 2P microscope stage.
29. When the microinjection has been completed, gently place the kidney back into the peritoneal cavity.
30. At this point, the animal may be used for short (below 8 h) and long term (above 8 h) imaging and/or non-imaging analyses (*see* **Note 19**).
31. For immediate imaging, transfer the animal to the 2PM stage (*see* Subheading 3.4).
32. Alternatively, for imaging at later time points or non-imaging analyses, close up the animal by suturing the retroperitoneum.
33. Single house the animal in the home cage and allow for recovery with sufficient food and water.
34. The kidney can be re-exposed for imaging at a later time.

3.4 Imaging Procedures

Numerous variants of 2PM imaging systems are available, and as each system has its own settings and nuances, the settings on the optics will not be discussed in detail here. Rather the reader is referred to [5, 6] (*see* **Note 20**). The system described in the methods below utilizes an inverted imaging system.

1. Prepare a raised scaffold inside a dish (50 mm dish with a 40 mm coverslip in the bottom) to stabilize the kidney on account for its curved surface. This can be achieved by stacking 4–7 pieces of 2 cm long strips of autoclave tape at the edge of the coverslip. Take care not to block the objective's light path.
2. To maintain the temperature of the animal, place 2 ReptiTherm pads on each side of the dish and a warming jacket blanket over the stage.
3. Position the rat such that the ventral side of the kidney contacts the base of the dish. The curvature of the kidney should be stabilized by the scaffold made in Subheading 3.4, **step 1**. Flood the dish with 0.9 % saline to maintain hydration of the exposed organ.
4. Check for motion in the field of view under the 10× or 20× objective. If significant motion is observed, adjust the rat to further stabilize the kidney (*see* **Note 21**), e.g., positioning thorax of the rat away from the coverslip bottom with the kidney close to the edge. Take notice to avoid hyperextending the vessels. An example of an infected nephron is shown in Fig. 2**.**

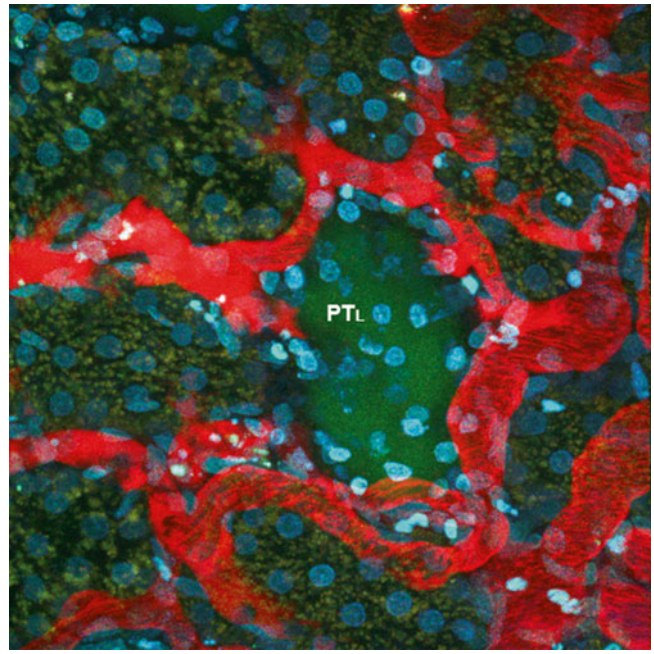

Fig. 2 2PM acquired fluorescence image of an infected kidney. UPEC (*green*) colonizing the proximal tubule are seen here to have completely obstructed the proximal tubular lumen (PT_L). Cell nuclei are labelled with Hoechst 33342 (*blue*). Blood flow is shown by the intravenous injection of fluorescent 500 kDa dextrans (*red*) which are retained in the circulation. Erythrocytes which do not take up conjugated dextrans appear as black streaks in the vasculature. The *dull green spots* represent the inherent autofluorescence of proximal tubular cells. The image is a 3D projection. Licensing information: (unpublished image)

3.5 Post-imaging Procedures

1. Upon completion of imaging and/or of experimentation: sacrifice the animal according to relevant local animal handling directives.
2. Streak 100 μl of blood, collected for example by the heart puncture method, on an LB agar plate and incubate at 37 °C overnight to analyze whether infection remained local or if systemic spread occurred.
3. Further analysis of bacterial dissemination can be performed by plating homogenates from biopsies or from organs, such as the liver and spleen (*See* **Note 22** and **23**).
4. The bacterial population present at the renal infection site can be estimated after isolating the tissue with a 5 mm biopsy punch, and subsequently performing colony counts on the homogenized tissue.

4 Notes

1. The use of a chromosomally located, constitutive active promoter that regulates the reporter gene expression is essential to (1) avoid use of antibiotics in the animal to maintain the plasmid, and (2) ensure constant reporter (GFP+) expression in the microenvironment [20].
2. Most dextran preparations are polydisperse, containing a range of dextran sizes. Dialyzation is essential to ensure that no small molecular weight molecules are present which become filtered into the nephrons.
3. Maintenance on agar plates also allows for easy identification of contamination of the culture.
4. Repeated washing steps are recommended to remove immunogenic culture debris such as lysed bacterial components and LPS.
5. The presence of hairs severely reduces the image quality during the subsequent 2PM imaging. Hairs present as intense cylindrical shadows.
6. Constant monitoring of body temperature is essential as the temperature of the animal can drop drastically during surgical preparation of the animal as well as during imaging.
7. Extreme care has to be taken with the micropipettes. The sharpened tips are highly brittle and break easily. If chipped or broken, they are more likely to cause tissue damage rather than a clean infection.
8. The inoculum may be introduced into the micropipette by either drawing the respective solution from the tip, or via a PE-60 tubing and syringe from the back end of the pipette.
9. Do not allow the presence of air bubbles within the inoculum segment of the micropipette.
10. Crystallized forms of Sudan Black require a day or two to completely dissolve in castor oil. In addition, the solution needs to be filtered before use (Also *see* **Notes 8** and **9**).
11. To avoid motion of the kidney during the 2PM imaging procedure, incisions should be made as small as possible, but can be enlarged if required.
12. Adipose tissue is highly autofluorescent and must accordingly be removed at positions, which would affect imaging. However, it is important to leave a few patches as sites to safely adjust the kidney with forceps.

13. Take caution not to overexpose the kidney to light; lamps may emit heat which can desiccate and damage the tissue.
14. The infection site must be carefully chosen to ensure optimal imaging. Ensure that the site contacts the glass surface of the dish just above the microscope objective in the inverted microscope setup. The kidneys, as most organs, have a good degree of curvature, which will impede the imaging process if infection site is not carefully chosen.
15. Upon encountering resistance from the kidney capsule, advance the needle in short intermittent pauses. The breaking of the capsular layer will be sudden to which the needle may puncture through both walls of the tubule.
16. Hydration of the kidney should be liberal (*see* Subheading 3.3, **step 15**). If dehydrated, the kidney capsule becomes plastic-like and no longer offers a firm resistance to the micropipette tip. Instead, application of the micropipette will result in deep depression of the kidney without penetration of the capsule. In this event, when the capsule is finally penetrated, the micropipette will stab far deeper beyond the intended surface located tubules.
17. If microinjection has punctured or was performed mistakenly into a blood vessel, the withdrawal of the needle will be accompanied by the release of blood on to the kidney surface. Perforation of the blood vessel in this step results in a non-local infection, which contradicts the aim of this model.
18. If more than one set of micromanipulators and micropumps is available, infusion of bacterial inoculum and Sudan-Black castor oil may be performed simultaneously.
19. The measure of tissue oxygen tension is an example of non-imaging analysis which we have performed. The procedure involves first the setting up of a tail cuff or direct arterial line with a pressure transducer to monitor blood pressure throughout the procedure. This is followed by performing a two point calibration of the Clark type electrodes with either $Na_2S_2O_5$ saturated H_2O or in air at 37 °C. After the infections with UPEC and sham have been initiated, insert one microelectrode into each tubular lumen while working under the stereoscopic microscope. Readings can then be collected for the desired duration, to which the data should be presented showing the comparison of the PO_2 at both infected and sham sites along with the blood pressure within the time frame of the experiment.
20. We would instead like to draw attention to the design of the imaging system, more specifically whether the optic system is upright or inverted. This is critical as the animal preparation

differs greatly between the two systems. In the upright system, the exposed kidney is placed in a kidney cup held above the exposed peritoneal cavity. To accommodate the associated fixtures, the incisions are typically larger. Whereas unwanted tissue motion is minimized in the upright system, the tendency for mortality is increased. In the inverted system, which is described below, the rat is positioned on its left side, with the kidney dipped into a saline filled petri dish placed over the objective. The kidney will be inherently stabilized since the weight of the animal is positioned over the organ (Fig. 1). Unwanted movements are thus kept to a minimum.

21. Motion is highly detrimental to live imaging. However, there is a limit to the extent of tissue-stabilizing surgical procedures that can be performed without resulting in mortality. One should also keep in mind that a basal level of motion is unavoidable. This includes motion due to breathing as well as pulsations from the heart and systemic circulation. If problem with motion, one may use 2PM systems with high image capture rates, or apply post imaging software that compensates for image drift. Our system is custom designed with a capture rate of one frame per second for a 512 by 512 pixel image and one frame per 2 ms for a line scan.
22. A key feature of the intravital model described here is the high degree of spatial and temporal precision. We have found this to promote an exceptional reproducibility between experiments in the live setting. Knowing the exact position of the infection site allows for analyses by other techniques to supplement the imaging data (*see* **Note 1**). Alternatively, the dynamic real-time monitoring of the infection site can be combined with endpoint studies since tissue biopsies containing the foci of infection can be accurately obtained. The biopsy can be used for other immunohistochemical analyses, or for transcriptomics analyses (*see* **Note 23**). The precise dissection of the infection site minimizes any dilution from uninfected tissue, thus allowing for total RNA extraction and transcriptomic analysis. Collectively, the multiple data sets that can be obtained and combined with real-time 2PM imaging aid in creating the full picture of the integrated pathophysiology of infection.
23. Accurate isolation of the infected tissue is highly beneficial for RNA extraction and microarray studies. The spatial control of our model allows for the amount of uninfected tissue in the biopsy to be minimized, which greatly increases the possibility of capturing infection-specific molecular details in the transcriptomics assays. The temporal control of our model enables harvesting of biopsies at well-defined time points, resulting in a precise description of the host response during infection [21].

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Part II

Cellular Aspects of Host-Bacteria Interactions

Chapter 6

Cre Reporter Assay for Translocation (CRAfT): A Tool for the Study of Protein Translocation into Host Cells

Amke den Dulk-Ras, Annette C. Vergunst, and Paul J.J. Hooykaas

Abstract

Many pathogenic bacteria introduce virulence proteins, also called effector proteins, into host cells to accomplish infection. Such effector proteins are often translocated into host cells by bacterial type III (T3SS) or type IV secretion systems (T4SS). To better understand the molecular mechanisms underlying virulence, it is essential to identify the effector proteins and determine their functions. Several reporter assays have been established to identify translocated effector proteins and verify T3SS- or T4SS-dependent transport into host cells. Here we describe a protocol to monitor the translocation of candidate effector proteins using Cre recombinase as a reporter, and more specifically how this Cre Reporter Assay for Translocation (CRAfT) can be used to detect translocation of Vir proteins from *Agrobacterium tumefaciens* into yeast and plant cells. The assay can be adapted for the study of the T3SS or T4SS of human pathogens.

Key words Protein translocation, Cre recombinase, CRAfT assay, Type IV secretion system, T4SS, Agrobacterium, Arabidopsis, Yeast

1 Introduction

The plant pathogenic soil bacterium *Agrobacterium tumefaciens* causes tumors on dicotyledonous plants [1]. During the infection process a single-stranded DNA segment (T-strand) copied from the tumor inducing (Ti) plasmid is transported from *Agrobacterium* into plant cells via a VirB/VirD4 type IV secretion system (T4SS), where it is integrated in the genome. Subsequent expression of the genes located on the T-DNA causes the tumorous overgrowth (*see* [2–4] for reviews). Besides the T-strand with VirD2 covalently attached, a number of effector proteins including the Vir proteins VirF, VirE2, VirE3, and VirD5, are independently translocated through the same T4SS channel from the bacterium into the host cell [5–7], where these effectors fulfil functions in favor of the pathogen [8–11]. The identified *A. tumefaciens* effectors have been shown to contain a C-terminal transport signal for

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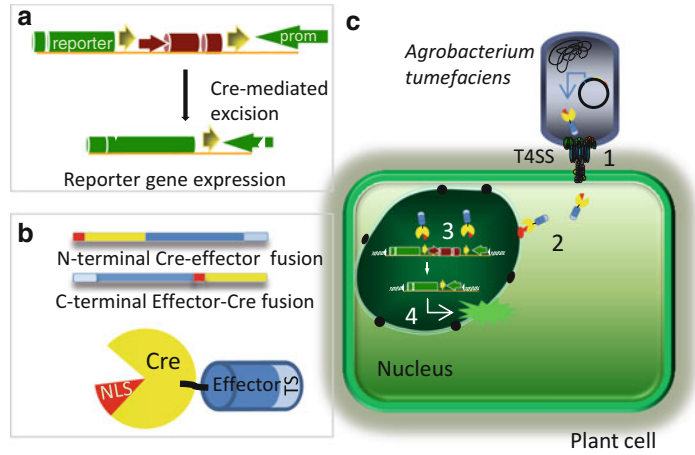

Fig. 1 Schematic representation of the CRAfT assay, indicated for *Agrobacterium-mediated* transformation of plant cells. (**a**) Transgenic plant cells carry the coding region of a reporter gene separated from its promoter sequence (e.g., p35S) by a f*lox*ed DNA sequence, thereby preventing the expression of the reporter gene. This f*lox*ed sequence can be another reporter gene, or a stopper sequence. *Lox-sequences* are indicated by (*yellow*) boxed arrows, and present in a direct repeat orientation, allowing for the excision of the f*lox*ed DNA sequence by a Cre-mediated recombination reaction at the *lox*-sites. Precise Cre-mediated recombination will result in expression of the reporter gene. (**b**) Cre fusions to the NH_2 and COOH-terminal portion of the putative effector protein, expressed in *Agrobacterium*, should be analyzed in translocation experiments (*see* **Note 4**). An SV40 NLS sequence has been included to enhance nuclear uptake of the fusion protein once delivered into the host cell, a prerequisite for Cre-mediated recombination at the target locus. (**c**) Simplified representation of [1] the T4SS-dependent delivery of a Cre-effector fusion protein into plant cells mediated by its translocation signal (TS in **b**), nuclear uptake, enhanced by the NLS sequence [2], Cre-mediated recombination at the target locus [3], resulting in constitutive expression of a reporter gene that can be detected through antibiotic selection (plant line 3043), or microscopical analysis of GFP expression (plant line CB1), *see* **Note 1**. For yeast, a f*lox*ed URA gene allows for the positive selection for loss of the marker by growth of yeast on medium containing fluoroorotic acid (FOA), toxic only in the presence of the gene product

translocation by the T4SS [5, 6], which is most likely recognized by the VirD4 coupling protein that is part of the VirB transport channel [12]. Earlier, we identified that the transport signal of the *Agrobacterium* effector proteins is hydrophilic and has a net positive charge with the consensus motif R-X [7]-R-X-R-X-R-XX(n)>[7].

Protein translocation can be monitored by using the CRAfT assay (*see* Fig. 1), which employs the Cre/*lox* site specific recombination system from bacteriophage P1 [13]. For this, the coding sequence of the *cre* gene is fused translationally to the coding sequence of the (candidate) effector protein and expressed in *Agrobacterium*, generally from a plasmid. Detection of protein

translocation requires transgenic recipient cells that contain a *lox-flanked* (*flox*ed) DNA segment that prevents the expression of a marker gene that will become expressed only after Cre-mediated excision of the f*lox*ed DNA sequence. The marker gene can be an antibiotic resistance gene, allowing for positive selection for recombination events, or for instance a fluorescence marker, allowing for visualization of Cre-recombination events using fluorescence microscopy (*see* **Note 1**). Alternatively, the counter selectable marker *URA3,* flanked by *lox*-sites, can be used in yeast as readout for protein translocation by growth of yeast on medium containing fluoroorotic acid (FOA), which is toxic only in the presence of the gene product [6]. Expression, or absence, of the marker gene after cocultivation of host cells with *Agrobacterium* is thus indicative of effector protein translocation.

Agrobacterium can be used as a donor to show T4SS-dependent translocation of its own natural effector proteins, but also of candidate effector proteins translocated by T4SS of other organisms (*see* **Note 2**). We have successfully used the CRAfT assay to show T4SS-dependent transport of the effector proteins Msi059 and Msi061of the plant symbiont *Mesorhizobium loti* into *Arabidopsis* [14]. Also, the AnkA protein of the human pathogen *Anaplasma phagocytophilum* [15] turned out to contain transport signals that allowed for recruitment to and translocation by the *A. tumefaciens* VirB T4SS. The *M. loti* Msi059 and Msi061 proteins harbor the C-terminal consensus transport motif that has been identified in the *A. tumefaciens* Vir proteins, but this is not apparent in the AnkA protein. Reversely, the Ank200 protein from *Ehrlichia chaffeensis* has a positively charged C-terminus which, however, is not recognized by the T4SS of *Agrobacterium*, and evidence was obtained that secretion takes place by a T1SS [16]. Also, we were unable to detect translocation by the *A. tumefaciens* VirB system of the *Legionella pneumophila* RalF effector protein (our unpublished results), which was shown to require a critical leucine at the -3 position for its transport by the *dot/icm* system [17]. Interestingly, De Jong et al. [18] showed that a C-terminal positively charged transport signal of the *Brucella suis* T4SS effector VceC, with a leucine residue at the -3 position, was recognized by the cognate *Legionella dot/icm* system [18] as measured by translocation of a β-lactamase-VceC reporter fusion protein. However, several other *Brucella* virulence proteins that are translocated in a VirB-dependent manner into host cells have recently been shown to contain an N-terminal Sec-signal, similar to the *Bordetella pertussis* toxin proteins [19, 20], indicating a periplasmic intermediate step. These findings suggest that different mechanisms of translocation may exist between different, and possibly by an individual, type IV secretions system(s).

Although a C-terminal recognition signal has emerged as a common theme in the identified transported substrates of the

T4SS of different bacterial pathogens, additional elements are sometimes present that may be necessary for recruitment to the cognate T4SS. Using the CRAfT assay, it has been shown for instance that effectors of the *Bartonella henselae* VirB T4SS contain a bipartite signal and that, besides a positively charged C-terminus, an additional 142 residue region named the BID domain, is also required [21]. Further, in the *Helicobacter pylori* CagA protein a C-terminal domain is essential but not sufficient for translocation [22]. In contrast to the other *Agrobacterium* effectors also the *Agrobacterium* relaxase VirD2, which mediates translocation of the T-strand through the T4SS has a more complex bipartite translocation signal consisting of a C-terminal domain and an internal part [23].

The CRAfT approach has also been used to detect protein transfer from one bacterium into another [24, 25]. Although for the study of protein translocation into mammalian cells other reporter proteins, including adenylate cyclase (Cya) and β-lactamase, are used frequently, the CRAfT assay can be easily adapted for use in human pathogens, as exemplified by *Bartonella* Bep effector translocation into HUVEC cells [21], and SopE protein translocation by the *Salmonella* T3SS [26] into COS-2 cells.

This chapter describes a detailed protocol to detect translocation of effector proteins from *Agrobacterium* into yeast and plant cells by application of the CRAfT system [5–7].

Due to its general applicability, we included some suggestions that apply to studies of protein translocation from human pathogens.

2 Materials

2.1 Bacterial Strains, Cre Constructs, Transgenic Reporter Hosts

1. *Agrobacterium* strain LBA1100 (*see* **Note 3**).
2. *Cre* fusion plasmids (s*ee* **Note 4,** Fig. 1).
3. *Arabidopsis thaliana* C24 reporter lines 3043 and CB1 [5, 7]. (*see* **Note 1**).
4. *Ura flox*ed yeast host strain LBY2 [6].

2.2 Stock Solutions for Agrobacterium Media

Prepare the stock solutions by dissolving powders in demineralized (Milli-Q) water, autoclave for 20 min at 120 °C and store at room temperature unless indicated otherwise.

1. M-N: 30 g $MgSO_4 \cdot 7H_2O$, 15 g NaCl/L
2. 1% $CaCl_2$: 10 g $CaCl_2 \cdot 2H_2O$/L
3. K buffer pH 4.8: adjust 1.25 M KH_2PO_4 (170.1 g/L KH_2PO_4) to pH 4.8 with 1.25 M K_2HPO_4 (217.8 g/L K_2HPO_4).
4. K buffer pH 7: 205 g K_2HPO_4, 145 g KH_2PO_4.
5. Fe sulfate: 100 mg $FeSO_4 \cdot 7H_2O$/L (*see* **Note 5**).

6. Microelements: 100 mg each of Na_2MoO_4, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and H_3BO_3/L.
7. 20 % NH_4NO_3: 200 g NH_4NO_3/L.
8. 20 % Glucose: 200 g glucose/L. Filter-sterilize (0.22 μm filter).
9. 1 M MES pH 5.5: 195.2 g MES/L pH 5.5 (5 N NaOH). Filter-sterilize (0.22 μm filter), and store at room temperature in the dark.
10. 0.2 M Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone): 39.24 mg/mL in dimethyl sulfoxide (DMSO). Do not sterilize, store at –20 °C. (*see* **Note 6**).
11. Rifampicin: 10 mg/mL in methanol. Do not sterilize, store at 4 °C.
12. Gentamycin: 40 mg/mL. Store at –20 °C.
13. Spectinomycin: 125 mg/mL. Store at –20 °C.

2.3 Agrobacterium Growth

1. LC medium: dissolve 10 g Bacto-Tryptone, 5 g yeast extract, and 8 g NaCl in 1 L Milli-Q H_2O. For solid medium in plates add 18 g agar. Autoclave for 20 min at 120 °C.
2. Minimal medium (MM): 20 mL M-N, 1 mL 1 % $CaCl_2$, 10 mL K buffer pH 7, 2.5 mL 20 % NH_4NO_3, 10 mL Fe sulfate, 5 mL microelements, and 10 mL 20 % Glucose in 1 L Milli-Q H_2O under sterile conditions.
3. Induction medium (IM): 20 mL M-N, 1 mL 1 % $CaCl_2$, 2.5 mL 20 % NH_4NO_3, 10 mL Fe sulfate, 5 mL microelements, 0.8 mL K-buffer (pH 4.8), 40 mL 1 M MES (pH 5.5), 5.7 mL sterile 87 % glycerol, and 10 mL 20 % glucose (for Liquid IM) or 5 mL glucose (for solid IM) (*see* **Note 7**) in 800 mL sterile Milli-Q H_2O (for liquid IM) or in 800 mL autoclaved Milli-Q H_2O containing 16 g/L agar (for cocultivation plates) (*see* **Note 8**). Make up to 1 L with sterile Milli-Q H_2O. Check the pH of the medium, which should be pH 5.3 (*see* **Note 9**).
4. 30 °C incubator.
5. 10 mL tubes, sterile (100 ml flasks, sterile).
6. Orbital shaker, 30 °C.
7. Spectrophotometer.
8. Eppendorf centrifuge.

2.4 Stock Solutions for Plant Media

Prepare the stock solutions by dissolving powders in demineralized (Milli-Q) water, do not sterilize, store at 4 °C unless indicated otherwise.

1. B5 macroelements 1 (10×): 25 g KNO_3, 2.50 g $MgSO_4 \cdot 7H_2O$, 1.50 g $NaH_2PO_4 \cdot H_2O$, 1.34 g $(NH_4)_2SO_4$/L.

2. B5 macroelements 2 (10×): 1.50 g $CaCl_2H_2O$/L.
3. B5 microelements (1,000×): 300 mg H_3BO_3, 1,000 mg $MnSO_4{\cdot}H_2O$, 200 mg $ZnSO_4{\cdot}7H_2O$, 75 mg KI, 25 mg $Na_2MoO_4{\cdot}2H_2O$, 2.5 mg $CuSO_4{\cdot}5H_2O$, 2.5 mg $CoCl_2{\cdot}6H_2O$/100 mL.
4. FeNa-EDTA (100×): 3.67 g/L.
5. B5 vitamins (100×): 1,000 mg thiamine–HCl, 100 mg pyridoxine–HCl, 100 mg nicotinic acid/L.
6. Myo-inositol (10×): 10.0 g/L.
7. 2,4-D (2,4-dichlorophenoxyacetic acid): 10 mg/mL in DMSO (*see* **Note 7**). Store at –20 °C in 1 mL aliquots.
8. Kinetin (6-furfurylamino purine): 5 mg/mL in DMSO. Store at –20 °C in 1 mL aliquots.
9. 2-iP (6-(dimethylallylamino)-purine): 20 mg/mL in DMSO. Store at –20 °C in 1 mL aliquots.
10. IAA (indole-3-acetic acid): 1 mg/mL in DMSO. Store at –20 °C in 1 mL aliquots.
11. 0.2 M acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone): 39.24 mg/mL in DMSO. Do not sterilize, store at –20 °C.
12. Kanamycin: 100 mg/mL. Filter-sterilize (0.22 μm filter), store at –20 °C.
13. Timentin: 100 mg/mL. Filter-sterilize (0.22 μm filter), store at –20 °C.

2.5 Plant Culture and Root Transformation

1. B5 medium (liquid): mix 100 mL B5 macroelements 1, 100 mL B5 macroelements 2, 1 mL B5 microelements, 10 mL FeNa–EDTA, 10 mL B5 vitamins, 10 mL myo-inositol, 20 g glucose, and 0.5 g MES in approximately 700 mL Milli-Q H_2O. Adjust to pH 5.7 with 1 N KOH. Make up to a volume of 1 l, autoclave for 20 min at 110 °C, store at 4 °C.
2. Callus inducing medium (CIM): B5 with 0.8 % agar. Use liquid B5 medium that is not yet autoclaved. Poor 500 mL aliquots in bottles which already contain 4 g agar (*see* **Note 10**). Autoclave for 20 min at 110 °C, let the temperature of the autoclaved medium cool down to 60 °C, add 50 μL/L 2,4-D stock and 10 μL/L kinetin. For cocultivation plates additionally add 500 μL/L 0.2 M acetosyringone (final concentration 20 μM). Mix well and poor CIM plates both with and without acetosyringone in 94 × 16 mm petri dishes with vents. Dry the plates in the laminar flow cabinet with the lid open for 45 min. Close them with gas-diffusible tape (Urgopore or 3M Micropore tape) and store at 4 °C.
3. Shoot inducing medium (SIM): Use 60 °C sterile B5 agar medium, add 250 μL/L 2iP, 150 μL/L IAA, 500 μL/L

kanamycin, and 1 mL/L timentin. Prepare plates as described for CIM medium.

4. Eppendorf tubes.
5. 70 % ethanol.
6. Pipette man (P1000) and tips.
7. 1 % hypochlorite solution containing 0.1 % Tween-20, freshly prepared.
8. 250 mL Erlenmeyer flask capped with aluminum foil and sterilized by autoclaving.
9. Orbital shaker.
10. Plant growth chambers (21 °C, 16 h light–8 h dark, 2,000 lx).
11. Plant growth chambers (25 °C, 16 h light–8 h dark, 1,500–2,000 lx).
12. 15 cm petri dishes.
13. Scalpel and forceps.
14. Gas-diffusible tape (Urgopore or 3M Micropore tape).
15. Microbiological safety cabinet.
16. High sided petri dishes. (90 × 25 mm).
17. Sterile filter paper.
18. Sterile sieve (*see* **Note 16**).
19. Fluorescence microscope (e.g., Leica MZ 16 FA) with GFP filter.

2.6 Stock Solutions for Yeast Media

Prepare the stock solutions by dissolving powders in demineralized (Milli-Q) water, autoclave for 20 min at 120 °C, and store at room temperature unless indicated otherwise.

1. MY Salt solution for MY medium: 100 g KH_2PO_4, 50 g $MgSO_4 \cdot 7H_2O$, and 100 g $(NH_4)_2SO_4$/L.
2. MY Microelements: 2 mL concentrated HCl, 0.5 g H_3BO_3, 10 g $CaCl_2 \cdot 2H_2O$, 0.4 g $ZnSO_4 \cdot 7H_2O$, 0.04 g $CuSO_4 \cdot 7H_2O$, 0.4 g $MnCl_2 \cdot 4H_2O$, 0.1 g KJ, 0.3 mL 60 % $FeCl_3$, and 0.2 g $NaMoO_4$/L. Filter-sterilize and store at room temperature.
3. MY Vitamin solution: 0.02 g biotin, 20 g myo-inositol, 4 g Ca-pantothenate, 4 g pyridoxine–HCl, 4 g thiamine, 4 g nicotinic acid, 4 g p-amino benzoic acid/L. Filter-sterilize and store at 4 °C.
4. 5 N KOH: 280.6 g/L. Do not sterilize, store at room temperature.
5. Cefotaxime: 200 mg/mL cefotaxime sodium salt (100× stock).
6. L-leucine: 3 g/L (100×).
7. Uracil: 2 g/l (100×).
8. FOA: powder.

2.7 Yeast Growth and Transformation

1. YPD medium: dissolve 10 g yeast extract, 20 g Bacto-Peptone, and 20 g glucose in 1 L Milli-Q H_2O. Adjust to pH 6.5 with 5 N KOH. For solid medium add 16 g agar. Autoclave for 30 min at 110 °C.
2. CY medium: dissolve 5 g yeast extract, 5 g Bacto-Peptone, and 10 g glucose in 1 L Milli-Q H_2O. Adjust to pH 6.5 with 5 N KOH. Autoclave for 30 min at 110 °C.
3. MY medium: mix 10 mL MY salt solution, 1 mL MY microelements, 1 mL MY vitamin solution, and 10 g glucose/L Milli-Q H_2O. Adjust to pH 6.5 with 5 N KOH. For solid medium additionally add 16 g/L agar, autoclave for 30 min at 110 °C.
4. 100 mL flasks.
5. 500 ml flasks (conical with narrow bottle neck).
6. Physiological salt solution: 0.9% NaCl.
7. Vortex.
8. Cellulose nitrate filter: pore size 0.45 μm, Sartorius Stedim.
9. Sterile 50 ml disposable centrifuge tubes.
10. Table top centrifuge.

3 Methods

3.1 Plant Assay

Day 1 (Thursday or Friday (*see* **Note 15**)): Preparation of *Arabidopsis* cultures.

1. Weigh about 3 mg seeds from *Arabidopsis* C24 line 3043 or line CB1 in a 1.5 mL Eppendorf tube for each 250 mL root culture (*see* **Note 11**).
2. Add 1 mL 70 % ethanol, shake the tube and let it stand for 1 min. The seeds will sink to the bottom of the tube. Carefully remove the ethanol with a P1000 tip (*see* **Note 12**).
3. Add 1 mL of a freshly prepared 1 % hypochlorite solution containing 0.1 % Tween-20. Make sure all seeds are in contact with the solution. Let stand for 10 min and remove the hypochlorite solution.
4. Rinse seeds 4 times in sterile Milli-Q water. After the last washing step leave seeds in a small volume of sterile water.
5. Pipette the seeds with the water into 50 mL sterile liquid B5 medium in a 250-mL Erlenmeyer flask capped with aluminum foil. Put flask with seeds at 4 °C in the dark for 4 days to allow for synchronized and efficient germination.

Day 5 (Tuesday): Culture *Arabidopsis*.

6. Put flask with seeds on a rotary shaker (80/100 rpm) in a growth room (21 °C, 16 h light–8 h dark, 2,000 lx) for 10 days. (*see* **Note 13**).

Day 12 (Tuesday): Preparation of *Agrobacterium*.

7. Streak the *Agrobacterium* strain(s) containing the cre::protein fusion construct on a fresh LC agar plate supplemented with the antibiotics rifampicin (50 µL/25 mL), spectinomycin (50 µL/25 mL), and gentamycin (25 µL/25 mL). (*see* **Note 14**). Incubate for 3 days at 30 °C, store at 4 °C.

Day 15 (Friday): Preparation and pre-culture of roots.

8. Use the 10 days old *Arabidopsis* cultures for separating the roots from hypocotyls, cotyledons, and leaves. The seedlings will have formed a clump that can be taken out of the flask with the aid of curved forceps. Place the clump on a 15 cm petri dish. Pull the seedlings out of the clump with the forceps and cut off the roots with a scalpel. Collect the roots in a drop of B5 medium on the plate.
9. Transfer the roots to a CIM agar plate (without acetosyringone) and spread them with the forceps. Make sure all the roots are in good contact with the medium. Close the plates with gas-diffusible tape. Incubate the roots for 3 days in a growth room at 25 °C and 1,500–2,000 lx.

Day 17 (Sunday): Pre-culture of *Agrobacteria*.

10. Inoculate cultures of the *Agrobacterium* strain(s) from the fresh plates in 5 mL liquid LC medium with appropriate antibiotics and grow overnight at 29 °C at 200 rpm (*see* **Note 15**).

Day 18 (Monday): Cocultivation of *Agrobacteria* and *Arabidopsis* (work in a microbiological safety cabinet).

11. Measure the OD_{600} of the (10 fold diluted) overnight *Agrobacterium* culture. Pellet such an amount of the bacterial cells in a sterile 1.5 mL eppendorf tube (2 min, 16,000 × *g*) that will result in an OD_{600} of 0.1 in 20 mL B5 medium, remove supernatant and resuspend the pellet in 1 mL B5.
12. Transfer the roots from the 3 days incubated CIM plates (**step 9**) to a petri dish (high) containing 19 mL liquid B5 medium.
13. Add the bacteria obtained in **step 11**. The final OD_{600} in 20 mL B5 should be 0.1.
14. Incubate the bacteria and the roots for 2 min, while shaking from time to time.
15. Collect the roots and place them in the lid of the petri dish as a bundle of approximately 5 cm. Cut the bundle in small pieces with a sharp blade, arrange the roots, and repeat this step. Finally cut the roots by tapping them with the blade yielding pieces of 3–5 mm (named explants).

16. Dry the root explants briefly on two layers of sterile filter paper and place them on CIM agar plates containing 100 μM acetosyringone. Spread the explants so they will be in close contact with the medium.
17. Incubate the cocultivation plates for 3 days in the growth room (25 °C, 2,000 lx).

Day 21 (Thursday): Detection of excision events resulting from protein translocation.

18. Translocation into *Arabidopsis* C24 line CB1 can be visualized by observing GFP fluorescence directly on the cocultivation plates using a fluorescence microscope with GFP filter. When *Arabidopsis* C24 line 3043 is used, root explants are collected with a forceps (bacteria will have overgrown the explants) and transferred to a sterile sieve (*see* **Note 16**) that is placed in a petri dish with liquid B5 medium. Wash root explants carefully by shaking the sieve. Repeat the washing step in fresh B5.
19. Blot the root explants dry on two layers of sterile filter paper (Root explants should be dry but NOT dried out) and transfer them to SIM-agar plates with 50 mg/L kanamycin and 100 mg/L timentin. Again be sure that all explants are in good contact with the medium. Do not plate the root explants too densely (*see* **Note 17**). Two petri dishes are used per cocultivation. Count the number of explants per plate so that protein translocation efficiencies can be calculated.
20. Transfer the plates to 3,000–4,000 lx at 25 °C. After 2–3 weeks the number of green calli can be counted under a fluorescence microscope.
21. Calculate the efficiency of protein transfer by dividing the number of Km resistant calli by the number of explants counted in **step 19** per plate (*see* **Note 18**). That the kanamycin resistance is indeed caused by fusion of the 35S promoter region to the *nptII* coding region due to Cre activity can be verified by PCR.

3.2 Yeast Assay

Day 1 (Friday (*see* **Note 15**)): Preparation of *Agrobacterium* and yeast strains.

1. Streak *Agrobacterium* strains containing the cre:: protein fusion constructs on fresh LC agar plates supplemented with rifampicin (50 μL/25 mL), spectinomycin (50 μL/25 mL), and gentamycin (25 μL/25 mL) (*see* **Note 14**). Streak the *Saccharomyces cerevisiae* strain on a fresh YPD agar plate. Incubate the plates for 3 days at 30 °C.

Day 4 (Monday): Start pre-cultures of *Agrobacterium* and yeast strains.

2. Inoculate the freshly grown *Agrobacterium* strains into a 100 mL flask containing 5 mL MM supplemented with the

same antibiotics used in the LC agar plates and inoculate the yeast strain into 10 mL CY medium. Incubate overnight at 30 °C on a shaker at ~180 rpm.

Day 5 (Tuesday): Start cocultivation.

3. Harvest cells from 2 mL *A. tumefaciens* overnight culture by centrifugation for 3 min. at 16,000 × *g* in an eppendorf centrifuge. Remove supernatant and resuspend cells in 200 μL IM.
4. Transfer cells to 5 mL IM containing 10 mM glucose (*see* **Note 7**) supplemented with 5 μL acetosyringone (final conc. = 200 μM) and 5 μL gentamycin at an OD600 of 0.25. Culture the cells at 28 °C and ~180 rpm for 5–6 h. (*see* **Note 19**).
5. Dilute yeast cells from the overnight culture 10 times in a 500 mL flask with 50 mL fresh CY medium and incubate at 30 °C on a shaker at ~180 rpm for 5–6 h.
6. During the 5–6 h incubation time prepare cocultivation plates: poor IM agar plates with 5 mM glucose (*see* **Note 7**), 25 μL/25 mL acetosyringone, 25 μL/25 mL gentamycin, 250 μL/25 mL leucine and uracil. (*see* **Note 20**) Dry the plates for 15 min by placing them upside down with open lid in a 55 °C incubator. Cut 47 mm round 0.45 μm cellulose nitrate filters into four equal pieces using sterile scissors and place 3 or 4 pieces on a cocultivation plate.
7. Harvest yeast cells from **step 4** in a sterile 50 mL disposable centrifuge tube by centrifugation for 10 min at 3,000 g. Remove supernatant and resuspend the cells in 2 mL IM without glucose. Pellet cells again by centrifugation and resuspend in 250 μL IM without glucose.
8. Transfer 60 μL of the pre-induced *Agrobacterium* strain to a sterile 1.5 mL eppendorf tube. Add 60 μL washed yeast cell suspension and mix well.
9. Carefully spot 100 μL of this cocultivation mix onto a cellulose nitrate filter piece placed on a cocultivation plate as described in **step 5**. Take care that the suspension stays on the filter. Do not move the plate for at least half an hour allowing the cocultivation mix to dry slightly. Incubate the plates for 6 days at 22 °C.

Day 11(Monday): Detection of excision events resulting from protein translocation.

10. Transfer the filter with the 6 days incubated cocultivation mix to a 2 mL eppendorf tube containing 2 mL sterile 0.9% NaCl with the aid of a forceps. The filter must be folded double to fit into the eppendorf tube. Vortex vigorously to resuspend the cells.
11. Make 10^{-1}, 10^{-2}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions of the well mixed suspension in physiological salt.

12. Plate 100 µL of undiluted, 10^{-1} and 10^{-2} dilutions on MY selective plates containing cefotaxime (25 µL/25 mL), leucine (250 µL/25 mL), uracil (250 µL/25 mL), and 0.1% FOA (*see* **Note 21**) to determine the number of colonies that lost the *Ura* gene by excision as a result of Cre activity.
13. Plate 100 µL of 10^{-4} and 10^{-5} dilutions on YPD or MY + Ura and Leu with cefotaxime plates to determine the output number of yeast.
14. Plate 100 µL of 10^{-5} and 10^{-6} dilutions on LC agar plates containing gentamycin to determine the output number of *Agrobacterium*. (*see* **Note 22**).
15. Incubate all plates for 3–5 days at 30°C until colonies appear.
16. Count the number of FOA resistant (Ura^-) colonies and of output yeast cells. Calculate the translocation efficiency by dividing the number of FOA resistant colonies by the number of output yeast cells. (*see* **Note 23**).

4 Notes

1. In this chapter we use two *Arabidopsis lox*-reporter lines, CB1 and 3043 [5, 7]. Figure 1 shows a schematic representation of the CRAfT assay. Transgenic line 3043 has kanamycin resistance as a readout for translocation, whereas line CB1 contains a GFP reporter gene that becomes expressed after Cre-mediated excision in the host cell. An advantage of the Cre recombinase as a reporter to detect translocation into host cells is that transfer of a small number of protein molecules can potentially mediate a stable recombination event that will result in the continuous expression of the marker gene. This makes it easy to detect even low amounts of translocated proteins, compared to reporter systems based on β-lactamase or adenylate cyclase that are often used for studies of protein translocation from human pathogens into mammalian cells [27]. Like the β-lactamase assay, the Cre reporter assay does not require lysis of infected cells for detection of translocation events which can thus be monitored in individual host cells. This gives an indication of the translocation efficiency compared to overall transformation or infection efficiency. In addition, although not useful for pathogens such as *Agrobacterium* and *Helicobacter* that "inject" their effectors into the host cell from the extracellular milieu, for intracellular pathogens such as *Brucella* and *Salmonella*, this allows for the simultaneous visualization of fluorescently labelled intracellular bacteria. A limiting step in the Cre assay is that the fusion protein has to enter the nucleus for the recombination reaction to take place.

Therefore, a strong SV40 NLS sequence is placed in the Cre-fusion proteins to enhance nuclear entry (*see* **Note 4**); however, the presence of very strong putative subcellular localization signals (e.g., ER, Golgi) in the effector protein under study may reduce nuclear uptake, and thus reduce detection levels of translocation.

2. To unambiguously show T4SS-dependent translocation, as a negative control Cre-Vir fusion transport should be analyzed from a *virB* deficient strain. For *Agrobacterium* and other pathogens that can survive equally well in the absence of a type IV secretion system as the wild type strain in the context of infection, the comparison of reporter gene expression after infection with the *virB* mutant and the wild type will give unbiased proof that the T4SS is important for translocation. However, it is harder to provide proof of T4SS-dependent translocation from bacteria such as *Brucella*, for which the T4SS is essential for bacterial intracellular survival. In these cases it is important to show that similar numbers of viable bacteria are present at the assay times for wild type and the *virB* mutant.
3. We use LBA1100 as a donor for protein translocation studies. This strain has a pTiB6 plasmid in which the T-DNA, *tra* and *occ* genes have been replaced by the spectinomycin resistance marker [28]. Our unpublished results show that the use of oncogenic strains, including LBA1010 [29] results in severely reduced protein translocation detection rates compared to transfer from LBA1100, possibly through competition between the T-strand and the effectors for the T4SS. Such reduced transfer rates are not detected when a binary T-DNA plasmid is used. We therefore suggest the use of an *Agrobacterium* strain from which the oncogenic T-DNA has been removed.
4. A. Cre-vir fusion plasmids are introduced into the *Agrobacterium* helper strain LBA1100 by electroporation [30]. These plasmids are made by cloning the coding sequence of the *cre* recombinase gene in frame to the sequence encoding the protein of interest under control of a *vir* promoter, so that the fusion protein is expressed upon induction of the *Agrobacterium* virulence system [5–7]. Plasmid pSDM3197 is used as a backbone for cloning Vir coding sequences after amplification by PCR [5]. An important question is whether to express Cre at the NH2- and/or COOH-terminal end of the effector protein. Translocation of *Agrobacterium* VirF and VirE2 proteins could only be detected when Cre was fused to the NH2-terminal region, and not the C-terminal region of the virulence proteins [5]. Even small tags added to the C-terminus of VirE2 interfered with its VirB-dependent transfer [31]. This is in agreement with our finding of a

C-terminal transport signal for *Agrobacterium* effectors [7], indicating the C-terminal portion of the effector protein should be "free" for recognition by the T4SS apparatus. Further, a 50 C-terminal signal of the VirE2, VirD5, and VirE3 proteins is sufficient for translocation into host cells in a T4SS dependent manner, and for VirF even 10 amino acids could direct Cre into host cells, albeit at lower efficiency. However, for the VirD2 protein, 50 amino acids were not sufficient to detect translocation, yet translocation of the full length protein was detectable. Later it was shown in our lab that an internal region in VirD2 is needed besides the C-terminus [23]. In addition to the VirD2 relaxase, other relaxases [25, 32] and effector proteins from other T4SS, including those from *Bartonella* and *Helicobacter*, have bipartite signals [21, 22], requiring other regions in the protein important for translocation. We suggest analyzing fusions of full length proteins, both N- and C-terminal to the reporter, before reaching the conclusion that a protein is not a translocated effector (*see* Fig. 1b).

B. To enhance nuclear uptake, the sequence encoding a SV40 nuclear localization sequence (NLS) is included in frame to the N-terminus of the Cre coding sequence to optimize nuclear translocation of the fusion protein (*see* Fig. 1b). The size and the folding of the fusion protein may influence the translocation efficiency. The largest fusion protein detected to be translocated in a CRAfT assay so far is 238 kDa [14]. We and others have been unable to detect translocation of a full length GFP protein into host cells, even after adding the strong 37 AA C-terminal peptide of VirF, possibly because the GFP protein has a strong globular fold, and the T4SS only supports transfer of unfolded substrates.

C. The presence of an incQ plasmid, such as RSF1010, in *A. tumefaciens* blocks virulence in plants [33]. This was shown to be due to substrate competition for the T4SS [34]. In agreement with these reports, we found that incQ plasmids also block translocation of Vir proteins (our unpublished data), and we therefore suggest to avoid using incQ vectors for cloning purposes.

D. The minimal transport signal required for translocation of a given protein (e.g., MobA encoded by incQ) may vary for recognition by different T4SS. IncQ plasmids are versatile plasmids that can be mobilized by different T4SS with different substrate specificity, including those of *Agrobacterium* and *Legionella*. The MobA relaxase, covalently bound to and directing DNA through the T4SS channel, must therefore have adopted several different signals to be recruited by these different T4SS. MobA has been shown to be a substrate of the Legionella *dot/icm* system, and could also be transported in the absence of incQ DNA [24]. We have shown that the C-terminal 48 amino acids of MobA were sufficient for its translocation

into eukaryotic host cells by the *Agrobacterium* T4SS [7]. However, Parker and Meyer [35] showed that transport of the primase region of MobA required both a large N-terminal portion of the primase domain and an adjacent leader region in conjugation and T4SS assays using *Escherichia coli* MV10.

5. After autoclaving $FeSO_4 \cdot 7H_2O$ a red precipitate will appear in the solution. This is normal and has no negative influence on the bacterial growth.
6. Use filter-sterilized DMSO (dimethyl sulfoxide) from Sigma to dissolve acetosyringone and phytohormones. Do not sterilize and do not flame DMSO. DMSO is toxic, so handle with care.
7. Prepare liquid IM without glucose. IM w/o glucose is needed to wash the yeast strain before cocultivation with *Agrobacterium* (*See* Subheading 3.2, **step 7**). Glucose has to be added to the liquid medium for the pre-induction of *Agrobacterium*. In solid IM less glucose is used because *Agrobacterium* and yeast cocultivation on high glucose leads to the death of *Agrobacterium* probably due to the production of toxic components, such as ethanol, by the yeast.
8. Not all sources of agar are suitable for *Agro/Yeast* cocultivations. Agars that give good results are "Micro Agar" from Duchefa (The Netherlands) or "Select Agar" from Invitrogen.
9. The correct pH is critical because the induction of the *Agrobacterium vir* genes by acetosyringone is optimal at pH 5.3.
10. Not all agars are suitable for *Arabidopsis* growth and efficient root transformations. "Daichin Agar" from Brunswich Chemie (The Netherlands) gives good results. If not, we suggest testing different agars. Do not autoclave the agar media at higher temperature than 110 °C or longer than 20 min and use an autoclave that does not need a long time to heat up and/or cool down. When the medium is kept at high temperature too long the plates will not be solid enough. Soft agar makes it very difficult to spread the root explants.
11. Approximately 3 mg of seeds is sufficient for 400–600 explants after cocultivation. Use one 250 mL culture for each root transformation. It is advisable to initiate more root cultures than needed because cultures are sometimes lost due to contamination. The experimental procedure for line 3043 has more steps than that of line CB1. Differences are indicated in the different steps in Subheading 3.
12. Filter the liquid from all rinsing steps over a filter paper covering a beaker. Floating seeds will be collected on the filter paper so that the transgenic seeds can be destructed, according to GMO regulations, apart from the liquid.

13. Philips TL 83HF light tubes which emit light in which the orange/red part of the spectrum is well represented or Sylvania Luxline Plus F36W/840 light tubes are convenient. Do not culture other plants species (such as *Catharanthus* cell cultures) in the same tissue culture room, because *Arabidopsis* is very sensitive for volatiles produced by other plants. The efficiency of transformation and protein translocation will decrease drastically in the presence of such, so far uncharacterized volatiles. Assays are also highly sensitive to volatiles from painting or welding activities close to the growth chamber.
14. The resistance for rifampicin is due to a chromosomal mutation in *Agrobacterium* strain LBA1100, spectinomycin resistance is carried by the pTi helper plasmid and gentamycin is the marker of the wide host range vector pRL662 [5], a pBBR-derived plasmid, into which the fusion proteins are cloned. Gentamycin must not be omitted from the medium because pRL662 is not stable in *Agrobacterium* and loss of this plasmid will reduce translocation efficiency. In each experiment, a strain which contains pRL662 expressing a non-fused Cre recombinase should be included as a negative control. In the plant assay with line 3043 cocultivation with this strain does not give false positive Km^r calli; however, in the yeast assay a high number of Ura^- colonies can be found, probably caused by homologous recombination (which is very efficient in yeast) between the *lox* sites. Plasmid pRL662 does not carry *mob* and *oriT* functions, and can only be transferred into *Agrobacterium* by electroporation. The use of a non-mobilizable plasmid can be desired when DNA transfer needs to be excluded in the protein translocation experiments.
15. The day schedule used for this protocol is based on avoiding experimental activities during the weekends. On Sunday, *Agrobacterium* cultures for the cocultivation have to be set up. For this the cultures can be inoculated on Friday and kept at 4 °C before transferring them to the shaker on Sunday.
16. Stainless steel sieves with 100 mesh screens (purchased from Sigma) are used. After contamination with bacteria, sieves are sterilized at 120 °C for 20 min in a beaker together with the contaminated B5 medium. The sieves are then cleaned with H_2O (do not use soap or other detergent!), allowed to dry, and sterilized again for further use.
17. Best results are obtained when 150–200 explants per petri dish are spread in a way that they are not making contact with each other (this may take a lot of time and patience). In this way, growth of Km^r calli (or GFP positive calli) and counting can be enhanced.
18. Using the described protocol it is possible to obtain a translocation frequency of 1.3 ± 0.6 kanamycin resistant calli per

root explant [5]. Km^r calli are not detected when roots are cocultivated with *Agrobacterium* expressing *cre* as a control, showing that the Cre protein does not contain any cryptic transport signals.

19. During this incubation time the *Agrobacterium* virulence genes will be induced resulting in the expression of the Cre::protein fusion and Vir proteins forming the Type IV secretion system. The induction temperature is very critical. In fact, induction is optimal at 22 °C but a higher temperature is used in the pre-culture to obtain sufficient *Agrobacterium* biomass. Temperatures exceeding 28 °C will result in decreased transformation efficiencies.
20. The antibiotics and amino acids used in the cocultivation plates are based on cocultivations with yeast strain LBY2 (Leu^-, Ura^-) and *Agrobacterium* containing pRL662 [5, 6] expressing the Cre::protein fusion. In case other strains are used different antibiotics or amino acids must be added.
21. FOA is difficult to dissolve in high concentrations used for stock solutions. Therefore, it is more convenient to add the (unsterilized) powder to the warm, but not hot, MY agar medium before pouring the plates. Shake the medium well until all powder is dissolved, which can take a few minutes.
22. Output *Agrobacterium* is determined to verify that the bacteria survived during the cocultivation. Low efficiency of protein translocation can be caused by starvation of the bacteria. When this is observed it is advised to repeat the experiment with freshly prepared media.
23. The efficiency found for protein transfer is in the order of 10^{-5} to 10^{-2} [5–7, 14]. The efficiency of the Cre negative control is lower than 10^{-6} (*see* **Note 11**). Quite some variations can be found between independent experiments. Therefore, it is wise to include internal controls in each experiment, such as a Vir protein of which the efficiency is known.

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Chapter 7

Detection of the Interaction Between Host and Bacterial Proteins: Eukaryotic Nucleolin Interacts with *Francisella* Elongation Factor Tu

Monique Barel and Alain Charbit

Abstract

Dissecting the interaction between bacterial and host proteins is fundamental in understanding pathogenesis. It is also very helpful for exploring new therapeutic approaches, either preventive or curative. Here, we describe different techniques, which allowed us to detect new molecules involved in the binding and infection of the bacterium *Francisella tularensis*, on human cells. This facultative intracellular pathogen is the causative agent of tularemia and is considered as a bio-threatening agent. The privileged host cells are monocytes and macrophages. We used both "in vitro" and "in vivo" experiments to explore the modulation of *F. tularensis* infection and thereafter determine a bacterial ligand and its host receptor molecule.

Key words *F. tularensis*, EF-Tu, Nucleolin, Infection, Human macrophages

1 Introduction

Francisella tularensis, the causative agent of tularemia, is one of the most infectious human bacterial pathogens [1]. After its phagocytosis, by immune cells, such as monocytes and macrophages, it escapes from phagosomes to multiply in the cytoplasm [2, 3]. The precise mechanisms that initiate bacterial uptake have not yet been elucidated. Participation of C3 [4], CR3 [5], class A scavenger receptors [6] and mannose receptor [7] in bacterial uptake has been reported. However, contribution of an additional, as-yet-unidentified receptor for *F. tularensis* internalization has also been suggested [7]. Our goal was to characterize these new molecules, both on bacterial and eukaryotic host cells. Here we describe a protocol for the confirmation of the specificity of such host protein–bacterial protein interactions using different methods, including (1) pull-down assay of bacterial membrane proteins using specific proteins (or peptides) followed by immunoelectroblotting with specific antibodies (Western blotting [8]); (2) fluorescence and

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confocal microscopy; and (3) binding of potential ligands to receptor proteins expressed on human cells and their effect on cell infection by bacteria. Combination of these "in vitro" and "in vivo" experiments allowed us to demonstrate that cell-surface-expressed nucleolin is a receptor for elongation factor Tu (EF-Tu) of *Francisella tularensis* Live Vaccine Strain (LVS). Nucleolin has been shown to mediate internalization of specific ligands, including HIV particles [9]. In response to binding of ligand, ligand–nucleolin complexes become internalized, thereafter allowing intracellular import of the ligand. Nucleolin has also been recently described as a receptor for the adhesin of *E. coli* O157:H7 [10]. The moonlighting function described for EF1, the eukaryotic analog of EF-Tu [11] led us to assume that EF-Tu, one of the major LVS antigenic protein in murine infection [12], could be a ligand for nucleolin. This interaction indeed promoted infection of human monocyte-like THP-1 cells [13]. As EF-Tu is also expressed on the surface of *F. novicida* and of *F. tularensis* Type A strain SchuS4 [14], this interaction may be a general mechanisms for *F. tularensis* and exist in other bacteria. Indeed, EF-Tu has been found also on the membrane of *Pseudomonas aeruginosa* where it binds to complement proteins [15] and on *Mycoplasma pneumoniae* membrane where EF-Tu binds [16]. Therefore, our methods completely supported the demonstration that EF-Tu may interact with various host molecules. It also confirmed that in addition to its known "house-keeping" function in peptide elongation during protein synthesis process, EF-Tu presents multifaceted roles and is involved in multiple cellular functions.

2 Materials

2.1 Infection of Human Cells with F. tularensis Bacteria

1. Human monocyte-like cell line THP-1 (ATCC® Number: TIB-202™).
2. RPMI 1640 medium containing stabilized glutamine and supplemented with 10 % heat-inactivated fetal calf serum (FCS) without antibiotics (*see* **Note 1**).
3. RPMI 1640 with 5 % FCS (RPMI-FCS).
4. RPMI 1640 containing 10 μg/mL gentamicin from a 10 mg/mL stock.
5. 37° incubator with 5 % CO_2.
6. 25 cm^3 cap-ventilated sterile culture flasks for growth of bacteria.
7. 75 cm^3 cap-ventilated sterile culture flasks for growth of human cells.
8. 12-well sterile microtiter plates.
9. Trypan Blue (1 mL NaCl at 42.5 g/L plus 4 mL trypan blue at 2 g/L).

10. Malassez cell.
11. *F. tularensis* ssp. Live Vaccine Strain, LVS.
12. Schaedler medium (Biomérieux), containing vitamin K3, with or without 10 μg/mL antibiotics.
13. Bacterial glycerol stocks kept at −80 °C in vials (150 μL glycerol/850 μL bacterial culture) (*see* **Note 2**).
14. Human serum with AB blood type from PAA handled in a manner to preserve complement activity (*see* **Note 3**) [5].
15. All experiments involving human cells and bacteria must be performed under a Class II Type A2 Microbiological Safety Cabinet.
16. 5 mL sterile tubes with caps.
17. Distilled sterile water.
18. Sterile 0.15 M NaCl (9 g/L in distilled water) (*see* **Note 4**).
19. Chocolate agar plates (Biomérieux).
20. *LVS-GFP* bacteria [13].

2.2 Preparation of Subcellular Fractions of LVS

1. Fast-Prep FP120 Cell Disrupter (Thermo Savant) and Fast prep beads.
2. Centrifuge (Jouan).
3. Beckmann Optima Max ultracentrifuge.
4. TNE: 50 mM (6.06 g/L) Tris–HCl, 150 mM (9 g/L) NaCl, 1 mM EDTA (0.3 g/L). Adjust pH to 8.0 with 5 N HCl (*see* **Note 5**).
5. TNE containing 1 % NP40 (10 mL from a 10 % stock solution), 1 % Triton X100 (10 mL from a 10 % stock solution). One tablet Complete Protein Inhibitor for 50 mL TNE, one tablet PhoStop (cocktail of phosphatase inhibitors) for 50 mL TNE.
6. BCA assay from Pierce.

2.3 Immunoblotting

1. Recombinant proteins (*see* Subheading 3.6, **step 1**), proteins from human cells (*see* Subheading 3.6, **step 3**) or present in the different subcellular fractions of LVS (*see* Subheading 3.5, **steps 1–9**), and proteins recovered from pull-down assays (*see* Subheading 3.5, **step 18** and Subheading 3.6, **step 6**).
2. SDS-PAGE A 10 % running gel is prepared by mixing 3.33 mL 30 % liquid acrylamide, 2.5 mL lower gel solution (17 g Tris, 4 mL 10 % SDS 60 mL distilled water) with pH adjusted to 8.8 with 5 N HCl (then add distilled water *qs* 100 mL). The running gel is loaded in a Mini-Protean 2 system (Bio-Rad). Polymerization of gel is obtained by adding 60 μL 10 % ammonium persulfate (APS) and 40 μL Tetramethylethylenediamine (TEMED) (*see* **Note 6**). After polymerization, 1 mL of stacking gel is loaded on top of the running gel (6.06 g Tris, 4 mL 10 %

SDS, 60 mL distilled water) with pH adjusted to 6.8 with 5 N HCl (then add distilled water *qs* 100 mL). Samples are diluted with sample buffer containing 1.25 mL lower gel solution, 1 mL 10 % SDS, 1 mL 100 % glycerol 1 mL 0.2 % bromophenol blue, 0.5 mL β-mercaptoethanol *qs* 10 mL with distilled water, and run in gel with TGS×1 buffer (Bio-Rad). After migration, gels are installed in a Trans-Blot Semi-Dry Bio-Rad apparatus on three sheets of Whatmann paper 3 M cut at the dimensions of the gels. A piece of nitrocellulose membrane is layered on the gel and three sheets of paper cover the membrane. Transfer is performed for 2 h at 14 V.

3. PBSX 1 (PAA).
4. PBS/0.05 % Tween-20 (PBS-T).
5. PBS-T containing 5 % powdered skimmed milk.
6. ECL Plus kit (Amersham).
7. Rotating wheel.
8. Rotating platform.

2.4 Fluorescence Experiments

1. Phorbol myristate acetate (PMA) (*see* **Note 7**).
2. 4 % paraformaldehyde (PFA) (*see* **Note 8**).
3. 50 mM NH_4Cl (*see* **Note 9**).
4. Mowiol mounting media (Calbiochem).
5. Slides and round glass coverslips.
6. Forceps.
7. Fluorescence microscope (Zeiss Axioplan 2) at 63× magnification and Qimaging Digital camera and analysis software is Q Capture Pro Fluorescence.
8. Confocal experiments with Zeiss LSM 5 Pa confocal microscope with argon (458/488 nm) and helium neon (543 nm) lasers. Analysis with LSM analysis software.
9. DAPI.

2.5 Antibodies and Peptides

1. Antibodies: rabbit anti-nucleolin (Abcam), anti-GST (Oncogen). Mouse monoclonal (MAbs) anti-nucleolin (clone D3) (MBL); murine polyclonal antibodies, specific for LVS EF-Tu [13]; HRP-linked secondary Abs (Dako). Alexa Fluor 546-labeled GAR.
2. Peptide construct HB-19, synthesized using Fmoc-protected D-Arg residue as described previously [17, 18] specifically binds to carboxy-terminal RGG domain of nucleolin.
3. Peptide construct 9Arg (9R), synthesized using Fmoc-protected D-Arg residue as described previously [17, 18]. This protease-resistant basic 9R peptide is used as a negative control as it does not interact with nucleolin.

4. Synthetic p63 peptide synthesized according to the last 63 amino acid residues of human nucleolin [19].
5. Biotin-labeled p63 peptide, an Fmoc Lys-biotin derivative at the N-terminus of the peptide.

2.6 Sepharose Beads

1. Glutathione sepharose beads (GST) (Amersham).
2. Avidin-agarose (ImmunoPure Immobilized Avidin, Pierce).

3 Methods

3.1 Interaction of LVS on Human Cells Is Decreased by Nucleolin Peptides

1. Incubate LVS-GFP bacteria grown in Schaedler medium (*see* **Note 10**) with 10 % human serum AB (*see* **Note 11**) for 30 min at 37 °C with constant shaking in 25 cm^3 sterile culture flask.
2. Wash THP-1 cells in RPMI and suspend in RPMI-FCS at 1×10^6 cells/mL (*see* **Note 12**).
3. Dispose the cells in sterile 12-well microtiter plates.
4. Incubate cells with 5 μM peptide HB-19 or 9R for 2 h at 37 °C.
5. Add LVS-GFP (Multiplicity of infection, MOI, of 100:1, *see* **Note 13**) for 30 min at 37 °C.
6. Wash cells with RPMI without gentamicin by centrifugation for 10 min at 250 × *g* in 15 mL centrifuge tubes.
7. Add on pelleted cells 400 μL 4 % paraformaldehyde (PFA) for 15 min at RT.
8. Add 50 mM NH_4Cl for 10 min at RT.
9. Centrifuge the cells.
10. Suspend pellet in 10 μL Mowiol.
11. Dispose the suspended pellet on a slide and cover with a glass coverslip, hold with forceps.
12. Analyze 15 different fields showing LVS bacteria expressing GFP (LVS-GFP) bound on THP-1 cells, using fluorescence microscopy. Count the number of bacteria per 100 cells using Q Capture Pro Fluorescence software (Fig. 1a). The difference in the mean number of bacteria bound per cell in the non-treated versus peptide-treated samples measure the effect of the peptide on cell–bacteria interaction.
13. As a negative control, use the protease-resistant basic 9Arg peptide (9R) that does not interact with nucleolin.

3.2 Infection of Human Cells by LVS Is Decreased by Nucleolin Peptides

1. Incubate LVS bacteria grown in Schaedler medium (*see* **Note 10**) with 10 % human serum AB (*see* **Note 11**) for 30 min at 37 °C with constant shaking in 25 cm^3 sterile culture flask.
2. Wash THP-1 cells in RPMI and suspend in RPMI-FCS at 1×10^6 cells/mL.

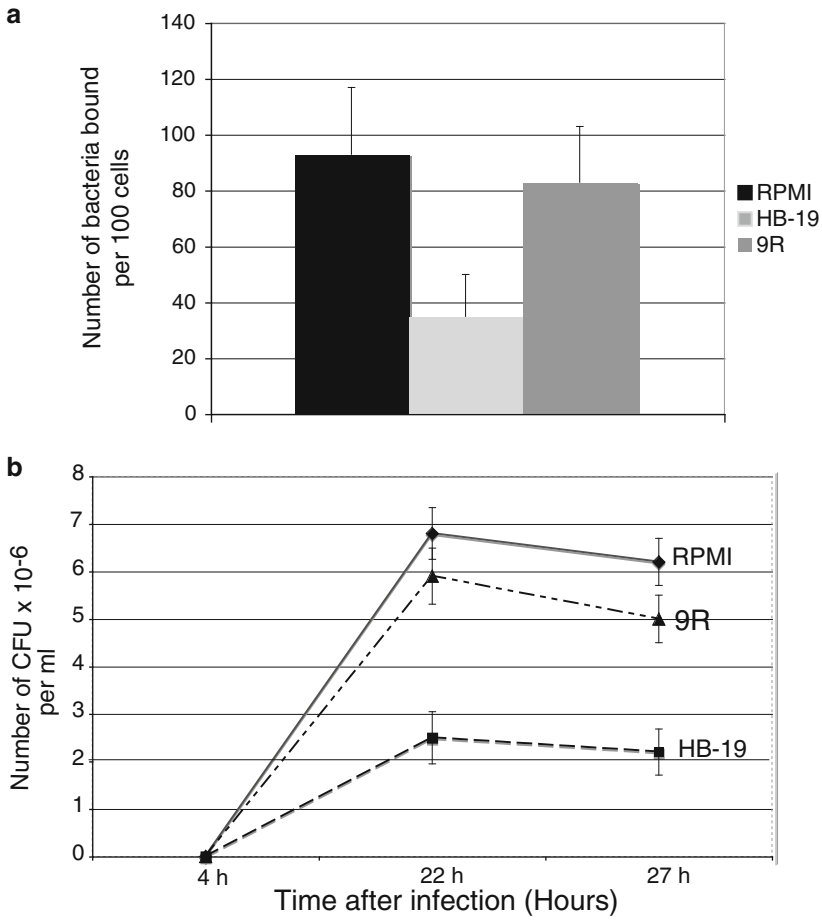

Fig. 1 Interaction of nucleolin with LVS is involved in binding (**a**) and infection (**b**) of human monocyte-like THP-1 cells. THP-1 cells were incubated in RPMI (*filled diamond*), in the absence or in the presence of 5 μM HB-19 pseudopeptide (*filled square*) or 9R control peptide (*filled triangle*) and infected with bacteria. (**a**) Fluorescence microscopy. The mean number of bacteria bound per cell was almost one bacterium per cell. Pre-incubation of THP-1 cells with HB-19 resulted in a 63 % decrease in the number of bound LVS-GFP bacteria. (**b**) Quantification of intracellular bacteria. Results show mean from three independent experiments, each performed in triplicate ± SD values indicated as error bars. A 63 % decrease in LVS infection was observed at 22 h, with 9R peptide without significant effect on LVS infection of THP-1 cells

3. Incubate cells with 5 μM peptide HB-19 for 30 min at 37 °C.
4. Add opsonized LVS (MOI of 100:1) for 30 min at 37 °C.
5. Wash cells by centrifugation for 10 min at 250 × *g* in 15 mL centrifuge tubes with RPMI containing gentamicin to remove extracellular bacteria.
6. Suspend pelleted cells in 2 mL RPMI containing gentamicin.
7. Dispose cells in 12-well sterile microtiter plates.
8. Incubate at 37 °C in a 5 % CO_2 incubator for times indicated on the figures.

9. Withdraw 100 µL of each suspension and add to tube containing 0.9 mL sterile H_2O for 30 min at RT.
10. Vortex for 10 s.
11. Withdraw 100 µL of the suspension as described in **step 9** and add to tube containing 0.9 mL 0.15 M NaCl.
12. Continue serial dilutions (*see* **Note 4**).
13. Finally plate 100 µL of each dilution 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} on chocolate agar without antibiotics (pre-warmed in a 37 °C incubator).
14. Incubate for 48–72 h at 37 °C to observe growth of LVS grey colonies.
15. Count the number of colonies per plate.
16. The effect of HB19 peptide on LVS infection is shown in Fig. 1b. The difference in the mean number of bacteria counted on plates in the non-treated versus peptide-treated samples measure the effect of the peptide on infection of cells after bacteria binding.
17. Use 9R peptide as negative control.

3.3 Interaction of LVS on Human Cells Is Decreased by Anti-EF-Tu Ab

1. Incubate LVS-GFP bacteria grown in Schaedler medium (*see* **Note 10**) with 10 % human serum AB (*see* **Note 11**) for 30 min at 37 °C with constant shaking in 25 cm^3 sterile culture flask.
2. Wash THP-1 cells in RPMI and suspend in RPMI-FCS at 1×10^6 cells/mL.
3. Dispose in a 12-well microtiter plate.
4. Add 1 mL opsonized LVS-GFP (MOI of 100:1) for 30 min at 37 °C, pre-incubated either with RPMI, or anti-EF-Tu Ab for 30 min at 37 °C.
5. Wash cells in RPMI without gentamicin.
6. Add 400 µL of 4 % PFA and incubate for 15 min at RT to fix the cells.
7. Pellet the cells by centrifugation, aspirate the PFA, replace with 400 µL of 50 mM NH_4Cl and incubate for 10 min at RT.
8. Pellet the cells by centrifugation and suspend the cell pellet in 10 µL Mowiol.
9. Dispose on a slide and cover with a glass coverslip hold with forceps.
10. Count the number of LVS bacteria expressing GFP (LVS-GFP) bound per 100 human THP-1 cells in 15 different fields (Fig. 2a), using a fluorescence microscopy. The difference in the mean number of bacteria bound per cell in the RPMI-treated versus antibody-treated samples measure the specific effect of the antibody on cell–bacteria interaction.
11. As a negative control, use non-immune serum (NIS).

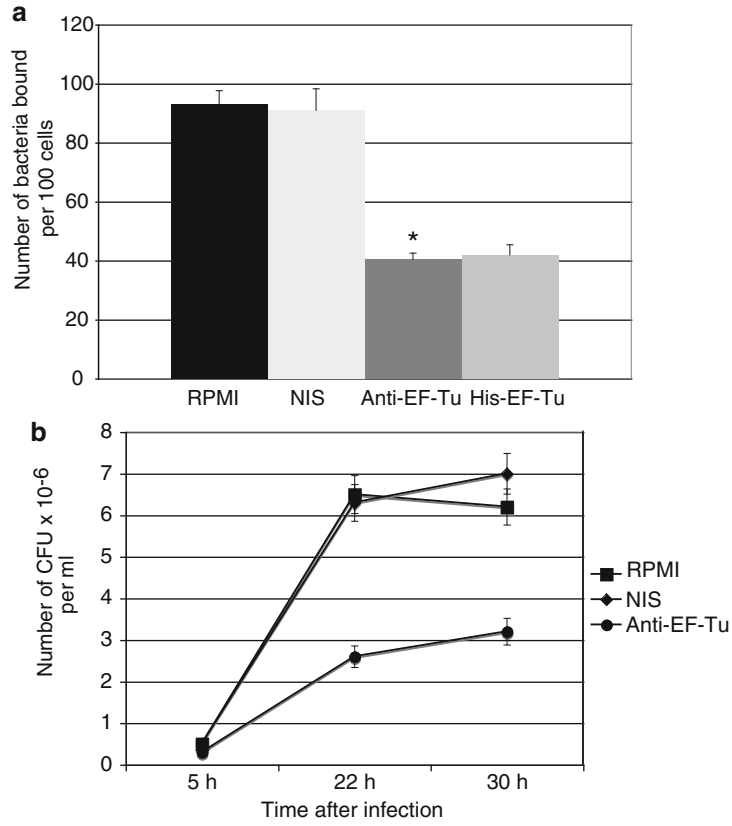

Fig. 2 Elongation factor Tu of LVS participates in (**a**) binding to human monocyte-like THP-1 cells and (**b**) in their infection. THP-1 cells were infected for 30 min by LVS that had been pre-incubated either with RPMI, NIS or anti-EF-Tu Ab (diluted 1/2,000). THP-1 cells were also pre-incubated with 50 μg His-EF-Tu. (**a**) Fluorescence microscopy. The mean number of bacteria bound per cell was almost one bacterium per cell. Maximum binding of 95 LVS-GFP bacteria per 100 human cells was recorded, in the presence of RPMI or NIS. When surface-exposed EF-Tu was blocked by anti-EF-Tu Ab, the number of bacteria bound per 100 cells was decreased by 58 %. Incubation of THP-1 cells with 50 μg His-EF-Tu, before infection with opsonized LVS-GFP also decreased LVS binding by 57 %. (**b**) Intracellular replication of LVS. RPMI (*filled square*), mouse non-immune serum (NIS) (*filled diamond*) or anti-EF-Tu Ab diluted 1/100,000 (*filled circle*). A maximum decrease (60 %) in bacterial number was observed at 22 h when bacteria were pre-incubated with anti-EF-Tu Ab, which corresponded to a decrease from 11 to 4 intracellular bacteria per cell

3.4 Infection of Human Cells by LVS Is Decreased by Anti-EF-Tu

1. Incubate LVS bacteria grown in Schaedler medium (*see* **Note 10**) with 10 % human serum AB (*see* **Note 11**) for 30 min at 37 °C with constant shaking in 25 cm^3 sterile culture flask.
2. Incubate opsonized bacteria with anti-EF-Tu diluted 1/2,000 for 30 min at 37 °C.

3. Wash THP-1 cells in RPMI and suspend in RPMI-FCS at 1×10^6 cells/mL.
4. Infect human cells with opsonized bacteria, as described in **step 2** for 1 h at 37 °C at an MOI of 100:1 (bacteria/cell).
5. Wash cells by centrifugation for 10 min at 250 × *g* in 15 mL centrifuge tubes with RPMI containing gentamicin to remove extracellular bacteria.
6. Suspend pelleted cells in 2 mL RPMI containing gentamicin.
7. Dispose cells in 12-well microtiter plates.
8. Continue as described in Subheading 3.2, **steps 8–14.**
9. Effect of anti-EF-Tu Ab on LVS infection is shown in Fig. 2b. The difference in the mean number of bacteria counted on plates in the non-treated versus antibody-treated samples measure the effect of the antibody on infection of cells after bacteria binding.
10. As a negative control, use non-immune serum (NIS).

3.5 Pull-Down Assays with Biotin-Avidin Beads

1. Pellet 150 mL of a 24 h LVS culture by centrifugation in a centrifuge for 15 min at 3,000 × *g*.
2. Suspend in 12× 1 mL 50 mM Tris–HCl pH 8.0.
3. Lyse 12× 1 mL bacteria in 12 tubes containing Fast-Prep beads by using Fast-Prep FP120 Cell Disrupter from Thermo Savant with four cycles (each cycle lasts 30 s and is run at speed 6.5) (*see* **Note 14**).
4. Eliminate undisrupted microbes by centrifugation for 5 min at 3,000 × *g*.
5. Centrifuge supernatant 60 min at 105,000 × *g* at 4 °C in a Beckmann Optima Max Ultracentrifuge.
6. Wash pellet, which contains bacterial membranes, twice by centrifugation 15 min, at 15,000 × *g* in a Beckmann Optima Max ultracentrifuge.
7. Solubilize for 45 min at 4 °C with 4 mL TNE containing 1 % NP40, 1 % Triton X100, protein and phosphatase inhibitors.
8. Centrifuge at 15,000 × *g* for 20 min at 4 °C to separate soluble membrane proteins present in the supernatant from membrane-associated proteins present in the pellet.
9. Determine protein concentration of different fractions by BCA assay (*see* **Note 15**).
10. Incubate 500 µg LVS membrane proteins for 2 h at 8 °C with different concentrations of biotinylated p63 peptide on a rotating wheel, in a 1.5 mL microtube.
11. Add 100 µL avidin-agarose for 2 h at 8 °C.

12. Centrifuge the beads for 1 min at 12,000 × *g* (*see* **Note 16**).
13. Add 1 mL PBS.
14. Centrifuge the beads for 1 min at 12,000 × *g*.
15. Aspirate the supernatant.
16. Repeat **steps 13–15** three times.
17. Heat the beads in 50 µL sample buffer (*see* Subheading 2.3, **item 2**) at 95 °C for 10 min.
18. Centrifuge to collect the supernatant.
19. Load a 10 % SDS-acrylamide gel and run as described in Subheading 2.3, **item 2**.
20. Electrotransfer for 2 h at 14 V with a semi-dry Trans-Blot apparatus on nitrocellulose membranes as described in Subheading 2.3, **item 2**.
21. Saturate the membrane overnight at 8 °C with PBS-T (*see* Subheading 2.3, **item 4**) containing 5 % skimmed milk.
22. For antibodies incubation 1 h at RT in PBS-T with 5 % skimmed milk on a rotating platform.
23. Incubate the membranes with mouse anti-EF-Tu antibody diluted 1/100,000 in PBS-T containing 5 % skimmed milk.
24. Incubate with HRP-linked secondary Antibodies (diluted 1/1,000 for goat anti-mouse antibodies containing 5 % skimmed milk) for 1 h at RT.
25. After incubation with antibodies, the membranes are washed extensively by adding PBS-T for 10 min on the rotating platform, throwing away the buffer and adding again PBS-T. Repeat for 1 h.
26. Detection is done with ECL chemioluminescent technics (*see* **Note 17**).
27. An example is shown in Fig. 3a. The bacterial protein(s)-biotinylated p63 peptide complexes are recovered after passage through avidin-sepharose columns (For specificity of the interaction, *see* **Note 18**).

3.6 Pull-Down Assays with GST-Beads

GST recombinant proteins used in a pull-down assay also demonstrate the specificity of EF-Tu interaction with nucleolin

1. Incubate 5 µg GST-EF-Tu, prepared as described in [13] with 50 µL glutathione-Sepharose beads for 4 h at 4 °C on a rotating wheel.
2. Wash extensively in PBS, as described in Subheading 3.5, **steps 13–16**.
3. Add 500 µg human membrane proteins, prepared as previously described [20] by solubilizing THP-1 cell membranes with

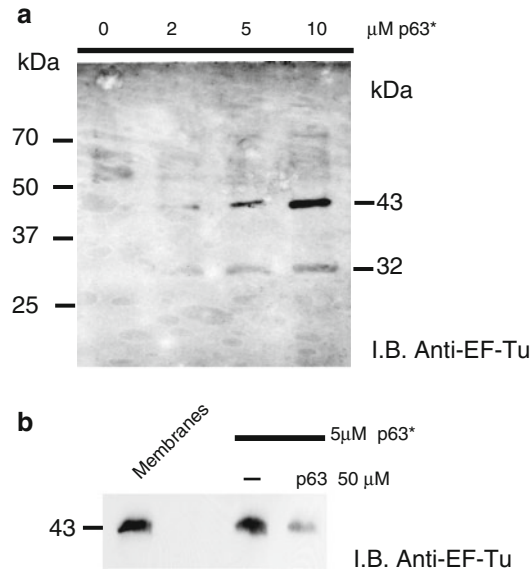

Fig. 3 Identification of bacterial ligands, for nucleolin. (**a**) 500 µg LVS membrane proteins and different concentrations of biotinylated p63 peptide were used for purification using avidin-agarose. Immunoblotting (I.B.) was performed with anti-EF-Tu Ab. The EF-Tu protein interacting with the biotinylated p63 peptide complexes is recovered. (**b**) 500 µg LVS membrane proteins were pre-incubated without (*lane* -) or with 50 µM unlabeled p63 peptide, before incubation with 5 µM biotinylated p63 peptide (p63*). Immunoblotting with anti-EF-Tu Ab diluted 1/100,000. Control: 50 µg LVS membrane proteins run directly. The specificity of the interaction between EF-Tu and the p63 peptide, which corresponds to the carboxy-terminal domain of nucleolin is confirmed by inhibiting this interaction by a 10 M excess of the unlabeled peptide, p63

1 % NP40 in TNE, containing protease and phosphatase inhibitors.

4. Incubate for 2 h at 8 °C.
5. Wash five times with TNE containing 1 % NP40, as described in Subheading 3.5, **steps 13–16**.
6. Continue with steps as described in Subheading 3.5, **steps 17–22.**
7. Incubate the membrane with anti-nucleolin MAb diluted 1/10,000.
8. Figure 4 shows that nucleolin, present in solubilized proteins from THP-1 cell membranes, interacted with recombinant EF-Tu.
9. Use GST as a negative control.

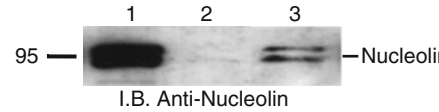

Fig. 4 Recombinant EF-Tu specifically interacts with nucleolin. 5 μg GST (*lane 2*) or GST-EF-Tu (*lane 3*) bound on glutathione-Sepharose beads were incubated with 500 μg solubilized membrane proteins. Control: 50 μg proteins solubilized from THP-1 membranes, run directly without incubation with beads (*lane 1*). Immunoblotting was performed with anti-nucleolin MAb, diluted 1/10,000. A protein doublet at 95 kDa, corresponding to human nucleolin, was detected only in GST-EF-Tu sample (*lane 3*). Nucleolin was not detected with GST (*lane 2*). *Lane 1* shows the amount of nucleolin detected in 50 μg total membrane proteins

3.7 Confocal Microscopy

Confocal miscroscopy demonstrates the co-localization of cell-surface-expressed nucleolin with EF-Tu expressed on surface of *F. tularensis*

1. Dispense a round coverslip in the wells of a 12-well sterile microtiter plate.
2. Add 1×10^6 THP-1 cells in 2 mL RPMI-FCS per assay in each well.
3. Add PMA at 200 ng/mL (from the 1 mM stock, *see* **Note 7**) 48 h at 37 °C in a CO_2 incubator, before infection.
4. Infect with 1 mL LVS-GFP (*green*), for 30 min at 37 °C.
5. Wash cells by adding 2 mL PBS and aspirating medium.
6. Repeat three times.
7. Incubate cells for 30 min at RT with 0.5 mL 5 % goat serum in PBS.
8. Incubate cells for 45 min at RT with 200 μL rabbit anti-nucleolin Ab, diluted 1/200.
9. Wash cells extensively as described in **steps 3** and **4.**
10. Incubate cells for 45 min at RT with 100 μL Alexa Fluor 546-labeled GAR (*red*) in the dark.
11. Wash as described in **steps 3** and **4.**
12. Fix cells with 400 μL 4 % PFA in PBS for 15 min at RT.
13. Incubate cells with 400 μL 50 mM NH_4Cl for 10 min at RT.
14. Wash as described in **steps 3** and **4.**
15. Add 2 mL PBS with 1 μL DAPI to color the nuclei in blue.
16. Write the name of the assays on the slide, with two assays per slide.
17. Dispense 10 μL Mowiol on slide for each assay.
18. Put glass coverslip with the cells facing the Mowiol (*see* **Note 19**).

19. Visualize cells (*see* Fig. 5) at the appropriate wavelengths under a confocal microscope with argon (458/488 nm) and helium neon (543 nm) lasers with analysis software. Co-localization is materialized, by merging of red with green resulting in yellow (*see* **Note 20**).

4 Notes

1. Antibiotics should be avoided in cell culture so that infection by *Francisella* bacteria will not be prevented.
2. Bacterial glycerol stocks are prepared from an overnight culture of bacteria in a shaking incubator at 37 °C. For 1 mL glycerol stock, 0.85 mL bacteria are gently but thoroughly mixed with 0.15 mL 100 % glycerol. Each mL is dispensed in a vial and immediately put at −80 °C until further use.
3. 100 mL of human serum are thawed at RT under sterile conditions in a Type 2 microbiological safety cabinet. Thawed serum is put immediately on ice. Once completely thawed, serum is dispensed in sterile tubes by 4 mL aliquots, refrozen, and conserved at −20 °C. When required an aliquot is thawed and is used only once.
4. For serial dilutions of bacteria-infected cells, dispense 0.9 mL distilled sterile water in one 5-mL sterile tube with caps and 0.9 mL 0.15 M NaCl in four 5-mL sterile tubes with caps.
5. Tris has a p*K*a of 8.1. So be careful, when adjusting pH with HCl to add it drop by drop, using a teated pipet. Fume of concentrate HCl is therefore avoided.
6. A layer of isopropanol is dispensed on the running gel, immediately after loading into the apparatus. This is to form a straight surface on the gel, to avoid differences in protein migration. When the gel has polymerized, rinse three times with water to eliminate the isopropanol.
7. Be careful to wear gloves, when manipulating PMA, which is a known potent tumor promoter. To avoid weighing, dilute the stock powder directly into the vial at a 1 mM concentration with distilled water, wearing a mask and under a hood.
8. Paraformaldehyde (PFA), a formaldehyde-releasing agent, is a suspected carcinogen. Therefore, use of 16 % PFA in sealed vials, to be diluted with sterile PBS is recommended. Manipulate with gloves.
9. Necessary for quenching residual aldehydes.
10. For all experiments with bacteria, one vial of frozen bacteria (*see* **Note 2**) is thawed, by adding directly into the vial 1 mL Schaedler containing K3. Then, the suspended bacteria are diluted

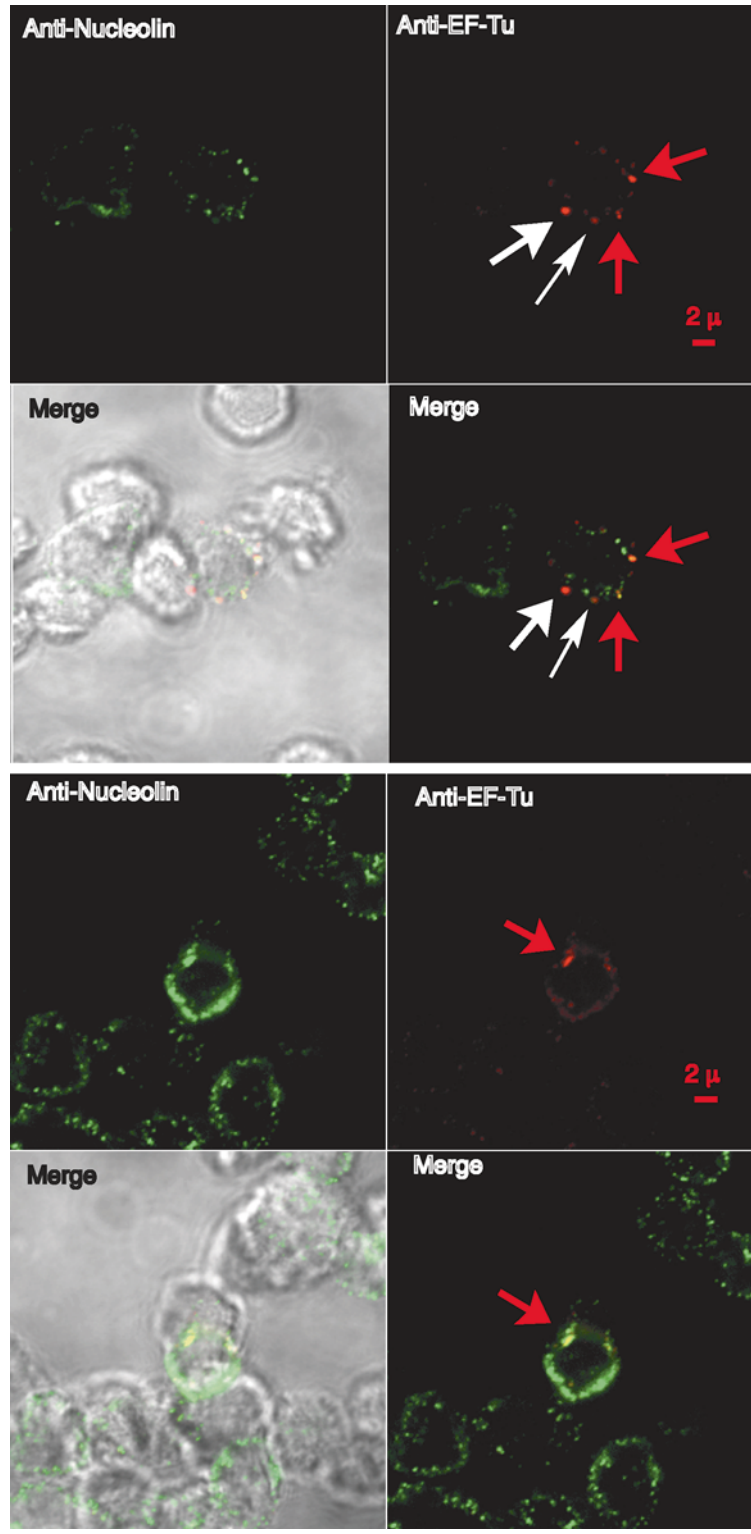

Fig. 5 Cell surface nucleolin co-localizes with LVS elongation factor Tu. Confocal microscopy at 63× magnification of THP-1 cells incubated with LVS. Human cell surface labeled with rabbit anti-nucleolin Ab diluted 1/200 (*green*). Bacteria labeled with murine anti-EF-Tu Ab diluted 1/2,000 (*red*). Merging observed with 3× Zoom either with fluorescence light (*right panel*) or as bright field (*left panel*).

in 13 mL Schaedler medium containing K3 and incubated overnight at 37 °C. Then bacteria are used for experiments.

11. Before infection of cells, bacteria are systematically incubated in the presence of human serum to obtain opsonized bacteria, due to the presence of active complement in the human serum [5], prepared as described in **Note 2**. This improves the binding of the bacteria on the cells [21].
12. For counting cells, 0.1 mL of cell suspension is drawn with a sterile pipette and dispensed in a 96-well microtiter plate. 0.1 mL of trypan blue solution is added. After mixing thoroughly, an aliquot is dispensed on a Malassez cell. Cells are counted under a light microscope with a cell counter. By counting two complete lines, the number read on the cell counter is multiplied by 10^4 and gives directly the number of cells as $\times 10^6$.
13. An MOI of 100:1 is necessary to improve the results as this method lacks in sensitivity.
14. Place bacteria on ice between each cycle, for 30 s to prevent the tubes from breaking because of the heat.
15. BCA assay was performed in a 96 well microtiter plate with flat bottom with Pierce kit. Standard curve was performed with 10 microL BSA at different concentrations (range from 100 to 1,600 mg/mL) in duplicate. Samples were tested in duplicate with 2 microL each in 10 microL RIPA lysis buffer. Add 200 microL mixture A:B (50:1) provided in the kit in each well. Calculate first the total volume needed and add three to four wells to the total number of assays, so as to add the same mixture to the whole test (to avoid problem of wrong volume with different pipets). Incubate for 30 min at 37 °C in the dark. Read in a densitometer at 560 nm.
16. To prevent the loss of beads after the multiple washes, be sure to put the 1.5 mL microtube in the centrifuge always with the opening of the tube facing the center of the centrifuge.
17. After last washes are over, membranes are put in a dry container. Add 8 mL ECL (mixture of ECL components A:B (1:40) for a 8×6 cm membrane) made extemporaneously on the membrane for 5 min, with rotation of the container. Then, membranes are put in a Hypercassette (Amersham), on a sheet of plastic, so that the membranes do not stick to the screens of

Fig. 5 (continued) *Red arrows* indicate co-localization (*yellow*) of LVS with nucleolin present on cell surface. *White arrows* indicate LVS bound on cell surface in the absence of nucleolin. The two photos are representative of five different experiments. Merging of nucleolin with LVS EF-Tu was observed only when EF-Tu was present on LVS bacteria meet clustered patches of nucleolin (*see red arrows*). No merging was observed when no nucleolin was present for LVS (*see white arrows*). This experiment demonstrates interaction of nucleolin with LVS EF-Tu at THP-1 cell surface

the cassette by direct contact. Then an autoradiography film is put in a dark room and the film is left for the first time for 5 min. After developing the film, the result is analyzed and depending on it, a new film may be put and left for shorter or longer times.

18. An interaction between two proteins may be demonstrated to be specific by using a peptide corresponding to a domain of one of the proteins, always used in a 10 M excess.
19. For best results in confocal analysis, dispense in a 12-well sterile microtiter plate 2 mL PBS in one well, and 2 mL distilled water in another well. With a forceps, hold one coverslip, plunge it rapidly first into PBS, then into water. The water rinses away the crystal salts present in PBS. If not done so, presence of crystal salts on the slide may damage the quality of confocal analysis. Water is drenched from the coverslip by capillarity on a sheet of paper. Then, the side of the coverslip with the cells is placed on the drop of Mowiol.
20. Quantification of co-localization may be performed using a special software such as Image J, which can be downloaded (http://rsb.info.nih.gov/ij).

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Chapter 8

Hijacking the Host Proteasome for the Temporal Degradation of Bacterial Effectors

Tomoko Kubori, Andree M. Hubber, and Hiroki Nagai

Abstract

To establish infection, intracellular pathogens need to modulate host cellular processes. Modulation of host processes is achieved by the action of various "effector proteins" which are delivered from the bacteria to the host cell cytosol. In order to orchestrate host cell reprogramming, the function of effectors inside host cells is regulated both temporally and spatially. In eukaryotes one of the most prominent processes used to degrade proteins is the ubiquitin-proteasome system. Recently it has emerged that the intracellular pathogen *Legionella pneumophila* is able to achieve temporal regulation of an effector using the ubiquitin-proteasome system. After establishing its replicative niche, the *L. pneumophila* effector SidH is degraded by the host proteasome. Most remarkably another effector protein LubX is able to mimic the function of an eukaryotic E3 ubiquitin ligase and polyubiquitinates SidH, targeting it for degradation. In this paper we describe a method to detect the polyubiquitin-modified forms of SidH in vitro and in vivo. Analyzing the temporal profile of polyubiquitination and degradation of bacterial effectors aids towards our understanding of how bacteria hijack host systems.

Key words Proteasome, Polyubiquitin, E3 ligase, Immunoprecipitation, *Legionella*, Infection, Effector, Metaeffector, Temporal regulation

1 Introduction

1.1 Background

Ubiquitin is one of the most conserved post-translational modifications of proteins in eukaryotic cells [1]. Ubiquitination of target proteins requires the transfer of free ubiquitin to a target protein in a successive process that requires at least three distinct steps catalyzed by enzymes: E1 activating enzymes, E2 conjugation enzymes, and E3 ubiquitin ligases [2]. E3 ubiquitin ligases are responsible for substrate specificity by directly interacting with target proteins [3]. Among the various cellular processes in which the ubiquitin system is involved, degradation of polyubiquitin-tagged proteins by 26S proteasome is one of the most widely observed processes [4, 5].

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Pathogenic bacteria have evolved specialized protein secretion systems to translocate a wide array of "effector proteins" into eukaryotic host cells. Distinct types of "nanomachines," referred to as type III, IV, and VI secretion systems, are utilized to directly inject bacterial proteins into the host cell cytosol. These effector proteins modulate many cellular processes essential for establishing a permissive infection. Host processes targeted by pathogens include those required for the uptake, trafficking, and degradation of pathogens inside cells. Effectors that function to induce cytoskeleton rearrangements, alter phagosome trafficking, interfere with cellular signaling, and escape host immune responses have been described (for recent reviews, *see* [6–8]). Therefore, effector proteins play a key role in hijacking of host cellular processes upon bacterial infection.

Salmonella enterica serovar Typhimurium type III secretion system effectors SopE and SptP are involved in host cell actin cytoskeleton rearrangement. The morphological change in host cell membrane induced by actin reorganization is required for *Salmonella* invasion to non-phagocytic cells. The effector proteins SopE and SptP target Rho family GTPases and possess guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP) domains, respectively. At the early stage of infection, the GEF activity of SopE activates the GTPases Rac1 and Cdc42 to induce the cellular responses leading to actin cytoskeleton rearrangement. This results in membrane ruffling which enables *Salmonella* to invade non-phagocytic cells [9]. At the later stage of infection, the GAP activity of SptP shuts off the membrane ruffling to regain the normal cellular morphology [10, 11] that is optimal for *Salmonella* growth inside cells. The timing of SopE and SptP function is achieved by the differential kinetics of proteasomal degradation of the proteins. Both SopE and SptP are polyubiquitinated in cells. SopE is degraded by the host ubiquitin-proteasome system immediately after host cell invasion, whereas SptP is degraded with a much longer half-life [12].

Legionella pneumophila uses a type IV secretion system, named Dot/Icm, to translocate at least 270 effector proteins into eukaryotic host cells [13]. Some of these effector proteins are implicated in modulating and/or hijacking the host ubiquitin pathway [14–18]. We found that LubX is one such effector that functions as a U-box-type E3 ubiquitin ligase [19]. U-box is a conserved domain for U-box type E3 ubiquitin ligases [20–22]. LubX contains two U-box domains. LubX U-box1 acts as a canonical U-box, which is a binding domain to E2 ubiquitin conjugation enzymes, whereas U-box-2 has a non-canonical function, as a substrate-binding domain [19]. We found that one LubX substrate is the bacterial effector SidH. In vitro binding experiments showed that SidH can directly bind to LubX, and modification of SidH with polyubiquitin was observed both in vitro and in vivo. Most significantly, ubiquitination of SidH depended on the catalytic activity of LubX;

mutant LubX with the amino acids mutation I39A (U-box 1 domain) failed to ubiquitinate SidH [23]. SidH is translocated into host cells soon after infection, whereas LubX expression/translocation is delayed [19]. Temporal regulation of SidH function is achieved by the delayed delivery of LubX at later stages of infection, as translocation of LubX correlates with the kinetics of SidH degradation. Presumably at the later stages of infection, the presence of SidH is somehow inhibitory to optimal bacterial reprogramming of cells. Thus LubX functions to eliminate at least one outlived effector during infection. This unexpected finding gives rise to a new term, "metaeffector," that can be used as a designation for an effector protein that regulates the function of another effector within host cells [23].

1.2 Overview of the Methods

This book chapter describes the protocol we used for detection of ubiquitinated SidH. This protocol consist of an in vitro ubiquitin ligase assay using purified proteins, as well as the detection of polyubiquitination in cells infected with *L. pneumophila*.

In the first section (Subheading 3.1), we describe in vitro ubiquitin ligation reaction using purified LubX E3 ubiquitin ligase and its substrate protein SidH. For detection of the ubiquitination of specific substrate protein, background signals derived from self-ubiquitination on E2 and E3 enzymes should be eliminated. This is achieved by the isolation of His-SidH by pull-down using nickel affinity resin after the in vitro ubiquitin ligase reaction. Eluted proteins are analyzed by SDS-PAGE and immunoblotting using antibodies against ubiquitin and SidH. This technique is applicable for other combinations of E3 ligases and substrate proteins by applying epitope-tagging to the proteins for pull-down and detection.

The second part is further divided into two subsections, Subheadings 3.2 and 3.3. In Subheading 3.2, we first describe a method to fractionate cell lysate containing translocated bacterial proteins after infection. We use Chinese hamster ovary (CHO)-FcγRII cells infected with *L. pneumophila*. In general, the amount of *Legionella* effector proteins translocated into host cells is not very high. To increase the chance of SidH detection in the cell lysate, we added a 3xFLAG epitope tag on the chromosome-encoded *sidH* gene. This strain was used as the parental strain to create a Δ*lubX* deletion derivative and a strain expressing LubX with the I39A mutation (*see* **Note 1** and Fig. 2 legend). At different time points after infection, 1 % (w/v) digitonin is added to infected cells, as digitonin permeabilizes eukaryotic but not bacterial cells. Centrifugation then separates the solution into supernatant (cell lysate) and pellet (insoluble materials and intact bacteria) fractions. The cell lysate should be free from any residual bacteria for specific detection of translocated bacterial proteins (not bacteria-residing proteins), so filtration of the cell lysate before further analysis is highly recommended. For detection of ubiquitinated proteins, a proteasome inhibitor such as MG132 should

be included throughout the entire procedure; otherwise the ubiquitin-modified proteins may be degraded by the proteasome. Indeed, proteasomal degradation of ubiquitin-modified proteins can be demonstrated by comparing the results with and without MG132 treatment. Adding *N*-ethylmaleimide (NEM), an inhibitor of deubiquitination, in the digitonin lysis buffer may help the detection of ubiquitinated proteins.

In Subheading 3.3, we describe an immunoprecipitation technique to pull-down a desired polyubiquitinated protein using the cell lysate obtained in Subheading 3.2, **step 1** and an antibody against the epitope-tagged protein. Here we describe the method using 3xFLAG-tagged SidH. We also successfully detected LubX using antibody against LubX (Fig. 2).

2 Materials

Prepare all solutions using sterilized Milli-Q water.

2.1 In Vitro Ubiquitin Ligase Assay

1. Recombinant human ubiquitin, recombinant rabbit E1 enzyme (E-302) and recombinant human E2 enzyme UbcH5a (E2-616).
2. Reaction buffer containing ATP (×2): 100 mM Tris–HCl, pH 7.5, 4 mM $MgCl_2$, 240 mM NaCl, 4 mM ATP, and 2 mM DTT.
3. Purified E3 ubiquitin ligases. In this case we used LubXΔC and LubXΔCI39A (*see* **Note 1**) prepared as described previously [19, 24].
4. Purified substrate proteins. In this case we constructed His-tagged SidH and purified it as previously described [23].
5. Pull-down buffer (PD buffer): 50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.1 % (w/v) TritonX-100.
6. Wash buffer (PDW buffer): 2.5 mM imidazole in PD buffer.
7. Elution buffer (PDE buffer): 250 mM imidazole in PD buffer.
8. 50 mM Tris–HCl, pH 7.5.
9. His-select Ni-Affinity Gel.
10. Antibody against ubiquitin (horseradish peroxidase-conjugated mouse monoclonal antibody to ubiquitin).
11. Antibody against the substrate protein or against epitope-tag peptide fused to the substrate protein. In this case, anti-serum against SidH peptide CQNIKGPEPVATPMETPE (SidH 2196 2212) was produced by MBL (Nagoya, Japan) and was purified by affinity chromatography using peptide-conjugated SulfoLink resins.
12. Antibodies against LubX and RalF as previously described [19, 25].

13. 30 °C heating block.
14. 14, Eppendorf tubes.
15. Ice.
16. Rotating wheel in cold chamber/room.
17. Refrigerated microcentrifuge.
18. 7.5 % polyacrylamide gel and gel apparatus for SDS-PAGE.
19. PVDF membrane for Western immunoblotting.

2.2 Fractionation of Legionella-Infected Cells for Detection of Ubiquitinated Effector Protein

1. CHO-FcγRII cells [26, 27] (*see* **Note 2**).
2. *L. pneumophila* strains. We use Lp01 derived strains expressing 3xFLAG-tagged SidH carrying wild-type *lubX* or *lubX* I39A mutation which destroys the E3 ligase activity of LubX [19, 23].
3. Charcoal-yeast extract (CYE) plates to grow *L. pneumophila* [28].
4. Tubes.
5. 37 °C incubator for bacterial plates.
6. α–MEM (Minimum Essential Medium) supplemented with heat-inactivated 10 % Fetal Bovine Serum (FBS).
7. DPBS (Dulbecco's Phosphate Buffered Saline).
8. Anti-*Legionella* antibody.
9. Dimethyl sulfoxide (DMSO).
10. MG132 (Calbiochem): 10 mM solution in DMSO.
11. Sterile water to suspend bacterial cells.
12. Spectrophotometer to measure optical density at 600 nm.
13. 10 % (w/v) digitonin solution dissolved in water.
14. Protease inhibitor cocktail (for mammalian cells and tissue extracts).
15. 10× DPBS (Dulbecco's Phosphate Buffered Saline).
16. 1 M *N*-ethylmaleimide (NEM) freshly dissolved in DMSO on the day of the experiment.
17. Cell scrapers.
18. Millex-HV 0.45 μm pore sized Syringe Driven Filter Unit (Low Protein Binding PVDF Membrane).
19. 1 mL syringes fitted to the Millex-HV filters.
20. 6 well plates.
21. 5 % CO_2 incubator.
22. Plate centrifuge.
23. Wet blotting transfer systems.

2.3 In Vivo Detection of Degradation of Ubiquitinated Proteins in Legionella-Infected Cells

1. nProtein A Sepharose 4 Fast Flow (GE Healthcare).
2. Immunoprecipitation (IP) buffer: 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1 mM Etylenediaminetetraacetic acid (EDTA), 2 % (w/v) Triton X-100, filtered.
3. 50 mM Tris–HCl, pH 7.5.
4. SDS sample buffer (2×): 125 mM Tris–HCl, pH 8.5, 4 % (w/v) Sodium dodecyl sulfate (SDS), 10 % (v/v) β-mercaptoethanol, 20 % (v/v) glycerol, trace amount of bromophenol blue (BPB).
5. Antibody against the FLAG-tag.
6. Boiling water bath.

3 Methods

3.1 In Vitro Ubiquitin Ligase Assay

1. Mix the following items in 25 μL total volume (adjust the volume with sterile Milli-Q water) in tubes placed on ice; 2 μL of 5 mg/mL recombinant human ubiquitin (10 μg), 0.56 μL of 4.5 μM of recombinant rabbit E1 enzyme (final concentration 100 nM), 2.0 μL of 2.5 μM recombinant human UbcH5a (final concentration 200 nM), 2.0 μL of 0.5 mg/mL (~22.0 μM) of purified LubXΔC or LubXΔCI39A (*see* **Note 1**) (final concentration ~1.8 μM), 4.0 μL of 1.0 mg/mL (~4.0 μM) of purified His-SidH (final concentration ~600 nM), and 12.5 μL of 2× reaction buffer containing ATP (2 mM final concentration).
2. Incubate the reaction mixture for 2 h at 30 °C.
3. Place the tubes on ice. Bring total volume of solution up to 1.1 mL with the cold PD buffer.
4. Take 100 μL of samples as "pre-pull-down samples" and keep them frozen in case of trouble shooting.
5. To each tube from the **step 3** add 30 μL of 50 % (v/v) suspension of His-select Ni-Affinity Gel, pre-equilibrated with the PD buffer.
6. Incubate the tubes on a rotating wheel with slow rotation at 4 °C for 1–2 h.
7. Wash the resin by centrifugation (5,000 × *g*, 30 s at 4 °C) and resuspension of the pellet with 1.0 mL of cold PDW buffer.
8. Repeat **step 7** three times.
9. Finally wash the resin with 1.0 mL of cold 50 mM Tris–HCl, pH 7.5. Remove supernatant.
10. Elute bound proteins from the resin by adding 50 μL of cold PDE.
11. Incubate the tubes on ice for 10 min.

12. Centrifuge the tubes (8,000 × *g*, 1 min at 4 °C) and recover the supernatant into a new tube on ice.
13. Apply an additional 30 μL of cold PDE on the resin, and repeat **steps 11** and **12**.
14. Combine the eluent (total comes to 80 μL).
15. Analyze the samples by SDS-PAGE and immunoblotting using antibodies against ubiquitin and SidH (*see* **Notes 3** and **4**). Amounts of samples subjected to SDS-PAGE analysis should be empirically determined according to antibodies used and sensitivity of your detection system for Western immunoblotting.

3.2 Fractionation of Legionella-Infected Cells for Detection of Ubiquitinated Effector Protein

1. Two days before infection, pick a single colony of *L. pneumophila* strain producing 3xFLAG-tagged SidH and spread uniformly on a CYE plate. Do the same for the strain carrying *lubX*I39A mutation (optional). Incubate plates at 37 °C for 48 h.
2. Plate CHO-FcγRII cells the day before infection at a density of 3×10^5 cells per well of 6-well tissue culture dish in 2 mL of medium (α – MEM with 10 % heat-inactivated FBS) and incubate in a 5 % CO_2 incubator at 37 °C. Typically two wells were used for infection per time point per *L. pneumophila* strain.
3. Next day, the CHO cells should be semi-confluent and *L. pneumophila* strains should grow heavily on CYE plates.
4. Exchange media 30 min prior to infection with pre-warmed fresh media (2 mL per well) with serum (without antibiotics) containing the opsonizing anti-*Legionella* antibody (*see* **Note 2**) at 1:3,000 dilution. In case you want to examine whether SidH is subjected to proteasomal degradation, add 10 μM (final concentration) MG132 (*see* **Note 5**) or the equivalent amount of DMSO (negative control).
5. Scrape a portion of heavy patch of *L. pneumophila* grown on CYE plates and resuspend bacterial cells with sterile water. Measure OD_{600} of the suspensions using a spectrophotometer, with appropriate dilution with water. Prepare suspensions of $OD_{600} = 1$ by dilution with water. The resulting suspensions contain approximately 1×10^6 bacterial cells/mL.
6. Infect the cells with *L. pneumophila* at a multiplicity of infection (MOI) of 30 (9 μL of the bacterial suspensions of the **step 5**) and immediately spin down the bacteria at 220 × *g* for 5 min at 25 °C.
7. Incubate the plate at 37 °C (*see* **Note 6**).
8. At 30 min post-infection, wash the cells with 2 mL each of pre-warmed (37 °C) DPBS three times. Then add 2 mL of pre-warmed (37 °C) fresh medium with serum. In case you

used MG132 or DMSO in the **step 4**, add 10 μM (final concentration) MG132 or the equivalent amount of DMSO (*see* **Note 5**).

9. Incubate further at 37 °C until desired time points (15 min to 10 h) to see kinetics of SidH ubiquitination and degradation in infected cells.
10. During infection, mix 100 μL of 10 % (w/v) digitonin stock solution (final 1 %(w/v)), 100 μL of 10× DPBS, 10 μL of 100× protease inhibitor cocktail, 1 μL of 10 mM MG132 (final 10 μM) or DMSO, and 10 μL of 1 M NEM (final 10 mM) in total volume of 1 mL (enough for one 6-well dish) adjusted with sterilized Milli-Q water. Place on ice until use.
11. Place the dishes on ice, and wash the cells with 2 mL per well of cold PBS three times.
12. Add 150 μL of the 1 % digitonin solution to each well.
13. Scrape the cells from the surface of the dishes using cell scrapers and collect into tubes on ice.
14. Centrifuge (13,000 × *g*, 10 min at 4 °C) the tubes to remove insoluble materials and recover the supernatant. The pellet fractions can be stored as "insoluble bacterial fractions" to examine whether bacteria-associated proteins are not released in digitonin soluble fractions in case of trouble shooting.
15. (Optional) To remove particulates which may interfere with the following immunoprecipitation processes, filter the supernatants using Millex-HV 0.45 μm pore filters with 1 mL syringes on ice.
16. Samples can be frozen at −80 °C or kept on ice for further processing (Subheading 3.3).

3.3 In Vivo Detection of Degradation of Ubiquitinated Proteins in Legionella-Infected Cells

All procedures should be carried out on ice or at 4 °C.

1. Thaw frozen cell lysates from Subheading 3.2, **step 16** slowly on ice, or use unfrozen lysates kept on ice.
2. To remove the possible aggregates formed during storage, centrifuge in a benchtop microfuge (max speed, 5 min at 4 °C) and recover the supernatant.
3. (Optional) Pre-absorption: Add 30 μL of 50 % (v/v) suspension of nProtein A Sepharose, equilibrated with IP buffer, to the cleared lysate.
4. Incubate the tubes on a rotating wheel with slow rotation at 4 °C for 2–3 h.
5. Centrifuge the tubes in a microfuge (500 × *g*, 3 min at 4 °C) and recover the supernatant. Keep a portion of the supernatant as a "pre-IP sample" in case of trouble shooting.

6. Immunoprecipitation: Add 10 μg of antibody against the FLAG-tag (for isolation of SidH) to each tube.
7. Incubate the tubes with slow rotation at 4 °C for 5 h to overnight (~18 h).
8. Add 30 μL of 50 % (v/v) suspension of nProtein A Sepharose equilibrated with IP buffer.
9. Incubate the tubes on a rotating wheel with slow rotation at 4 °C for 5–6 h.
10. Wash the resin with centrifugation in a microfuge (500 × *g*, 3 min at 4 °C) and suspend the pellet with 1.0 mL of cold IP buffer.
11. Repeat **step 10** another three times.
12. Wash the resin by centrifugation in a microfuge (500 × *g*, 3 min at 4 °C) and suspend the pellet with 1.0 mL of cold 50 mM Tris–HCl, pH 7.5.
13. Repeat **step 12** and suspend the pellet with 20 μL of 2× SDS sample buffer.
14. Boil the tubes for 5 min, and place the tubes on ice.
15. Centrifuge the tubes in a microfuge (10,000 × *g*, 3 min at 4 °C) and recover the eluent (supernatant).
16. Analyze the eluted samples by SDS-PAGE and immunoblotting (*see* **Notes 4** and **7**) using antibodies against the FLAG-tag (for detection of SidH).

4 Notes

1. LubX has a C-terminal domain which functions as translocation signal. The LubX truncation lacking the C-terminal domain (LubXΔC) is routinely used for our biochemical analyses because removing the C-terminal domain increased the solubility of the protein. LubXΔCI39A is a derivative of LubXΔC which lacks the catalytic activity [19].
2. *L. pneumophila* can only invade phagocytic cells. Non-phagocytic cell-lines can be used when Fcγ receptor II (FcγRII) is ectopically expressed, which enables *L. pneumophila* to be taken up via Fc-mediated phagocytosis [26, 27]. In this case, the use of opsonizing antibody is mandatory.
3. Expected results are shown in Fig. 1. You will see high molecular weight derivatives of His-SidH which react both with anti-His and with anti-ubiquitin antibodies. Ubiquitinated His-SidH should be detected in the sample containing wild-type LubXΔC, but not in that containing I39A mutant protein. Another effector protein RalF was not ubiquitinated in a LubX-dependent manner.

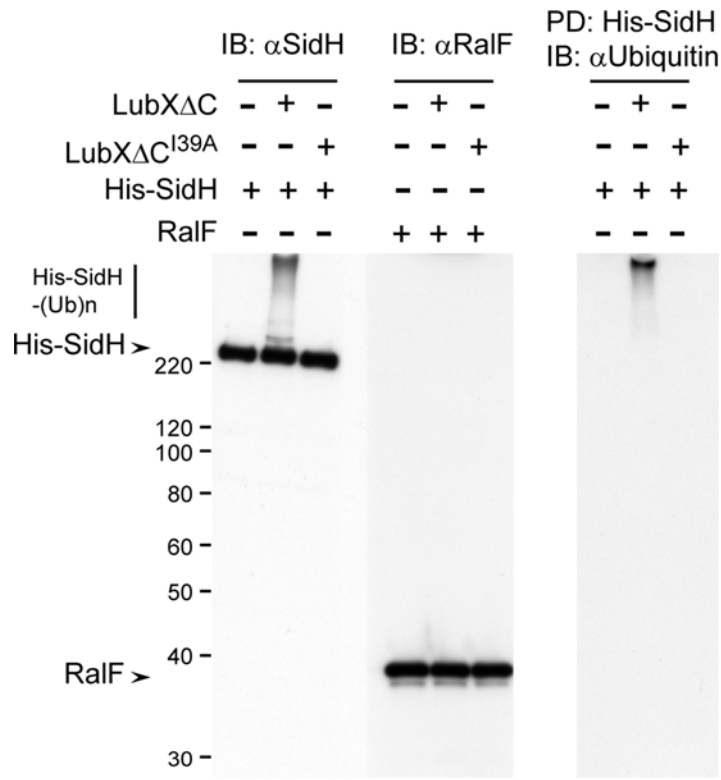

Fig. 1 LubX promotes ubiquitination of SidH in vitro. In vitro ubiquitin ligation reaction containing indicated E3 and substrate proteins were carried out. The reaction mixtures were analyzed by immunoblotting using anti-SidH or anti-RalF antibodies (RalF is a negative control protein which is not a substrate of LubX). Pulled-down material was analyzed by immunoblotting using an anti-ubiquitin antibody. *Numbers* at the *left side* of the images designate positions of molecular weight markers (in kDa). Polyubiquitin-modified proteins are detected as high molecular weight ladder bands

4. For detection of high molecular weight ladders typically observed for polyubiquitinated proteins, wet transfer systems are recommended over semi-dry transfer systems because of higher transfer efficiencies of high molecular weight proteins.
5. MG132 is a reversible proteasome inhibitor. Therefore, it has to be maintained in the cells throughout the entire procedure.
6. Especially for short time-course experiments, rapidly bringing the temperature of the media up to 37 °C is important in order to obtain reproducible results. For this purpose we float dishes in a 37 °C water-bath for 5 min. We then carefully remove the dishes from the water-bath, wipe off the residual water from the edges of the dishes with paper towels, and place the dishes into a 5 % CO_2 incubator at 37 °C.
7. Expected results are shown in Fig. 2. Because of limitation due to low amounts of translocated 3xFLAG-SidH in infected

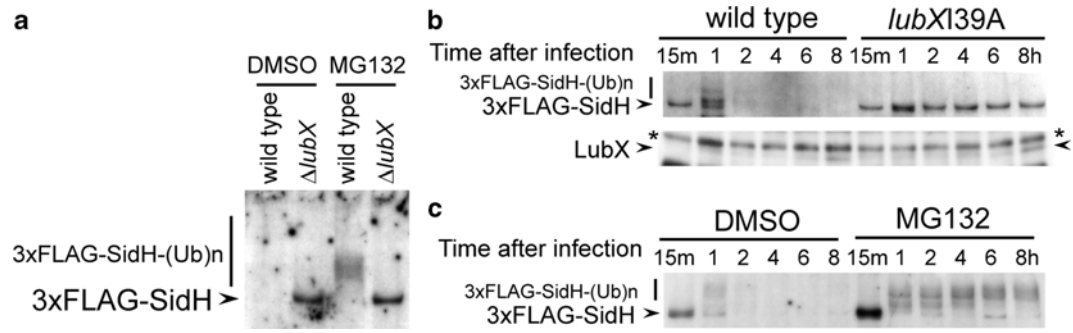

Fig. 2 LubX-mediated ubiquitination and proteasomal degradation of SidH within the host cells. (**a**) CHO-FcγRII cells were infected for 8 h with wild-type or *lubX* deletion *L. pneumophila* strains carrying the 3xFLAG-tagged *sidH* gene. 10 μM MG132 or an equivalent amount of DMSO was employed throughout the experiment. One percent digitonin extracts of the infected cells were subjected to immunoprecipitation with anti-FLAG antibody and immunoprecipitates were analyzed by immunoblotting using anti-FLAG antibody. (**b**) Time course of intracellular SidH and LubX levels after infection. CHO-FcγRII cells were infected for the times indicated with wild-type or *lubXI39A* U-box1 dead mutant *L. pneumophila* strains carrying the 3xFLAG-tagged *sidH* gene in the absence of MG132. Immunoprecipitation and immunoblotting were carried out as in (**a**) using anti-FLAG or anti-LubX antibody. The *asterisks* denote a nonspecific signal. (**c**) Shut down of SidH requires host proteasome. CHO-FcγRII cells were infected with a wild-type *L. pneumophila* strain carrying the 3xFLAG-tagged *sidH* gene in the presence of 10 μM MG132 or an equivalent amount of DMSO. Immunoprecipitation and immunoblotting were carried out as in (**a**) using anti-FLAG antibody

cells, it is difficult to show that high-molecular weight 3xFLAG-SidH derivatives react with anti-ubiquitin antibody using Western immunoblotting as in Subheading 3.1. However, you can see LubX-dependent ubiquitination of SidH (compare wild type and *lubX*I139A) and its proteasomal degradation (compare DMSO and MG132).

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Chapter 9

Live Cell Imaging of Phosphoinositide Dynamics During *Legionella* Infection

Stephen Weber and Hubert Hilbi

Abstract

The "accidental" pathogen *Legionella pneumophila* replicates intracellularly in a distinct compartment, the *Legionella*-containing vacuole (LCV). To form this specific pathogen vacuole, the bacteria translocate via the Icm/Dot type IV secretion system approximately 300 different effector proteins into the host cell. Several of these secreted effectors anchor to the cytoplasmic face of the LCV membrane by binding to phosphoinositide (PI) lipids. *L. pneumophila* thus largely controls the localization of secreted bacterial effectors and the recruitment of host factors to the LCV through the modulation of the vacuole membrane PI pattern. The LCV PI pattern and its dynamics can be studied in real-time using fluorescently labeled protein probes stably produced by the soil amoeba *Dictyostelium discoideum*. In this chapter, we describe a protocol to (1) construct and handle amoeba model systems as a tool for observing PIs in live cell imaging, (2) capture rapid changes in membrane PI patterning during uptake events, and (3) observe the dynamics of LCV PIs over the course of a *Legionella* infection.

Key words Amoeba, *Dictyostelium discoideum*, Effector proteins, *Legionella pneumophila*, Pathogen vacuole, Phosphoinositides, Real-time fluorescence microscopy, Type IV secretion

Abbreviations

ACES	*N*-(2-acetamido)-2-aminoethane-sulfonic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
icm/dot	Intracellular multiplication/defective organelle trafficking
MOI	Multiplicity of infection
PI	Phosphoinositide
PtdIns(4)*P*	Phosphatidylinositol-4-phosphate
T4SS	Type IV secretion system

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Methods in Molecular Biology, vol. 1197, DOI 10.1007/978-1-4939-1261-2_9,

1 Introduction

1.1 Subversion of Host Phosphoinositide Lipids by Legionella pneumophila

The environmental bacterium *Legionella pneumophila* is a natural parasite of protozoa, which upon inhalation reaches the human lung, replicates in alveolar macrophages and causes a severe pneumonia termed "Legionnaires' disease" [1, 2]. The mechanism of intracellular replication in amoeba and macrophages is evolutionarily conserved, and the process centers on the formation of a distinct membrane-bound compartment, the "*Legionella*-containing vacuole" (LCV). LCV formation is an astonishingly complex process that relies on the bacterial Icm/Dot type IV secretion system (T4SS) and more than 300 different "effector proteins" [3–6]. While the function of most *Legionella* effectors is unknown, some of them show intriguing novel biochemical activities and target small GTPases or phosphoinositide (PI) lipids [7].

Eukaryotic cells employ small GTPases and PI lipids to regulate vesicle trafficking, cytoskeleton dynamics and signal transduction [8, 9]. To this end, the *myo*-inositol carbohydrate head group of PI lipids is reversibly modified by kinases or phosphatases at the 3, 4, and/or 5 position, yielding seven distinct mono- or polyphosphorylated lipids, which define organelle identity and membrane dynamics.

Over the last couple of years, *L. pneumophila* has been shown to produce a number of effector proteins, which after translocation by the Icm/Dot T4SS selectively bind to specific PI lipids on the LCV membrane [10]. These effectors anchor to the cytoplasmic face of the pathogen vacuole and expose their "business end" to the cytoplasm of the infected host cell. Thus, PtdIns(4)*P*, a marker of the secretory pathway, is bound by the ER interactor SidC (and its paralogue SdcA) [11, 12], as well as by the Rab1 guanine nucleotide exchange factor (GEF) SidM (also termed DrrA) [13]. The endosomal PI PtdIns(3)*P*, on the other hand, is bound by the virulence factor LpnE possibly interacting with the PI phosphatase OCRL/Dd5P4 [14], as well as by the glycosyl transferase SetA [15]. Finally, the Icm/Dot substrate LidA preferentially binds mono-phosphorylated PI lipids [13].

PtdIns(4)*P* accumulates in an Icm/Dot-dependent manner on LCVs [11] and might be produced by the Icm/Dot-translocated effector SidF, a PI polyphosphate 3-phosphatase that specifically hydrolyzes the 3-phosphate of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [16]. In addition, the host PI 4-kinase IIIβ (PI4KIIIβ) [13] and the PI 5-phosphatase OCRL1/Dd5P4 [14] could contribute to the production of PtdIns(4)*P* on the LCV membrane.

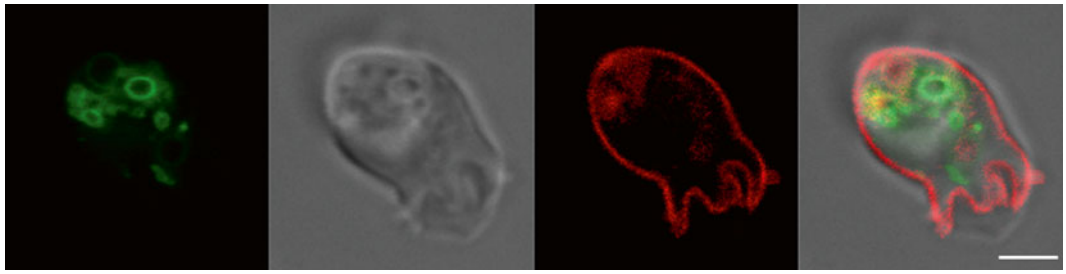

Fig. 1 Bright field and fluorescence images of dually transfected *Dictyostelium discoideum* amoeba. *D. discoideum* producing 2FYVE-GFP, labeling endosomal compartments, and $P4C_{SidC}$-mRFPmars, labeling primarily the plasma membrane pool of PtdIns(4)*P* with faint Golgi localization, is shown. Scale bar, 7.5 μm

1.2 The Amoeba Dictyostelium discoideum as a Model for Legionella Infection

The haploid social soil amoeba *Dictyostelium discoideum* is a powerful model to study cell biological and developmental processes [17], as well as host–pathogen interactions [18, 19]. *D. discoideum* can easily be cultivated and clonally grown to high numbers. A plethora of genetic tools are available for the amoeba, including the genome sequence, DNA microarrays, plasmids allowing constitutive or inducible gene expression, targeted deletions of (multiple) genes, and random mutants obtained by "restriction enzyme-mediated insertion" (REMI) mutagenesis. Specifically, a number of expression vectors for N- or C-terminal fusions with green or red fluorescent proteins are available for *D. discoideum*. These include either extra-chromosomal [20] or integrating plasmids [21, 22], encoding green fluorescent [20–22], red fluorescent [23], or green and red fluorescent proteins simultaneously [24] (Fig. 1).

Upon infection of *D. discoideum* producing calnexin-GFP (a marker of the endosomal reticulum and the LCV) with *L. pneumophila*, intact pathogen vacuoles were enriched by immunoaffinity purification and subsequently analyzed by proteomics [25]. The interactions between *D. discoideum* and *L. pneumophila* have also been studied in great detail by flow cytometry [26] or by fluorescence microscopy using amoeba strains that produce GFP fusion proteins [27]. To this end, either fixed samples were analyzed, or live cells were investigated by real-time microscopy [28].

1.3 Analysis of LCV PI Patterns by Live Cell Imaging

The use of live cell imaging for time lapse observation has clear advantages compared to fixed sections. It is the best way to view intact membrane and vesicle morphology while following the same group of cells through time. Whereas the use of antibody staining is mutually exclusive with live cells, labeling of organelle structures or cell compartments can be accomplished in addition to PI labeling by cells expressing two fluorescent fusion constructs (Table 1).

Table 1

Fluorescent protein probes for live cell imaging of phosphoinositides

Protein probes for PI lipids	Localization in live cell imaging	Reference
PtdIns(3,4,5)P_3		
PH_{GRP1}	Plasma membrane	[33]
PtdIns(3,4,5)P_3/PtdIns(3,4)P_2		
PH_{Akt}	Plasma membrane	[34]
PH_{CRAC}	Plasma membrane	[33, 35]
PtdIns(3,4)P_2		
PH_{TAPP1}	Plasma membrane	[33]
PtdIns(3,5)P_2		
No reliable probe for imaging	n/a	
PtdIns(4,5)P_2		
$PH_{PLC\Delta1}$	Plasma membrane	[36]
PtdIns(3)P		
FYVE	Early endosomes	[37]
PtdIns(4)P		
PH_{FAPP1}	Golgi, plasma membrane	[33]
$P4C_{SidC}$	Plasma membrane, some Golgi	[12]
PtdIns(5)P		
3 × PHD	Nucleus, plasma membrane	[38]

We developed a short term observation protocol to follow rapid and immediate changes of the PI pattern on a single cell level. This method allows the operator to capture events that would otherwise proceed too rapidly to be captured in fixed sections, and even if they could be, a wealth of sequential information would be missing. This protocol is ideal for observing PI changes upon bacterial uptake and the minutes following.

In contrast to the method for short rapid observation which examines single cells, the protocol for time lapse observation works on the basis of capturing hundreds of cells at a given time to generate statistics. The quality of the statistics is dependent on the uniform distribution of infecting *L. pneumophila* and a simultaneous infection of the cells. We recommend this protocol for observing slower processes of PI accumulation or loss, which occur over a timespan of hours.

Here, we describe a protocol to (1) construct and handle amoeba model systems as tools for observing PIs in live cell imaging, (2) capture rapid changes in membrane PI patterning during uptake events, and (3) observe the dynamics of LCV PIs over the course of a *Legionella* infection.

2 Materials

Prepare all solutions using deionized, distilled water and analytical grade reagents.

2.1 *Legionella pneumophila*

1. *Legionella pneumophila* Philadelphia 1 wild-type strain JR32 and the isogenic mutant strain GS3011 (Δ*icmT*, JR32 *icmT*3011::Kan) lacking a functional Icm/Dot T4SS are used [29].
2. AYE (ACES yeast extract) medium [30]: 10 g/L *N*-(2-acetamido)-2-aminoethane-sulfonic acid (ACES), 10 g/L Bacto™ yeast extract (Difco; *see* **Note 1**), 3.3 mM L-cysteine, 0.6 mM $Fe(NO_3)_3$. Add 10 g of ACES and 10 g of yeast extract in 950 mL of H_2O. Add filter sterilized 0.4 g/10 mL L-cysteine and 0.25 g/10 mL $Fe(NO_3)_3$ solutions (*see* **Note 2**). Adjust the pH to 6.9 with 10 M KOH. To select for plasmid pSW001 or pNT-28, add 5 μg/L chloramphenicol (Cam, stock: 30 mg/mL ethanol). Pass the medium several times through a glass fiber filter paper, followed by a 0.2 μm filter cartouche. Store the medium at 4 °C in the dark (*see* **Note 3**).
3. CYE (charcoal yeast extract) agar plates [31]: 10 g/L ACES, 10 g/L Bacto™ yeast extract (Difco; *see* **Note 1**), 2 g/L activated charcoal powder (puriss. p.a.), 15 g/L agar, 3.3 mM L-cysteine, 0.6 mM $Fe(NO_3)_3$. Dissolve 10 g of ACES and 10 g of yeast extract in 950 mL of H_2O and adjust the pH to 6.9 with 10 M KOH. Transfer the solution to a 1-L Schott bottle containing 2 g of activated charcoal powder, 15 g of agar and a stir bar. Autoclave and let the agar solution cool down to 50 °C. Add filter sterilized 0.4 g/10 mL L-cysteine and 0.25 g/10 mL $Fe(NO_3)_3$ solutions (*see* **Note 2**). To select for plasmid pSW001 or pNT-28 add 5 μg/L Cam. Mix the solution on a magnetic stirrer and pour plates. Dry plates to remove condensation water and store at 4 °C for up to 6 months.
4. 15 mL tubes.
5. Rotation wheel.
6. Inverted light microscope.

2.2 *Dictyostelium discoideum*

1. The axenic *D. discoideum* strains AX3 or AX2 are used.
2. HL5 medium, modified [32]: 5 g/L BBL™ yeast extract (Becton Dickinson; *see* **Note 1**), 5 g/L Bacto™ Proteose Peptone (Becton Dickinson; *see* **Note 1**), 5 g/L BBL™ Thiotone™ Peptone (Becton Dickinson; *see* **Note 1**), 11 g/L D(+)glucose monohydrate (*see* **Note 4**), 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4. Adjust the pH with 1 M KOH or 1 M HCl to 6.5 ± 0.1. Autoclave and store the medium at 4 °C. If necessary, add Penicillin/Streptomycin (Pen/Str) or Fungizone to

maintain sterility. The modified HL5 medium supports axenic growth of *D. discoideum*.

3. LoFlo medium (ForMedium) (*see* **Note 5**).
4. Standard Petri dishes or 75 cm^2 cell culture flasks.
5. Plasmid DNA (Table 1).
6. Antibiotics for cell culture: 10 μg/mL G418 (stock: 10 mg/mL H_2O), 10 μg/mL Blasticidin-S (stock: 10 mg/mL H_2O), 100 U/mL Penicillin (stock: 10,000 U/mL), 0.1 mg/mL Streptomycin (stock: 10 mg/mL H_2O). Filter sterilize and store the stock solutions at −20 °C. Consult manufacturer's instructions for storage duration and stability.
7. Electroporation buffer (EB): 50 mM sucrose, 10 mM KH_2PO_4, adjust pH to 6.1 with KOH. Store 50 mL aliquots at −20 °C.
8. Gene Pulser Xcell™ with CE module (Bio-Rad).
9. 2 mm gap electroporation cuvettes.
10. Refrigerated centrifuge with capacity for 50 mL tubes.
11. Freezing mixture: HL5 medium with 10 % (v/v) DMSO.
12. Cryogenic cell freezing box and 1.8 mL cryo-tubes.
13. Haemocytometer.
14. Incubator 21–23 °C.

2.3 Microscopy

1. Confocal laser scanning inverted microscope system with argon 488 nm laser line and near red 561 excitation.
2. Spinning disk confocal inverted microscope system with 488 nm (485–20/530–25 filter) and 568 nm (568–10/607–40 filter) laser lines or similar, and charge coupled device (CCD) or complementary metal oxide semiconductor (CMOS) camera.
3. Capture and analysis software.
4. 35 mm live cell and 8 well μ-slide imaging dishes (ibidi GmbH).
5. Cell culture centrifuge.
6. Objective immersion oil.
7. 70 % ethanol.
8. Lens cleaning tissues.

3 Methods

3.1 Growth of L. pneumophila for Infection

1. Growth on CYE agar or in AYE medium: streak out *L. pneumophila* from frozen glycerol stocks onto CYE plates containing 5 μg/mL Cam to maintain the plasmids pSW001 or pNT-28, and grow for 2 days at 37 °C (*see* **Note 6**). Pipette 3 mL AYE

medium containing 5 μg/mL Cam into a 15 mL test tube and inoculate to a final OD_{600} of 0.1. Incubate on a rotation wheel for 14–16 h at 37 °C, until bacteria reach their peak infectivity (final OD_{600} 3.0–3.4) (*see* **Note 7**).

2. Check cultures for fitness (namely motility and homogeneity of rod-shaped bacteria) by examining a few μL of culture on a slide with an inverted light microscope (40× objective) (*see* **Note 8**).

3.2 Culturing of D. discoideum

1. Axenic *D. discoideum* can be cultured in flasks or regular Petri dishes (*see* **Note 9**). Remove spent medium from a growing culture, and add 5 mL fresh HL5. Resuspend cells by repeated pipetting of the media over the plate, or by tapping the flask.
2. Determine cell concentration by counting the cells with a haemocytometer.
3. Seed cells at a density of 5×10^4/mL. At this density, cultures can be split every 2–3 days (*see* **Note 10**). Incubate cells at 21–23 °C, and grow to 70–80 % confluence (*see* **Note 11**).

3.3 Transformation of D. discoideum for Expression of Fluorescent Probes

1. Grow cells to 70–80 % confluence. Prepare one flask per two transformations.
2. Pre-cool a centrifuge with capacity for 50 mL tubes to 4 °C.
3. Remove old media from the culture flask and wash with 10 mL ice cold electroporation buffer (EB). Add and wash gently to avoid dislodging the adhering cells.
4. Add 10 mL EB buffer to resuspend cells. Transfer the suspension to a 50 mL tube. Count cells and add EB to 25 mL. Shake gently and centrifuge 5 min at 500 × *g*.
5. Meanwhile, add 2–10 μg of plasmid DNA to a 2 mm gap electroporation cuvette on ice (*see* **Note 12**).
6. Resuspend cells in EB (10^6 cells/100 μL). Add 200 μL to the electroporation cuvette. Pipette in and out gently to mix cells with the plasmid DNA.
7. Transform the cells using the following settings: 850 V, 10 μF, 0.6 ms pulse length, two pulses spaced by a 5 s interval, 2 mm cuvette gap distance. A resistor is not necessary.
8. Immediately put the cuvette back on ice for 3–5 min. During this time, add 3 mL HL5 medium to each of three wells in a 6-well plate.
9. Divide the contents of the cuvette evenly among the three HL5 wells. Incubate the plate at 21–23 °C (*see* **Note 13**).
10. Begin antibiotic selection 24 h after the transformation.
11. Change media the next day. The vast majority of the cells will have died and detached. Continue antibiotic selection in 3 mL HL5.

12. Change media every 2–3 days. Micro-colonies of antibiotic-resistant amoeba should be evident after 7–12 days from transformation (*see* **Note 14**).
13. Before the cells in the well reach confluence, wash off the cells using a pipette and transfer to a 10 mL culture in a 75 cm^2 flask. Continue antibiotic selection.
14. When the cells reach sub-confluence, check their fluorescence (production of GFP fusion protein) with a standard or confocal fluorescence microscope (*see* **Note 15**).
15. To freeze cells for future use, remove the medium from one culture flask, tap off the cells in 4 mL ice cold freezing mixture and distribute 1 mL aliquots to 1.8 mL cryo-tubes.
16. Place tubes in a cryogenic cell freezing box in a −80 °C freezer.

3.4 D. discoideum Seeding for Infection and Microscopy

1. *D. discoideum* amoeba should be harvested from 70 % confluent cultures. When splitting a culture, prepare an additional flask dedicated for experimentation.
2. Remove medium, wash with 5 mL LoFlo medium and resuspend cells in 5 mL LoFlo medium.
3. Count cells and seed $2–4 \times 10^5$/mL in 35 mm glass-bottom observation dishes or 8-well μ-slides (*see* **Note 16**). Cell adhesion typically takes 30 min.
4. After cells have adhered, carefully replace the LoFlo medium.
5. Make sure that the microscope stage thermostat is set to between 22 and 25 °C (*see* **Note 17**).

3.5 Real-Time Imaging of Rapid Infection Events

1. Use 60× or 100× oil objective and mount the sample (*see* **Note 18**).
2. Bring cells into focus using bright field. The focus is typically sharper around the center of the dish. Switch to fluorescence and find a patch of cells with bright fluorescence.
3. Calibrate microscope capture settings accordingly, so that recording can proceed in the following steps (*see* **Note 19**).
4. Introduce up to 5 μL of a diluted *L. pneumophila* culture by submerging a pipette tip directly above the objective position. Remove and replace the lid of the container without disturbing the settings. Refocus quickly and begin observation (*see* **Note 20**).
5. Motile *L. pneumophila* will arrive at the focal plane within seconds of addition (Fig. 2). Begin recording and maintain focus manually by fine-tuning. Should no desired events take place within the first few minutes, move on to another area of cells. Unless it is required to follow an event for >15 min, move to new dish and repeat the process.
6. Use an analysis software program to evaluate the results (*see* **Note 21**).

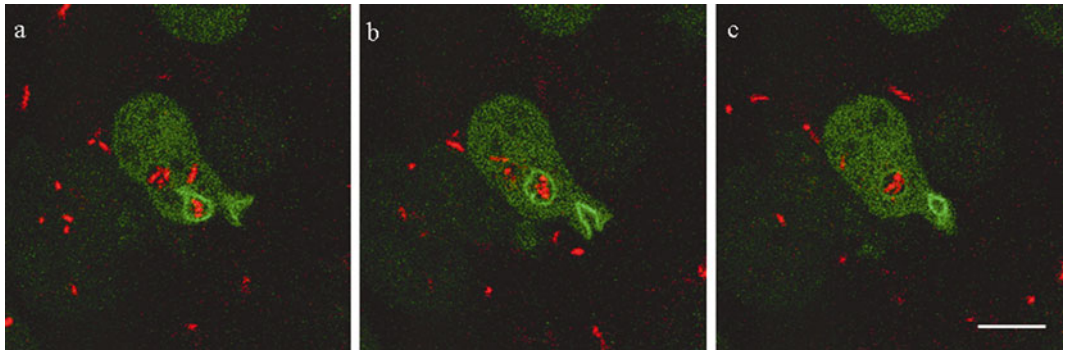

Fig. 2 Dynamics of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 in *L. pneumophila*-infected *D. discoideum*. Membrane acquisition and clearance of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 in *D. discoideum* producing PH_{CRAC}-GFP upon infection with DsRed-producing wild-type *L. pneumophila* (pSW001). (**a**) Phagosome formation upon internalization, (**b**) persistence of the PH_{CRAC}-GFP probe for about 45 s, (**c**) rapid disappearance of the probe signal from the phagosome. This series was captured with a confocal laser scanning microscope and a 100× oil objective. A 400 Hz scan with 512 × 512 pixel frame size allowed for one image capture every 2.5 s, sufficient to effectively follow uptake events. Scale bar, 5 μm

3.6 Long-Term Real-Time Observation of Dynamic Infection Events

1. Just prior to *L. pneumophila* cultures reaching early stationary growth phase, setup and program the microscope. Use the 60× objective to calibrate the settings by imaging uninfected *D. discoideum* (*see* **Note 22**).
2. Calculate the number of bacteria and dilute to give a multiplicity of infection (MOI) between 10 and 20. The MOI must be fine-tuned for a specific application. Introduce bacteria, mix gently to distribute them as evenly as possible, and centrifuge for 5 min at 1,000 × *g* (*see* **Note 23**).
3. Mount cells on the microscope stage. Bring them into focus and be prepared to image the first time point.
4. Capture three representative images at user-defined time points, which best represent the process analyzed (Fig. 3). As it is not possible to capture all images simultaneously, start imaging so that capturing is half completed when the specific time point is reached. Be consistent with the imaging time range and try to keep it minimal.
5. Continue manual time lapse capture, refocusing before every new image (*see* **Note 24**).
6. Use an analysis software program to evaluate the results (*see* **Note 25**).

4 Notes

1. The source and quality of yeast extract and peptone affect the physiology of *L. pneumophila* and *D. discoideum*. For a high reproducibility of virulence traits and growth characteristics,

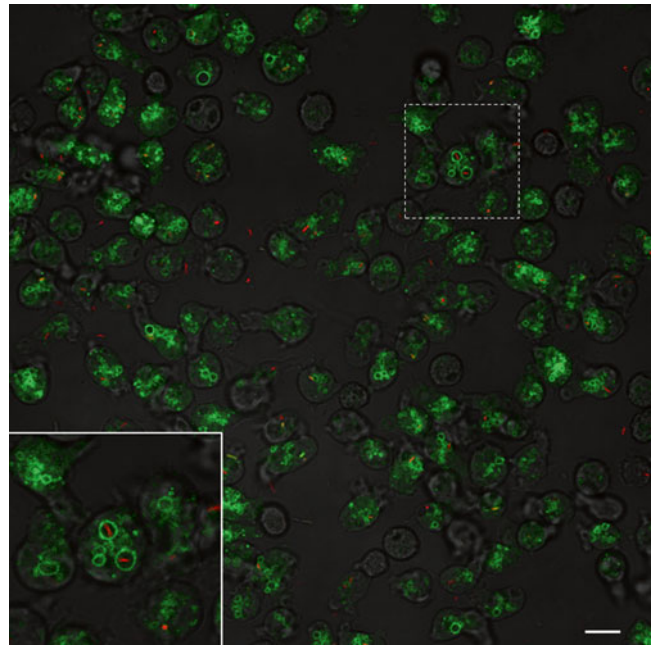

Fig. 3 Dynamics of PtdIns(3)*P* in *L. pneumophila*-infected *D. discoideum*. Membrane localization of PtdIns(3)*P* in *D. discoideum* producing 2FYVE-GFP 1 h post infection with DsRed-producing *L. pneumophila* Δ*icmT* (pSW001). Many avirulent Δ*icmT* mutant bacteria localize inside of PtdIns(3)*P*-positive compartments. The *inset* (*bottom left*) is an enlarged view of the field marked by the *dash-line box*. This confocal scanning image was captured with a 63× oil objective, 1.6× digital magnification, 1,200 × 1,200 pixel frame size and two-line average at 100 Hz. Scale bar, 30 μm

the components should be tested beforehand, and the same suppliers and batches should be used for all experiments.

2. Dissolve L-cysteine and $Fe(NO_3)_3$ each separately in 10 mL of H_2O in a 15 mL tube. Stir the medium and slowly add the L-cysteine solution first, followed by the iron solution to prevent precipitation.
3. Pre-filter the medium 6–8 times through a glass fiber filter paper to remove precipitates. L-cysteine is light sensitive.
4. Glucose caramelizes upon autoclaving in combination with the medium. Suspend the D(+)glucose in 50 mL of pre-warmed H_2O, filter sterilize (0.2 μm) and add to the autoclaved medium.
5. The LoFlo low fluorescence medium for *D. discoideum* is ideal for microscopic observation of live cells, but not conducive to cell multiplication.
6. *L. pneumophila* grown for 2 days (instead of 3 days) on plate yields liquid cultures growing faster and morphologically more

homogeneous. The bacteria are notoriously filamentous on agar plate, and therefore, are grown in liquid culture as homogeneous motile rods. The motile bacteria make contact with host cells without centrifugation just seconds after their introduction to the observation dish. Thus, the initial interaction events will not be missed.

7. Under our conditions, an OD_{600} of 3.0 corresponds to approximately 2×10^9 CFU/mL, where the CFU number equates to the number of bacteria. The exact correlation of the OD_{600} with the bacterial concentration depends on laboratory conditions and strain used and must be determined experimentally. Since early stationary phase cultures are at their peak virulence this is a critical issue.
8. It is generally a good idea to inoculate several cultures for a given strain using more and less inoculum. Compare the motility of the bacteria and select the culture with the fastest and highest percentage of motile rods. Be aware that it is not unusual upwards of 90 % of culture to appear sessile.
9. For general maintenance of a cell line, standard Petri dishes are an inexpensive and convenient way to culture *D. discoideum*. Cell culture flasks are well suited for applications such as transformation to simplify washing and cell recovery.
10. Be careful not to over-dilute cells when passaging, as such will result in slower replication rates, and the culture will take several days longer to reach sub-confluence.
11. Aim to split cultures when the cells are evenly spaced in exponential growth phase. Never allow the cells to reach confluence, as this will drastically reduce transformation efficiency and cause other physiological changes.
12. 10 μL is a good volume in which to have the DNA. High delivery volumes only dilute the electroporation buffer.
13. Dividing up the transformation will ensure that resulting micro-colonies all arise from different transformants.
14. Micro-colonies can be difficult to spot at first. Some may appear as early as 4 days or as late as 2 weeks after transformation. Appearance of micro-colonies is different for each construct and can be influenced by the selection marker used.
15. In case the cell population produces the GFP fusion protein in a heterogeneous manner, it might be necessary to select for clones with a high production level of the protein of interest. To this end, cells can be singularized in 96-well plates at a density of approximately 0.5 cells/well, and the production of the GFP fusion protein of individual clones can be assessed by fluorescence microscopy and/or Western blot.

16. 35 mm observation dishes are practical with a large viewing area, but we prefer 8-well μ-slides. The advantages are that they eliminate switching between dishes (for different strains or conditions) and are a convenient way to make replicates.
17. *D. discoideum* is extremely sensitive to temperatures above 25 °C. Beyond this temperature, cells will round up and die.
18. A 60× objective should be used with a scanning microscope, as a higher magnification will generally not transmit enough light to view the sample effectively. Compensate with digital magnification and screen resolution adjustments. A 100× objective can be used effectively with a spinning disk microscope for close-up capture of rapid events.
19. The rate of capture will depend on the expected speed of the event. For rapid, short term observation, a spinning disk microscope is best suited. An image in full screen resolution and two channels can typically be captured in less than 1 s. The exposure time necessary to capture a good signal from each channel is dependent on the fluorescence brightness, and will ultimately determine the capture rate. Setup for a scanning microscope can prove trickier, as the scan rates are fixed for each frame size at a given scan frequency and number of photomultiplier tubes engaged. As a starting point, a capture resolution of 512 × 512 pixels and scan frequency of 400 Hz will provide one two-channel image every 2.5 s. Higher quality capture can be obtained by lowering the scan frequency (to make slower scan passes) and line-averaging two scans. However, it is not recommended that the combined scan time exceed 5 s. Motile *L. pneumophila* are very quick relative to the scan and may give the appearance of being sheared as they change location during a capture.
20. Dilute a few microliters of the AYE culture 1:10 in LoFlo medium. The introduced bacteria will spread like a cloud and remain suspended. This phenomenon is caused by filamentous *L. pneumophila* in bundles and aggregates. Growth in liquid AYE culture reduces filament formation and provides smaller, more uniform rods, but it is not perfect. Filaments remain suspended well above the focal plane and do not generally interfere with imaging, while the motile bacteria (*see* **Note 8**) swim down to the focal plane within seconds of their introduction.
21. For more obvious changes, movies and pictures can effectively describe what is happening. Changes in general can be quantified and represented graphically. For rapid events, one might find a feature that is common to all cells and use it as a reference point. Take for example closure of a phagocytic cup relative to the appearance/disappearance of the PI being monitored.
22. In this case, a 60× objective provides a better overview of events. It is desirable to capture a minimum of 100 cells at a

time. For this application, examining slow changes, speed is not essential and a scanning microscope may be preferred. The scanning microscope has the advantage that image size is not limited by the resolution (number of pixels) of the camera's CCD. Instead, images can be made quite large (with a tradeoff for proportionally increased scan times). A recommended capture size is 1,200 × 1,200–1,600 × 1,600 pixels. Scans can be made at 100 Hz and should always feature a two-line average. Such scans can be expected to take 40–60 s.

23. Even distribution of bacteria is the key to simultaneous infection. Although bacteria will be taken up at different times over the course of the infection, the bulk will be taken up in the first few minutes. The better the synchronization, the better the statistical representation. For multiple strains or conditions, consider staggering infections so that imaging of two or more samples does not need to occur simultaneously. The multiplicity of infection (MOI) represents the number of bacteria relative to the number of cells.
24. This procedure requires the operator to be present for imaging at each time point. The use of an autofocus function is not recommended, as cells may change shape and position quite significantly compared to their original state. As an intrinsic feature of the *L. pneumophila* infection, we observe an Icm/Dot-dependent rounding and detachment of infected cells with onset 30–45 min post infection. This phenomenon normally presents a technical difficulty, but in this case can be advantageous, as the infected cells line up orderly for imaging in the center of the observation dish.
25. Using this method, one is most likely interested in quantifying and characterizing PI patterning on the LCV or other related cellular compartments. Most software programs are equipped with a line tool that generates a fluorescence intensity histogram. This tool allows the characterization of the intensity of a signal around the LCV and to compare it to the baseline fluorescence of other cellular components labeled by the same probe. It is important that the signal captured is never saturated. State-of-the-art software applications are equipped with tools for finding discrete objects, such as vacuoles. For statistical analysis, the images should hold raw data for counting over 200 events per time point for each sample.

Acknowledgments

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Chapter 10

Investigating Interference with Apoptosis Induction by Bacterial Proteins

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Abstract

The modulation of host cell apoptosis by bacterial pathogens is critical for their intracellular survival. Several intracellular bacteria achieve this by secreting proteins that interact with apoptosis pathways to inhibit host cell apoptosis. *Anaplasma phagocytophilum*, which causes human granulocytic anaplasmosis, is such bacterium. The protein Ats-1, translocated from *A. phagocytophilum* by the bacterial type IV secretion system, localizes to host cell mitochondria, and interferes with apoptosis induction. In this chapter, we present a protocol applied to investigate an anti-apoptotic effect of Ats-1.

Key words *Anaplasma phagocytophilum*, Apoptosis, Ats-1

1 Introduction

Apoptosis of infected cells is one of the important innate immune responses against intracellular pathogens, including viruses, bacteria, and parasites [1]. *Anaplasma phagocytophilum* is an obligatory intracellular bacterium that primarily infects short-lived neutrophils [2]. Neutrophils typically undergo spontaneous apoptosis within 6–12 h after release into the blood circulation from the bone marrow [3]. *A. phagocytophilum* extends the life span of infected neutrophils to complete its life cycle by inhibition of neutrophil apoptosis [4–6]. Two main apoptotic pathways are defined, intrinsic (mitochondria-mediated apoptosis) and extrinsic (death receptor-mediated apoptosis) [7]. *A. phagocytophilum* blocks both intrinsic and extrinsic mode of apoptosis [6]. *A. phagocytophilum* has a type IV secretion system, which translocates effector molecules to host cells to exert their activity to favor bacterial infection [8–11]. Ats-1 (*Anaplasma* translocated substrate-1) is one of these effectors, and has been found to be imported into mitochondria and interfere with mitochondria-mediated intrinsic apoptosis [10]. In mitochondria-mediated apoptotic pathway, after cells receive intrinsic apoptotic signals, such as DNA damage, the proapoptotic

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Bcl-2 family members, including Bax, translocate to mitochondrial membranes, and cause the opening of mitochondrial permeability transition pore, leading to the release of apoptogenic proteins, including cytochrome *c*, apoptosis inducing factor (AIF), and endonuclease G [7]. Released cytochrome *c*, together with procaspase 9 and apoptosis protease activating factor, forms apoptosome, leading to the activation of caspase 9 and downstream effector caspase 3, which executes apoptosis by cleaving a number of cellular proteins including nuclear lamins, poly-ADP-ribose-polymerase (PARP), and actin. One of the hallmarks of apoptosis is chromosomal condensation, which is partially contributed to the released AIF and endonuclease G [7]. Since yeast cells have the ability to respond to human Bcl-2 family proteins, and it is easy to transform the yeast cells with genes encode human proteins, they are used as a simpler model to study the apoptosis-regulating proteins of higher organisms [12]. Here we describe in detail the protocol that we applied to investigate the effect of Ats-1 on host cell apoptosis, including determination of cell and nuclear morphology, translocation of Bax into mitochondria, PARP cleavage in etoposide-induced apoptosis in mammalian cells, and the effects of Ats-1 on human Bax-induced apoptosis in yeast cells.

2 Materials

2.1 Cultures

1. *A. phagocytophilum*-infected HL-60 cells (*see* **Note 1**).
2. Monkey endothelial RF/6A cells.
3. Yeast haploid strain YPH499.

2.2 Culture Media and Materials

1. RPMI 1640.
2. Complete RPMI 1640: 500 mL RPMI 1640, 55 mL fetal bovine serum (FBS), 5.5 mL 200 mM L-glutamine.
3. Complete advanced MEM (Minimum Essential Medium): 500 mL advanced MEM, 55 mL fetal bovine serum, 5.5 mL 200 mM L-glutamine.
4. Cell dissociation reagent: TrypLE express.
5. YPD liquid medium (per liter): 50 g YPD medium. Add deionized water to 1,000 mL, and autoclave at 121 °C for 15 min. Keep at 4 °C.
6. YPD agar medium (per liter): 50 g YPD medium, and 20 g agar. Add deionized water to 1,000 mL, and autoclave at 121 °C for 15 min. Dispense agar medium into petri dishes (25 mL each). Keep at 4 °C.
7. Yeast synthetic dextrose (SD) minimal agar medium with leucine and uracil dropout (SD/–Leu/–Ura) (per liter): 26.7 g minimal SD base, 620 mg Leu/Trp/Ura dropout supplement,

20 mg L-Tryptophan, and 20 g agar. Add deionized water to 1,000 mL, and autoclave at 121 °C for 15 min. Dispense agar medium into petri dishes (25 mL each). Keep at 4 °C.

8. Yeast synthetic dextrose minimal agar medium with leucine dropout (SD/–Leu) (per liter): 26.7 g minimal SD base, 690 mg Leu dropout supplement, and 20 g agar. Add deionized water to 1,000 mL, and autoclave at 121 °C for 15 min. Dispense agar medium into petri dishes (25 mL each). Keep at 4 °C.
9. Yeast synthetic dextrose minimal liquid medium with leucine dropout (SD/–Leu) (per liter): 26.7 g minimal SD base, 690 mg Leu dropout supplement. Add deionized water to 1,000 mL, and autoclave at 121 °C for 15 min. Keep at 4 °C.
10. Yeast synthetic dextrose minimal liquid medium with leucine and uracil dropout (SD/–Leu/–Ura) (per liter): 26.7 g minimal SD base, 620 mg Leu/Trp/Ura dropout supplement, and 20 mg L-Tryptophan. Add deionized water to 1,000 mL, and autoclave at 121 °C for 15 min. Keep at 4 °C.
11. Yeast synthetic galactose minimal liquid medium with leucine and uracil dropout (SG/–Leu/–Ura) (per liter): 6.7 g yeast nitrogen base without amino acids, 20 g galactose, 620 mg Leu/Trp/Ura dropout supplement, 20 mg L-Tryptophan. Add deionized water to 1,000 mL, and autoclave at 121 °C for 15 min. Keep at 4 °C.
12. 75 cm^2 flasks.
13. 15 and 50 mL centrifuge tubes.
14. 6 well cell culture plates.
15. Glass slides.
16. Autoclaved glass coverslips (18 × 18 mm) (*see* **Note 2**).
17. Petri dishes (100 mm × 15 mm).

2.3 Immunolabeling

1. Diff-Quik staining kit.
2. PBS (per liter): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4, pH 7.4. Autoclave at 121 °C for 15 min. Keep at 4 °C.
3. Paraformaldehyde solution. Add PBS to 0.4 g paraformaldehyde to 10 mL in a 15 mL centrifuge tube to make 4 % (w/v) stock solution. Keep the tube in a 60 °C water bath for 10 min, followed by vortexing for 2 min. Repeat the heat and vortexing steps until the paraformaldehyde is dissolved (about 2–3 rounds). Dispense 1 mL aliquots into Eppendorf tubes and store at –20 °C. Good for 2 months. When necessary, dilute to 2 % by mixing with 1 volume of PBS before use.
4. PGS solution (per 100 mL): 0.4 g bovine serum albumin (BSA), 0.2 g gelatin, and 0.3 g saponin in 100 mL PBS. Keep at 4 °C. Good for 1 week.

5. Primary antibodies: Rabbit anti-Ats-1 [11], horse anti-*A. phagocytophilum* [13], mouse monoclonal anti-Mn-Sod (clone MnS-1, Alexis), mouse monoclonal anti-Bax (Clone 3, BD Transduction Laboratory), mouse monoclonal anti-cytochrome *c* (clone 2G8, Santa Cruz biotechnology), mouse monoclonal anti-yeast porin (clone 16G9E6BC4, Invitrogen), rabbit anti-actin (Sigma), rabbit anti-PARP (Cell Signal Technology). Keep at −20 °C in 50 % glycerol.
6. Secondary antibodies: Cy3-conjugated goat anti-horse IgG (keep at −20 °C in 50 % glycerol), Alexa Fluor 488-conjugated goat anti-rabbit IgG (keep at 4 °C), Alexa Fluor 350-conjugated goat anti-mouse IgG (keep at 4 °C), Alexa Fluor 555-conjugated goat anti-mouse IgG (keep at 4 °C), peroxidase-conjugated goat anti-rabbit IgG (keep at −20 °C in 50 % glycerol), peroxidase-conjugated goat anti-mouse IgG (keep at −20 °C in 50 % glycerol).
7. DAPI. Dissolve 5 mg 4′,6-diamidino-2-phenylindole, dilactate (DAPI dilactate) in 1 mL deionized water to make 10.9 mM stock solution and keep frozen at −20 °C. To make working solution, add 1.38 μL DAPI dilactate stock solution into 50 mL PBS before use.

2.4 Plasmids

1. pEGFP-N1 (Clontech).
2. pAts-1: The gene encoding Ats-1 of *A. phagocytophilum* was cloned into pEGFP-N1 vector between SalI and NotI sites [11].
3. pGADT7 AD (Clontech).
4. pYAts-1. The gene encoding Ats-1 of *A. phagocytophilum* was cloned into yeast constitutive expression vector pGADT7 AD between HindIII and HindIII sites [11].
5. pBax. The cDNA encoding human Bax was cloned into yeast inducible vector pYES2/NT A (Invitrogen) between HindIII and NotI sites [11].

2.5 Other Reagents and Equipment

1. 50 mM Etoposide (a topoisomerase II inhibitor, an apoptosis inducer by causing DNA damage). Dissolve 14.71 mg etoposide in 500 μL DMSO.
2. SDS-PAGE sample buffer for PARP: 6 M urea, 62.5 mM Tris–HCl, 10 % glycerol, 5 % β-mercaptoethanol, 2 % SDS, 0.00125 % bromophenol blue, pH 6.8 [12].
3. 2× SDS-PAGE sample loading buffer: 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 0.004 % bromphenol blue, 0.125 M Tris–HCl, pH 6.8.
4. MitoTracker Red CMXRos. The MitoTracker Red CMXRos (Invitrogen) is provided as a kit of 20 vials, each containing 50 μg MitoTracker Red. Add 94 μL DMSO to a vial to dissolve

MitoTracker Red CMXRos to make 1 mM stock solution. Store frozen at –20 °C, and keep from light.

5. Lyticase. Dissolve Lyticase in deionized water to make the concentration of 2,000 U/mL.
6. DTT Buffer. Prepare 1 M Tris-H_2SO_4 (pH 9.4) stock solution, and store at room temperature. Before use, dilute tenfold and add dithiothreitol (DTT) to 10 mM, prewarm to 30 °C.
7. Lyticase Buffer: 1.2 M sorbitol, 20 mM potassium phosphate buffer, pH 7.4.
8. β-Mercaptoethanol.
9. Homogenization Buffer: 0.6 M sorbitol, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 % (w/v) bovine serum albumin. Precool the homogenization buffer on ice before use.
10. YEASTMAKER yeast transformation system (Clontech).
11. Hemocytometer.
12. Cytocentrifuge.
13. Spectrophotometer.
14. Refrigerated microcentrifuge.
15. Gene Pulser Xcell System (electroporator).
16. Cell culture incubator.
17. Light microscope and fluorescence microscope.
18. SDS-PAGE system.
19. Semi-dry blotting apparatus.
20. Enhanced chemiluminescence reagent (ECL).
21. Gel documentation system (Fujifilm LAS-3000 Imager and Fujifilm MultiGauge program).
22. Shaker incubator.
23. Super pap pen.
24. BCA protein assay kit.
25. Dounce homogenizer.
26. 0.2 cm electroporation cuvettes.
27. Sonicator.
28. Heating water bath or block (65 °C).
29. –20 °C methanol.
30. 1 % SDS.
31. Mounting medium.
32. Skim milk powder.
33. Parafilm.

34. 30 °C incubator.
35. Orbital shaker.
36. Forceps.
37. Eppendorf tubes.
38. Petri dishes (150 mm × 15 mm).

3 Methods

3.1 Immunolabeling of Infected Cells

1. Culture *A. phagocytophilum*-infected HL-60 cells at 3×10^6 cells/mL in complete RPMI 1640 medium in 75 cm^2 flasks at 37 °C under 5 % CO_2/95 % air in humidified incubator for 2 or 3 days.
2. Cytospin 100 μL cultured cells in a cytocentrifuge (300 × *g*, 2 min) and stain the cells by using Diff-Quik staining kit to determine the infection level.
3. When the cell infectivity is more than 80 %, count the cell number using a hemocytometer, and centrifuge 3×10^6 cells (250 × *g*, 5 min) in a 15 mL centrifuge tube.
4. Aspirate the culture medium, and resuspend the cell pellet with 600 μL 2 % (w/v) paraformaldehyde (*see* **Note 3**), transfer it to a 1.5 mL Eppendorf tube, and rotate the tube end-to-end for 30 min at room temperature to fix the cells.
5. Centrifuge the fixed cells (250 × *g*, 5 min), aspirate the liquid, and resuspend the cell pellet with 500 μL PBS to wash cells (*see* **Note 3**).
6. Repeat the **step 5** twice.
7. Centrifuge the fixed cells (250 × *g*, 5 min), aspirate the liquid, resuspend the cell pellet with 500 μL PGS solution (*see* **Note 3**), and rotate the cells end-to-end for 30 min at room temperature to permeabilize cell membranes (*see* **Note 4**).
8. Centrifuge cells (250 × *g*, 5 min), aspirate the liquid, resuspend the cell pellet with 300 μL PGS solution containing rabbit anti-Ats-1 antibody or preimmune rabbit IgG (1:100 dilution), mouse anti-Mn-Sod antibody or normal mouse IgG (1:100 dilution), and horse anti-*A. phagocytophilum* or preimmune horse serum (*see* **Note 5**), and rotate the cells end-to-end for 60 min at room temperature.
9. Centrifuge the cells (250 × *g*, 5 min), aspirate the liquid, and resuspend the cell pellet with 300 μL PBS to wash out unbound primary antibodies (*see* **Note 3**).
10. Repeat the **step 9** twice.
11. Centrifuge cells (250 × *g*, 5 min), aspirate the liquid, resuspend the cell pellet with 300 μL PGS solution containing Alexa

Fluor 488-conjugated goat anti-rabbit IgG (1:200 dilution), Alexa Fluor 350-conjugated goat anti-mouse IgG (1:200 dilution), and affipure Cy3-conjugated goat anti-horse IgG (1:100 dilution) (*see* **Note 5**), and rotate the cells end-to-end for 60 min at room temperature (*see* **Notes 6** and **7**).

12. Centrifuge the cells (250 × *g*, 5 min), aspirate the liquid, and resuspend the cell pellet with 300 μL PBS to wash out unbound secondary antibodies (*see* **Note 3**).
13. Repeat the **step 12** twice.
14. Take 50 μL stained cells to centrifuge down to a glass slide (300 × *g*, 2 min) by the cytocentrifuge.
15. Place 20 μL mounting medium on top of cells, and cover with a coverslip.
16. Observe and capture image under florescence microscope using blue (Alexa Fluor 350), green (Alexa Fluor 488), and red (Cy3) color channels (*see* **Note 8**).

3.2 Transfection

1. Culture 2.0×10^6 adherent RF/6A cells in 15 mL complete advanced MEM medium in a 75 cm^2 flask at 37 °C under 5 % CO_2/95 % air in humidified incubator 1 day before transfection.
2. Dissociate cells using 3 mL TrypLE express at 37 °C for 5 min.
3. Add 6 mL complete advanced MEM (double volume to TrypLE express) to inactivate TrypLE express.
4. Transfer detached cells to a 50 mL centrifuge tube, and centrifuge (200 × *g*, 5 min).
5. Aspirate the liquid, resuspend the cell pellet in 10 mL PBS, and count the cell number using hemocytometer.
6. Centrifuge the cells (200 × *g*, 5 min), aspirate the liquid, and resuspend the cell pellet with RPMI 1640 (no supplement) to cell density of 2×10^7/mL.
7. Add 80 μL cell suspension and 5 μg plasmid into a 0.2 cm electroporation cuvette, and mix well.
8. Put autoclaved glass coverslips into 6-well cell culture plate with a sterile forceps.
9. Electroporate the cells by Gene Pulser Xcell System with the setting of voltage 100 V and capacity 1,000 μF, quickly add 1 mL complete advanced MEM (prewarmed to 37 °C) (*see* **Note 9**), aspirate the cells gently from the cuvette and transfer to one of the wells in a 6 well cell culture plate, and add 2 mL complete advanced MEM to each well (*see* **Note 10**).
10. Incubate the cells at 37 °C under 5 % CO_2/95 % air in a humidified incubator.

3.3 Apoptosis Induction

1. Distribute pAts-1-transfected, or sham (pEGFP-N1)-transfected RF/6A cells into 6-well plates at 1.6×10^5 cells/well in 3 mL complete advanced MEM medium (from Subheading 3.2, **step 9**), four wells for each transfection.
2. Incubate cells at 37 °C under 5 % CO_2/95 % air in humidified incubator for 20 h. Take a coverslip from a well in each transfection with sterile forceps and immunostain the cells to observe pAts-1- and pEGFP-N1-transfected cells under fluorescence microscope to determine transfection efficiency (follow Subheading 3.4, **steps 2–12**).
3. When the transfection efficiency is more than 50 % (*see* **Note 11**), add 6 μL 50 mM etoposide solution (*see* **Note 12**) to 3 mL medium in other three wells of pAts-1- and sham-transfected cells, to a final concentration of 100 μM.

3.4 Immunostaining of Apoptosis-Induced Cells

1. Incubate cells for one more day at 37 °C under 5 % CO_2/95 % air in humidified incubator after etoposide treatment (from Subheading 3.3, **step 3**).
2. Take the coverslips out from the wells of plates and wash the cells on coverslips with PBS once.
3. Fix transfected RF/6A cells on the coverslip by immersing coverslips in drops of 400 μL 2 % paraformaldehyde with the cells facing up on a piece of parafilm for 30 min at room temperature (*see* **Note 13**).
4. Rinse the coverslip with PBS once.
5. Permeabilize the cell membranes by immersing coverslips in drops of 500 μL PGS solution with the cells facing up on a piece of parafilm for 10 min.
6. Aspirate the PGS solution, and add drops of 400 μL PGS solution containing rabbit anti-Ats-1 and mouse anti-cytochrome *c*, or rabbit anti-Ats-1 and mouse anti-Bax on a piece of parafilm in a covered petri dish (150 mm × 15 mm) with wetted filter papers inside (*see* **Notes 14** and **15**), followed by incubation at 37 °C for 45 min.
7. Wash the cells on coverslips with 20 mL PBS in petri dish (100 mm × 15 mm) on orbital shaker for three times, 2 min each.
8. Incubate the cells on coverslips with drops of 400 μL PGS solution containing Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200 dilution) and Alexa Fluor 555-conjugated goat anti-mouse IgG (1:200 dilution) on a piece of parafilm in a covered petri dish (150 mm × 15 mm) with wetted filter papers inside (*see* **Note 5**), at 37 °C for 45 min (*see* **Note 6**).
9. Wash the cells on coverslips with 20 mL PBS in petri dish (100 mm × 15 mm) on orbital shaker three times, 2 min each.

10. For the cells labeled with anti-Ats-1, anti-cytochrome *c*, or anti-Bax, incubate the coverslips in 400 μL PBS solution containing 300 nM DAPI on a piece of parafilm in a covered petri dish (150 mm × 15 mm) with wetted filter papers inside for 5 min at room temperature, followed by rinsing once in PBS.
11. Add 20 μL mounting medium on glass slides, and put coverslips over the mounting medium with cells facing down.
12. Observe and capture image under florescence microscope using blue (DAPI), green (Alexa Fluor 488), and red (Alexa Fluor 555) color channels (*see* **Note 16**).

3.5 Analysis of PARP Cleavage (See Note 17)

1. Incubate pAts-1, or sham (pEGFP-N1)-transfected RF/6A cells for 12 h at 37 °C under 5 % CO_2/95 % air in humidified incubator after etoposide treatment (from Subheading 3.3, **step 3**).
2. Aspirate the cell culture medium, which contains detached apoptotic cells, and transfer to 1.5 mL Eppendorf centrifuge tubes, followed by centrifugation (250 × *g*, 5 min).
3. Carefully discard the supernatant and resuspend the cell pellet in 50 μL SDS-PAGE sample buffer for PARP.
4. Add SDS-PAGE sample buffer for PARP to a well containing remaining adherent cells (150 μL/well), and use cell scraper to harvest cells.
5. Combine the sample of the same well from **steps 3** and **4** into a 1.5 mL Eppendorf centrifuge tube, and sonicate the cells until the samples are not viscous (about 15 s at power setting 2).
6. Heat the samples at 65 °C for 15 min.
7. Prepare 8 % acrylamide gel, and run samples on SDS-PAGE.
8. Transfer proteins from SDS-PAGE gel to nitrocellulose membrane using semi-dry blotting apparatus.
9. Block the nitrocellulose membrane with 5 % skim milk in PBS for 30 min at room temperature on orbital shaker.
10. Incubate overnight the nitrocellulose membrane with rabbit antibodies against PARP (1:1,000 dilution), actin (1:1,000 dilution), and Ats-1 (1:1,000 dilution) in 5 % skim milk at 4 °C on orbital shaker (*see* **Note 18**).
11. Wash the membrane three times with PBS, 5 min each on orbital shaker.
12. Incubate membrane with peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution) for 1 h at room temperature on orbital shaker.
13. Wash the membrane four times with PBS, 5 min each.
14. Develop the western blot reactions using ECL Western Blotting Substrate.

15. Capture the images by CCD camera (Fujifilm LAS-3000 Imager), measure the PARP, cleaved PARP and actin band densities using Fujifilm MultiGauge program, and calculate the relative band density of cleaved PARP to actin in each sample.

3.6 Localization of Ats-1 in Transformed Yeast Cells

1. Grow yeast strain YPH499 on YPD agar medium at 30 °C for 2 days.
2. Pick up one colony and culture in 50 mL YPD liquid medium.
3. Make competent YPH499 cells by YEASTMAKER Yeast Transformation System.
4. Transform YPH499 with plasmid, pYAts-1 (Ats-1 is constitutively expressed), or control plasmid pGADT7 AD using YEASTMAKER Yeast Transformation System.
5. Grow the transformants on yeast selective agar medium (synthetic dextrose minimal medium lacking leucine (SD/–Leu)) for 3 days at 30 °C.
6. Incubate one colony from each transformation into 3 mL SD/–Leu liquid medium and culture overnight at 30 °C in a shaking incubator (250 RPM shaking).
7. Transfer 100 μL culture to 3 mL fresh SD/–Leu liquid medium and continue to culture until OD (A600) reaches 0.5 measured with spectrophotometer, followed by incubation with 2.4 μL 1 mM MitoTracker Red CMXRos (final concentration, 800 nM) for 30 min in a shaking incubator (250 RPM shaking).
8. Centrifuge the yeast cells at 1,000 × *g* for 10 min, discard the supernatant and resuspend the cells in 3 mL fresh SD/–Leu liquid medium to wash cells.
9. Centrifuge the yeast cells at 1,000 × *g* for 10 min, discard the supernatant and resuspend the cells in 1 mL 4 % paraformaldehyde solution.
10. Transfer the cells to 1.5 mL Eppendorf tube, and rotate the tube end-to-end for 1 h at room temperature to fix the cells. Protect the cells from light.
11. Centrifuge the yeast cells in a microcentrifuge at 1,000 × *g* for 5 min, discard the supernatant and resuspend the cells in 1 mL lyticase buffer.
12. Repeat **step 11** two more times and resuspend the cells in 950 μL lyticase buffer.
13. Add 50 μL lyticase solution (100 U) and 1 μL β-mercaptoethanol to yeast cells in lyticase buffer. Mix and keep at room temperature for 30 min with occasional shaking to digest yeast cell wall.
14. Cytospin yeast cells in a cytocentrifuge (400 × *g*, 2 min) to glass slides.

15. Immerse the glass slides into −20 °C methanol for 5 min to permeabilize the cells.
16. Use super pap pen to draw lines around the cells (*see* **Note 19**).
17. Cover the cells on glass slides with 50 μL PGS solution containing rabbit anti-Ats-1 antibody (1:100 dilution) (*see* **Note 5**), and incubate in a covered humidified petri dish at 37 °C for 45 min to immunostain the cells.
18. Rinse the cells on glass slides with PBS for three times, 2 min each.
19. Incubate the cells with 50 μL PGS solution containing Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200 dilution) (*see* **Note 5**), and incubate at 37 °C for 45 min (*see* **Note 6**).
20. Rinse the cells on slides with PBS for three times, 2 min each.
21. Observe and capture image under florescence microscope using green (Alexa Fluor 488) and red (MitoTracker Red) color channels (*see* **Note 20**).

3.7 Ats-1 Rescues Yeast Cells from Bax-Induced Growth Arrest

1. Cotransform the yeast haploid strain YPH499 with two plasmids, pBax (human Bax expression is inducible by galactose) and pYAts-1 (Ats-1 is constitutively expressed) (*see* **Note 21**), or pBax and control plasmid pGADT7 AD using YEASTMAKER Yeast Transformation System.
2. Grow the yeast transformants on SD/−Leu/−Ura selective agar medium at 30 °C until colonies grow/appear (about 3 days).
3. Incubate one colony into 3 mL SD/−Leu/−Ura liquid medium and culture overnight at 30 °C (250 RPM shaking).
4. Centrifuge the yeast cells (1,000 × *g*, 5 min) to remove SD/−Leu/−Ura medium.
5. Aspirate the supernatant, and resuspend the cell pellet in 3 mL SG/−Leu/−Ura liquid medium.
6. Measure the OD value of yeast cell culture at A600 with spectrophotometer, and dilute the cells with SG/−Leu/−Ura medium to make the OD_{600} to 0.05.
7. Take 100 μL culture ($OD_{600} = 0.05$), and make eight 10× fold serial dilutions. Spread 40 μL yeast cells from each dilution to SD/−Leu/−Ura agar plate, and incubate them at 30 °C for 3 days. Count viable yeast cells from diluted samples, and calculate viable yeast cells in undiluted 40 μL culture (Day 0).
8. Continue to culture 3 mL yeast cells ($OD_{600} = 0.05$) in liquid SG medium at 30 °C for 5 days.
9. Take 100 μL culture, and make eight 10× fold serial dilutions. Spread 40 μL of yeast cells from each dilution to SD/−Leu/−Ura

agar plate, and incubate them at 30 °C for 5 days. Count viable yeast cells from diluted samples, and calculate viable yeast cells in undiluted 40 μL culture (day 5).

10. Calculate the ratio of yeast cell number of day 5 to that of day 0 in each cotransformed cells (*see* **Note 22**).

3.8 Analysis of Bax Translocation to Yeast Mitochondria

1. Cotransform the yeast haploid strain YPH499 with two plasmids, pBax, and pYAts-1, or pBax and control plasmid pGADT7 AD using YEASTMAKER Yeast Transformation System.
2. Grow the yeast transformants on SD/–Leu/–Ura agar medium at 30 °C until colonies show up (about 3 days).
3. Incubate one colony into 20 mL SD/–Leu/–Ura liquid medium and culture overnight at 30 °C (250 RPM shaking).
4. Centrifuge the yeast cells (3,000 × *g*, 5 min) to remove SD/–Leu/–Ura medium.
5. Discard the supernatant, and resuspend the cell pellet in 20 mL SG/–Leu/–Ura liquid medium.
6. Measure the OD value of yeast cell culture at A600, and dilute the culture with SG/–Leu/–Ura medium to make the OD_{600} to 0.4 in total 50 mL volume.
7. Culture the yeast cells for 12 h at 30 °C in a shaker incubator with 250 RPM shaking.
8. Pellet the yeast cells by centrifugation at 3,000 × *g* for 5 min, discard supernatant, and resuspend cells with deionized water (*see* **Note 23**).
9. Centrifuge at 3,000 × *g* for 5 min, discard supernatant, and weigh the pellets.
10. Resuspend the yeast pellets in prewarmed DTT buffer (2 mL/g pellet) and shake slowly at 30 °C for 20 min.
11. Centrifuge at 3,000 × *g* for 5 min and resuspend the pellet in lyticase buffer (about 7 mL/g pellet).
12. Centrifuge and resuspend pellet in lyticase buffer (7 mL/g pellet) containing 100 U/mL lyticase. Shake slowly at room temperature for 30–60 min.
13. Determine visually the progress of spheroplast formation by mixing 5 μL of cells with 5 μL of 1 % SDS (*see* **Note 24**). Spheroplasts have a ghost-like shape after treatment with SDS under phase contrast microscope.
14. When more than 80 % yeast cells become spheroplasts, harvest the cells by centrifugation at 3,000 × *g* for 5 min and resuspend the pellets with lyticase buffer.
15. Centrifuge and resuspend the pellets in the ice-cold homogenization buffer (6.5 mL/g pellet). Remove 100 μL spheroplasts

for future use to determine the Bax, Ats-1, and yeast mitochondrial protein (porin) levels (*see* **Note 25**) in spheroplasts by western blot analysis.

16. Homogenize the spheroplasts with 20 strokes using Dounce homogenizer on ice.
17. Dilute the sample twofold with the ice-cold homogenization buffer, and centrifuge at 4,000 × *g* for 5 min at 4 °C to pellet cell debris and nuclei.
18. Collect the supernatants, and centrifuge at high speed (12,000 × *g*, 4 °C) for 15 min to obtain crude yeast mitochondria pellet.
19. Resuspend the crude yeast mitochondria pellet with the ice-cold homogenization buffer.
20. Measure the protein concentration in crude yeast mitochondria and spheroplast fractions using BCA protein assay, and make the protein concentration equal in all samples.
21. Take 50 μL solution out from each sample and mix it with 2× SDS-PAGE sample loading buffer.
22. Heat the samples at 100 °C for 5 min on heat block.
23. Run western blot analysis (*see* Subheading 3.5, **steps 7–14**) for crude yeast mitochondria and spheroplasts using antibodies against Bax, porin, and Ats-1.
24. Capture the images by CCD camera (Fujifilm LAS-3000 Imager), measure the Bax and yeast porin band density using Fujifilm MultiGauge program, and calculate the relative band density of Bax to porin in each sample.

4 Notes

1. *A. phagocytophilum* invades host cells through caveolae-mediated endocytosis, and replicates in the membrane-bound compartment in the cytoplasm of infected host cells, resembling the early autophagosome [14]. *A. phagocytophilum* can be propagated in human promyelocytic leukemia cell line HL-60 and some endothelial cells, such as RF/6A cells. *A. phagocytophilum* has a developmental cycle. Infection starts with small infectious dense-cored cells (DCs), which convert to large replicating reticulate cells (RCs), and then goes back to DCs to initiate a new cycle of infection [2]. A characteristic morphology of growing *A. phagocytophilum* is called "morula" (mulberry in Latin), because it grows as aggregates (clumps). It takes *A. phagocytophilum* 3–4 days to complete the developmental cycle in a host cell.
2. Put the glass coverslips (18 × 18 mm) in autoclave pouches, sealed, and autoclave for 15 min at 121 °C.

3. Use wide-bore pipette tips to resuspend cells to prevent cell damage.
4. Saponin in the PGS solution binds to cholesterol in cell membranes, and forms pores in cell membranes.
5. Centrifuge the primary and secondary antibodies at 12,000 × *g* for 10 min (4 °C) to remove aggregates prior to use.
6. Keep the cells from the light during incubation with fluorochrome-conjugated secondary antibodies.
7. The secondary antibodies are affinity purified and preabsorbed with IgG from other species of animals to prevent cross reaction. Thus, different secondary goat antibodies can be mixed to detect primary antibodies from different species without cross reaction. However it is necessary to make sure that there is no cross reactions between antibodies and no cross-talk among different color channels by performing each pair of primary and secondary antibodies, separately. Also negative controls need to be set up to make sure there are no non-specific reactions of secondary antibodies to infected cells or *A. phagocytophilum*.
8. As Ats-1 is translocated from *A. phagocytophilum* into mitochondria, it is expected to see the Ats-1, which is not colocalized with *A*, *phagocytophilum* labeled with horse anti-*A*, *phagocytophilum* serum, colocalizes with Mn-Sod (mitochondria marker).
9. Complete advanced MEM prewarmed to 37 °C is quickly added into electroporation cuvettes after electroporation to obtain better cell viability.
10. Electroporation is used to transfect RF/6A cells, as this method produces higher transfection efficiency (~50 %) than chemical transfection agent (~20 %) for RF/6A cells.
11. The transfection efficiency needs to be checked before proceeding to the next step, because the signal change may not be significant in cells with low transfection efficiency.
12. Etoposide, a topoisomerase II inhibitor, causes DNA damage and induces apoptosis. It is toxic, and carcinogenic. Precautions to prevent inhalation, and skin contact should be taken by wearing gloves, lab coat, and face mask when handling.
13. Only the cells adhering to coverslips are fixed. Detached cells are removed by washing.
14. During mitochondria-mediated apoptosis, Bax translocates to mitochondrial membranes, and cytochrome *c* is released from mitochondria to cytosol, leading to the chromosomal condensation. Cytochrome *c* release, nuclear condensation, and Bax translocation to mitochondria are examined to determine apoptotic status of cells.

15. As negative control, the sham (pEGFP-N1)-transfected cells were also incubated with mouse anti-cytochrome *c*, or mouse anti-Bax antibodies.
16. It is expected to see that the cells, expressing apoptosis inhibitory proteins, including Ats-1, are more resistant to apoptosis induction, as indicated by fewer cells with condensed nuclei, released cytochrome *c* and Bax translocation to mitochondria, than sham-transfected cells.
17. PARP is one of the targets of apoptosis execution protease, caspase-3. PARP cleavage can serve as a marker of cells undergoing apoptosis.
18. Actin, a housekeeping protein in cells, is used to normalize the loading amount between samples.
19. Super pap pen is used to make a hydrophobic barrier to keep the antibody solution inside.
20. MitoTracker Red is used to label mitochondria in yeast cells, as there are not many antibodies which are commercially available to label yeast mitochondria in immunostaining. The mouse monoclonal antibody against yeast mitochondria porin from Invitrogen can only be used in western blotting.
21. pBax is the plasmid which expresses human Bax gene under the control of yeast inducible *GAL1* promoter in vector pYES2/NT A (Invitrogen). Bax is not expressed in medium containing glucose, but expressed in medium containing galactose. pYAts-1 is the plasmid which expresses *A. phagocytophilum* Ats-1 under the control of yeast constitutive *ADH1* promoter in vector pGADT7 AD (Clontech). Ats-1 is expressed in medium containing glucose or galactose.
22. Bax inhibits yeast cell growth by inducing apoptosis. Ats-1 has the ability to antagonize the action of Bax. Thus it is expected that compared to negative control (pBax and pGADT7 AD-cotransformed cells), pBax and pYAts-1-cotransformed cells have high numbers of viable cells at day 5 post-culture.
23. **Steps 8–19** is the procedure to isolate crude yeast mitochondria, which was described before [15].
24. SDS lyses spheroplasts, which have ghost-like shapes after treatment, observed by phase contrast microscopy. Before SDS treatment, solid gray content is observed inside spheroplasts with a microscope. After treatment, the inside of spheroplasts becomes clear.
25. The total protein amount of Bax, Ats-1, and yeast mitochondrial protein (porin) in spheroplasts is measured in order to compare them to those in isolated mitochondria.

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Chapter 11

Bimolecular Fluorescence Complementation for Imaging Protein Interactions in Plant Hosts of Microbial Pathogens

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Abstract

Protein–protein interactions mediate many aspects of cellular function. Scientists have developed numerous techniques to investigate these interactions, both in vitro and in vivo. Among these, the peptide complementation assay Bimolecular Fluorescence Complementation (BiFC) allows visualization of the subcellular sites of protein–protein interactions in living cells. BiFC comprises a "split GFP" system: GFP protein (or its derivatives) is split into two fragments, neither of which fluoresces on its own. Interacting proteins linked to these peptide fragments may bring them into proximity, allowing them to refold and restore fluorescence. Although this system was first exploited for use in animal cells, we have developed BiFC for use in plants. Pathogens transfer numerous effector proteins into eukaryotic cells and manipulate host cellular processes through interactions between effector and host proteins. BiFC can therefore facilitate studies of host–bacterial interactions. In this chapter, we describe the numerous BiFC vectors we have constructed, their uses, and their limitations.

Key words Fluorescence imaging, Peptide complementation, Fluorescent proteins, Subcellular localization, Plant vectors

1 Introduction

Protein function is often mediated through formation of transient or stable complexes with other proteins. Some proteins, such as kinases, phosphatases, F-box proteins, ubiquitin ligases, etc., associate transiently with their targets to effect protein modifications. Some enzymes must be activated by posttranslational modifications catalyzed by proteins with which they interact. Many proteins interact with numerous partners to form complexes necessary for function. Finally, many proteins, including translocated bacterial effector proteins, must interact with other proteins for proper intracellular localization. Thus, investigations of protein–protein interactions are essential to understand cellular functions and to gain a better understanding of host–pathogen interactions at the protein level.

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Scientists have developed numerous techniques to investigate protein–protein interactions. Several of these function in vitro using purified proteins or cellular extracts. These include structural studies on co-crystallized proteins, co-immunoprecipitation, "pull-down" assays using tagged proteins, and "far-western" blotting or gel overlay assays. Other "quasi-in vivo" assays use surrogate hosts to investigate protein–protein interactions. These include yeast and bacterial two-hybrid systems. Additional in vivo assays use the natural host in which particular proteins are normally produced. The advantages of these latter assays are that the proteins are made (and modified) in their natural environments, and one can often follow subcellular localization and trafficking of protein complexes. The disadvantage of these techniques is that they are conducted in a complex milieu, and that protein–protein interactions can be indirect, reflecting complexes with "bridging" molecules.

In vivo protein–protein interaction techniques include co-immunoprecipitation, tandem affinity purification of complexes (TAP tagging), Förster resonance energy transfer (FRET), and bioluminescence resonance energy transfer (BRET). Recently, peptide complementation assays using split luciferase, GFP, or RFP molecules, or their derivatives, have become popular [1–8]. Split GFP/RFP assays are relatively easy to conduct and, unlike several other techniques, do not require specialized equipment or computer algorithms other than a fluorescence or confocal microscope.

Bimolecular fluorescence complementation (BiFC) is a peptide complementation assay in which complementing fluorescent protein fragments, neither of which fluoresces on its own, are brought together in such a way that they can fold and restore fluorescence. In practice, this is accomplished by individually placing each of the two partial fluorescent protein fragments, as translational fusions, onto other proteins of interest. If the two proteins of interest interact, they may bring together the complementing fluorescent protein fragments, and fluorescence may be restored (Fig. 1). Scientists first developed BiFC in animal systems [1, 9], but quickly adapted it for use in bacterial [10, 11] and plant species [12–15]. In addition to indicating protein–protein interactions, BiFC can be used to localize the subcellular site of interaction [14, 16, 17]. The inclusion of alternatively colored fluorescent organelle markers helps identify these sites [18]. For reviews of BiFC, *see* [19–23]. Although BiFC can be practiced using proteins derived from DsRed (e.g., 3, 6–8), GFP derivatives form the basis of most BiFC systems. Several of the initially described systems used the enhanced Yellow Fluorescent Protein (EYFP), whereas later systems employed the brighter and more stable yellow derivative Venus [24]. Similarly, the brighter blue fluorescent protein Cerulean augmented the original blue Cyan Fluorescent Protein (CFP) [17, 25–27]. However, there are advantages and disadvantages of using each fluorescent protein. Scientists need to be open-minded in order to

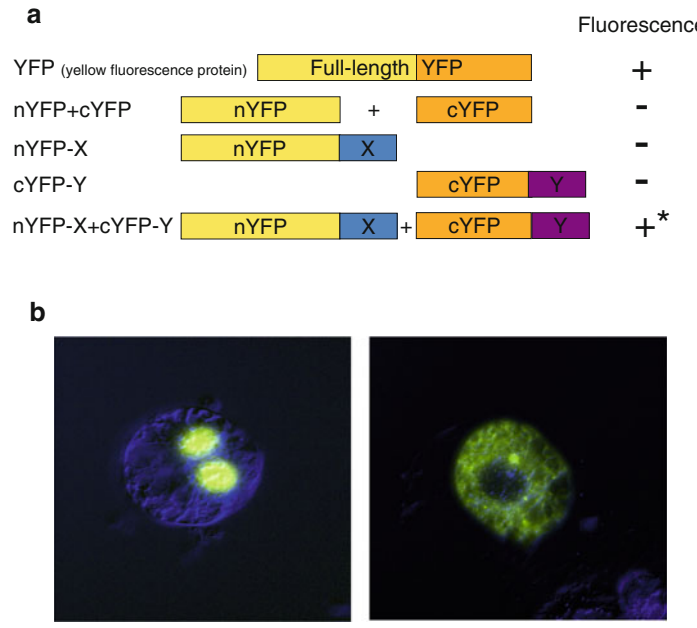

Fig. 1 (**a**) Schematic diagram of the BiFC process. A fluorescent protein (YFP is shown as an example) is split; one part is affixed to protein X, and the other to protein Y. If proteins X and Y interact, they may bring the two portions of YFP together such they will fold correctly and restore fluorescence (*asterisk*). (**b**) Examples of BiFC in tobacco BY-2 protoplasts. These are merged bright field images (pseudocolored in *blue*) with YFP fluorescence images (*yellow*). *Left panel*, interaction of *Agrobacterium* VirD2 protein with the *Arabidopsis* importin-α protein AtImpa-1. Note the *yellow fluorescence* signal in the nuclei; *right panel*, interaction of *Agrobacterium* VirE2 protein with VirE2. Note the *yellow fluorescence* signal in the cytoplasm. nYFP, N-terminal fragment of YFP; cYFP, C-terminal fragment of YFP

choose the best candidate with which to work. Table 1 presents information regarding the fluorescence characteristics and imaging of these proteins. The "split" in these 238 amino acid derivatives can be in one of several places. Initial systems used fragments split between amino acids 154 and 155, whereas more recent systems utilize splits between amino acids 173 and 174. An interesting variant of these systems employs an "overlap" of fluorescent protein fragments: the N-terminal fragment extends to amino acid 173, whereas the C-terminal fragment initiates at amino acid 155. This combination results in brighter fluorescence complementation [17, 25].

Several excellent BiFC systems exist for use in plants [8, 12, 13, 15, 27]. Below, we describe a system developed in our laboratory [14, 17] that we have used to show subcellular localization of several *Agrobacterium tumefaciens* virulence effector proteins that are translocated by the bacterial type IV secretion system into host

Table 1
Characteristics of fluorescent proteins used for BiFC

Protein	Excitation (nm)	Emission (nm)	Brightness[a]	Photostability[b]	p*K*a	Oligomerization
EGFP	488	507	34[c]	174	6.0	Weak dimer
EYFP	514	527	51[c]	60	6.9	Weak dimer
Venus	515	528	53[c]	15	6.0	Weak dimer
ECFP	433	475	18[c]	59	5.0	Weak dimer
Cerulean	433	475	27[c]	36	4.7	Weak dimer
DsRed	558	583	41[d]	16 (for monomer)	4.7 (4.7)	Tetramer
				Maturation rate at 37 °C: 9.9 h		
mRFP1	584	607	6[d]	9	4.5	Monomer
				Maturation rate at 37 °C: 0.2 h		
mRFP1-Q66T	549	570	16[d]	>9	7.5	Monomer
				Maturation rate at 37 °C: 0.6 h		
mCherry	587	610	16[c,d]	96	<4.5	Monomer
				Maturation rate at 37 °C: 0.25 h		

[a]Brightness values are the product of the extinction coefficient and quantum yield at pH 7.4 under maturation conditions, in mM^{-1} cm^{-1}. For comparison, free fluorescein at pH 7.4 has a brightness of ~69 $(mM\ cm)^{-1}$
[b]Time for bleaching from an initial emission rate of 1,000 photons/s down to 500 photons/s ($t_{1/2}$; for comparison, fluorescein at pH8.4 has $t_{1/2}$ of 5.2 s). Data for photostability are from [37]. Data for maturation rate are from [3, 38]
[c]Data are from [37]
[d]Data are from [3, 37, 38]

cells, and their interactions with host proteins. We give a step-by-step description of the transfection of tobacco BY2 cells, and the analysis of BiFC interactions using fluorescence microscopy. The Notes section contains much additional information on the methodology that will aid the reader in conducting research using BiFC.

2 Materials

1. pSAT-derived plant BiFC vectors (Table 2 describes the current BiFC vectors from our laboratory (*see* **Note 1**).
2. BY-2 medium: 4.3 g Murashige and Skoog (MS) salts, 1 mg vitamin B1, 370 mg KH_2PO_4, 2 mg 2,4-dichloro-phenoxyacetic acid (2,4-D), and 30 g sucrose in 950 ml water. Adjust pH to

Table 2
Fluorescent protein tagging vectors

Gelvin lab stock number	Plasmid name	Expression cassette flanking sites	Reference
BiFC Vectors contain a CaMV double 35S promoter and TEV translation enhancer, except when indicated, a nopaline synthase (nos) promoter is used			
nVenus-C			
E3228	pSAT1-nVenus-C	*Asc*I	[17]
E3229	pSAT4-nVenus-C	I-*Sce*I	[17]
E3230	pSAT6 -nVenus-C	PI-*Psp*I	[17]
nVenus-N			
E3308	pSAT1-nVenus-N	*Asc*I	[17]
E3231	pSAT1A-nVenus-N	*Asc*I	[17]
E3310	pSAT4-nVenus-N	I-*Sce*I	[17]
E3232	pSAT4A-nVenus-N	I-*Sce*I	[17]
E3233	pSAT6-nVenus-N	PI-*Psp*I	[17]
E3962	pSAT1-nVenus(155)-N	AscI	Unpublished
E3963	pSAT1-nVenus(155)I152L-N	AscI	Unpublished
cCFP-C			
E3242	pSAT1-cCFP-C	*Asc*I	[17]
E3243	pSAT4-cCFP-C	I-*Sce*I	[17]
E3244	pSAT6-cCFP-C	PI-*Psp*I	[17]
cCFP-N			
E3449	pSAT1-cCFP-N	*Asc*I	[17]
E3450	pSAT1A-cCFP-N	*Asc*I	[17]
E3451	pSAT4-cCFP-N	I-*Sce*I	[17]
E3347	pSAT4A-cCFP-N	I-*Sce*I	[17]
E3497	pSAT6-cCFP-N	PI-*Psp*I	[17]
nCerulean-C			
E3415	pSAT1-nCerulean-C	*Asc*I	[17]
E3416	pSAT4-nCerulean-C	I-*Sce*I	[17]
E3417	pSAT6-nCerulean-C	PI-*Psp*I	[17]
nCerulean-N			
E3307	pSAT1-nCerulean-N	*Asc*I	[17]
E3246	pSAT1A-nCerulean-N	*Asc*I	[17]
E3309	pSAT4-nCerulean-N	I-*Sce*I	[17]
E3247	pSAT4A-nCerulean-N	I-*Sce*I	[17]
E3248	pSAT6-nCerulean-N	PI-*Psp*I	[17]
nEYFP-C			
E3075	pSAT1-nEYFP-C1	*Asc*I	[14]
E3081	pSAT4-nEYFP-C1	I-*Sce*I	[14]
E2884	pSAT6-nEYFP-C1	PI-*Psp*I	[14]
E4054	pSAT1-Pnos-nYFP-C	*Asc*I	Unpublished

(continued)

Table 2
(continued)

Gelvin lab stock number	Plasmid name	Expression cassette flanking sites	Reference
nEYFP-N			
E3077	pSAT1-nEYFP-N1	*Asc*I	[14]
E3079	pSAT1A-nEYFP-N1	*Asc*I	[14]
E3083	pSAT4-nEYFP-N1	I-*Sce*I	[14]
E3085	pSAT4A-nEYFP-N1	I-*Sce*I	[14]
E2913	pSAT6-nEYFP-N1	PI-*Psp*I	[14]
E3960	pSAT1-nEYFP(155)-N	AscI	Unpublished
E3961	pSAT1-nEYFP(155)I152L-N	AscI	Unpublished
E4053	pSAT1-Pnos-nYFP-N	*Asc*I	Unpublished
cEYFP-C			
E3076	pSAT1-cEYFP-C1(B)	*Asc*I	[14]
E3082	pSAT4-cEYFP-C1(B)	I-*Sce*I	[14]
E3108	pSAT6-cEYFP-C1	PI-*Psp*I	[14]
E4020	pSAT1-Pnos-cEYFP-C	*AscI*	Unpublished
cEYFP-N			
E3078	pSAT1-cEYFP-N1	*Asc*I	[14]
E3080	pSAT1A-cEYFP-N1	*Asc*I	[14]
E3084	pSAT4-cEYFP-N1	I-*Sce*I	[14]
E3323	pSAT4A-cEYFP-N1	I-*Sce*I	[14]
E2914	pSAT6-cEYFP-N1	PI-*Psp*I	[14]
E3086	pSAT6A-cEYFP-N1	PI-*Psp*I	Unpublished
E4023	pSAT1-Pnos-cEYFP-N	*AscI*	Unpublished
cCFP′-C[a]			
E3596	pSAT1-cCFP′-C	*Asc*I	Unpublished
cCFP′-N[a]			
E3595	pSAT1A-cCFP′-N	*Asc*I	Unpublished
BiFC vectors with nopaline synthase (Pnos) promoter			
E3683	pSAT5-Pnos-cCFP-C	I-*Ceu*I	Unpublished
E3685	pSAT5-Pnos-cCFP-N	I-*Ceu*I	Unpublished
E3684	pSAT5-Pnos-nVenus-C	I-*Ceu*I	Unpublished
E3686	pSAT5-Pnos-nVenus-N	I-*Ceu*I	Unpublished
BiFC vectors with peptide tags			
T7-nVenus-C			
E3454	pSAT1-T7-nVenus-C	*Asc*I	Unpublished
E3455	pSAT4-T7-nVenus-C	I-*Sce*I	Unpublished
E3456	pSAT6-T7-nVenus-C	PI-*Psp*I	Unpublished
nVenus-T7-N			
E3727	pSAT1A-nVenus-T7-N	*Asc*I	Unpublished
E3726	pSAT4A-nVenus-T7-N	I-*Sce*I	Unpublished
E3728	pSAT6-nVenus-T7-N	PI-*Psp*I	Unpublished
T7-nCerulean-C			
E3723	pSAT1-T7-nCerulean-C	*Asc*I	Unpublished
E3724	pSAT4- T7-nCerulean-C	I-*Sce*I	Unpublished
E3725	pSAT6- T7-nCerulean-C	PI-*Psp*I	Unpublished

(continued)

Table 2
(continued)

Gelvin lab stock number	Plasmid name	Expression cassette flanking sites	Reference
myc-nCerulean-N			
E3734	pSAT1A-nCerulean-myc-N	*Asc*I	Unpublished
E3453	pSAT4A-nCerulean-myc-N	I-*Sce*I	Unpublished
Rare cloning sites (RCS) on high copy number plasmid			
E3074	pBluescript, ampicillin resistant	–	Unpublished
E3414	pUC119, ampicillin resistant	–	[17]
E3610	pRCIII, kanamycin resistant		Unpublished
Rare cloning sites (RCS) on T-DNA binary vectors			
E3185	*hpt*II for plant selection	–	Unpublished
E3184	*npt*II for plant selection	–	Unpublished
E3407	*bar* for plant selection	–	Unpublished
E3055	*bar* for plant selection	–	[17]
E3519	*bar* for plant selection	–	Unpublished
E4082	*hpt*II for plant selection	–	Unpublished
E4085	*npt*II for plant selection	–	Unpublished
E4145	*bar* for plant selection	–	Unpublished
Full-length fluorescent protein tagging vectors			
Cerulean-C			
E3528	pSAT1-Cerulean-C	*Asc*I	Unpublished
E3529	pSAT4-Cerulean-C	I-*Sce*I	Unpublished
E3530	pSAT6-Cerulean-C	PI-*Psp*I	Unpublished
Cerulean-N			
E3534	pSAT4A-Cerulean-N	I-*Sce*I	Unpublished
Venus-C			
E3542	pSAT1-Venus-C	*Asc*I	Unpublished
E3543	pSAT4-Venus-C	I-*Sce*I	Unpublished
E3544	pSAT6-Venus-C	PI-*Psp*I	Unpublished
E4041	pSAT1-Pnos-Venus-C	*Asc*I	Unpublished
Venus-N			
E3758	pSAT1-Venus-N	*Asc*I	Unpublished
E3533	pSAT4A-Venus-N	I-*Sce*I	Unpublished
E4042	pSAT1-Pnos-Venus-N	*Asc*I	Unpublished
EYFP-C			
E3150	pSAT6-EYFP-C1	PI-*Psp*I	Unpublished
EYFP-N			
E3225	pSAT6-EYFP-N1	PI-*Psp*I	Unpublished
mRFP-C			
E3026	pSAT6-mRFP-C1	PI-*Psp*I	[17]
mRFP-N			
E3025	pSAT6-mRFP-N1	PI-*Psp*I	[17]
mCherry-C			
E3275	pSAT6-mCherry-C	PI-*Psp*I	Unpublished
mCherry-N			
E3279	pSAT4A-mCherry-N	I-*Sce*I	Unpublished

[a]Mutant cCFP to diminish dimerization

5.7 using 1 N KOH. Top up with water to 1 L. Aliquot 50 ml into each of 20 250-ml flasks. Seal flasks with aluminum foil and autoclave the medium at 250 °F for 20 min.

3. Protoplast isolation solution: 7.4 g $CaCl_2 \cdot 2H_2O$, 1.6 g $NaOAc \cdot 3H_2O$, and 45 g mannitol in 950 ml water. Adjust pH to 5.7 using 1 N KOH. Top up with water to 1 L. Sterilize by autoclaving at 250 °F for 20 min.
4. Protoplast enzyme digestion solution: 0.48 g of Cellulase R10 (Onazuka, 1.2 %) and 0.24 g of Macerozyme (0.6 %) in 35 ml water. Stir until most of the powder is dissolved, adjust pH to 5.7 using 0.1 N HCl, and add water to make up to 40 ml. This solution needs to be prepared fresh immediately before use. Transfer the solution into a 50-ml conical tube, centrifuge the solution at $1600 \times g$ for 10 min to pellet the insoluble substances. Sterilize the clear supernatant fluid by passing the solution through a 0.2 μm syringe filter into a sterile tube.
5. Protoplast floating solution: 99 mg myo-inositol, 2.88 g L-proline, 100 mg enzymatic casein hydrolysate, 102.6 g sucrose, 97.6 mg MES, 4.3 g MS salts, 1 mg vitamin B1, 370 mg KH_2PO_4 in 950 ml water. Adjust the pH to 5.7 using 1 N KOH. Top up with water to 1 L. Filter-sterilize the solution using a 0.2 μm sterile filter unit (e.g., Nalgene or Millipore).
6. 40 % PEG solution (1 ml): 0.4 g of PEG4000 (Fluka), 0.5 ml of 0.8 M mannitol, 0.1 ml of 1 M $CaCl_2$, and 0.05 ml water. Warm the tube in a 55 °C water bath and vortex the solution from time to time to help the PEG dissolve completely. Always prepare this solution fresh immediately before use. Depending on the number of samples in your experiment, you may need to scale up the quantity of this solution by increasing all components proportionally.
7. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7. The solution can be sterilized by autoclaving. Store the solution refrigerated (*see* **Note 2**).
8. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7. Filter-sterilize using a 0.2 μm filter. Keep the solution refrigerated all the time (*see* **Note 2**).
9. Incubation solution: Same as preparation of BY-2 medium except add 72.9 g mannitol when making 1 L of BY-2 medium.
10. 1 % BSA (bovine serum albumin).
11. Shaker with clips for 250-ml flask, operate at room temperature.
12. Tabletop low-speed centrifuge with swing-bucket rotor.
13. Sterile 50 ml conical centrifuge.
14. 12 × 75 mm polypropylene tube.
15. 20 × 100 mm sterile plastic petri dishes.

16. Parafilm.
17. Aluminum foil.
18. Rotary shaker, operate at room temperature.
19. Inverted light microscope.
20. Glass slides and coverslips.
21. Hemocytometer.
22. BY-2 cells.
23. Epi-fluorescence microscope or confocal microscope equipped with filters for YFP, CFP, and RFP and 20× and 40× objective lens.
24. 24-well tissue culture plates.

3 Methods

3.1 Cloning Using pSAT-Derived Plant BiFC Vectors

1. Choose the appropriate vector (*see* **Notes 3–6**, Table 1 and Fig. 2).
2. Generate fusions of proteins of interest with fluorescent protein fragments using standard recombinant DNA techniques (*see* **Notes 7** and **8**).
3. Optional: Combine multiple BiFC expression cassettes into one vector using standard recombinant DNA techniques (*see* **Notes 9–12**) [17, 28]. Figure 3 shows maps of these vectors.

3.2 Tobacco BY-2 Protoplast Preparation

1. Tobacco BY-2 suspension cultures are maintained in BY-2 medium. Every 7 days, transfer 1.2 ml of BY-2 cells into 50 ml of fresh BY-2 medium in a 250-ml flask. Shake the cells on a shaker at 140 rpm at room temperature for 4–5 days before use (*see* **Note 13**).
2. Using sterile technique, transfer 20 ml of cells into a 50 ml sterile conical centrifuge tube, cap the tube, and centrifuge the cells at 190 × *g* for 5 min at room temperature.
3. With a sterile pipette, gently remove the supernatant solution (being careful not to disturb the rather loose pellet), and suspend the cells in 40 ml of Protoplast Enzyme Digestion Solution.
4. Transfer the suspended cells into two 20 × 100 mm sterile plastic petri dishes, tape the dishes with Parafilm, cover the dishes with aluminum foil and place them on a rotary shaker with gentle shaking (60 rpm) for about 4 h at room temperature (*see* **Note 14**).
5. To purify protoplasts away from non-protoplasted cells and cellular debris, collect digested cells in a sterile 50 ml conical tube. Cap the tube and centrifuge at 190 × *g* for 5 min.

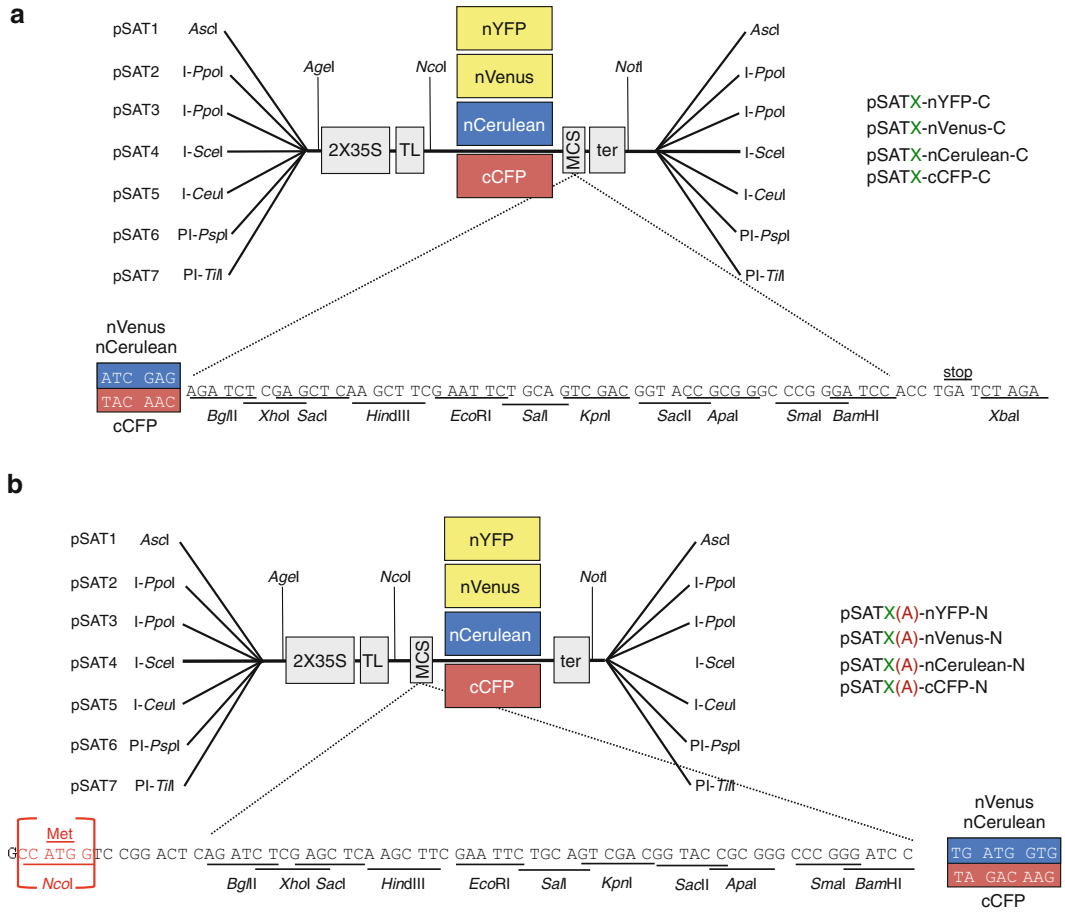

Fig. 2 Maps of the various vectors used for tagging proteins with split GFP derivatives. Protein reading frames are indicated by the indicated three-nucleotide codons. (**a**) Vectors for use in tagging proteins at their N-termini (i.e., the protein of interest has an N-terminal tag). (**b**) Vectors for use in tagging proteins at their C-termini (i.e., the protein of interest has a C-terminal tag). Note that in vectors denoted by pSATX(A), the *Nco*I site (depicted in brackets) upstream of the multiple cloning site has been deleted, resulting in loss of the upstream methionine codon

Fig. 3 (continued) resistance upon bacteria. *Arrows* indicate the known direction of transcription. The direction of transcription of the plant selection markers in E3184 and E3185 is not yet determined. LB/RB, T-DNA left/right border sequences; P, plant-active promoter; Term, polyA addition signal sequence; *ocs*, octopine synthase; *nptII*, neomycin phosphotransferase II gene conferring resistance to kanamycin; *hptII*, hygromycin phosphotransferase II gene conferring resistance to hygromycin; *bar*, gene conferring resistance to the herbicides Basta/bialophos/phosphinothricin. "E" numbers indicate strain numbers in the Gelvin laboratory *E. coli* stock collection. Sites marked in *black* are unique. Unique rare-cutting sites are marked in *red*. Sites that are present but are not unique are marked in *blue*

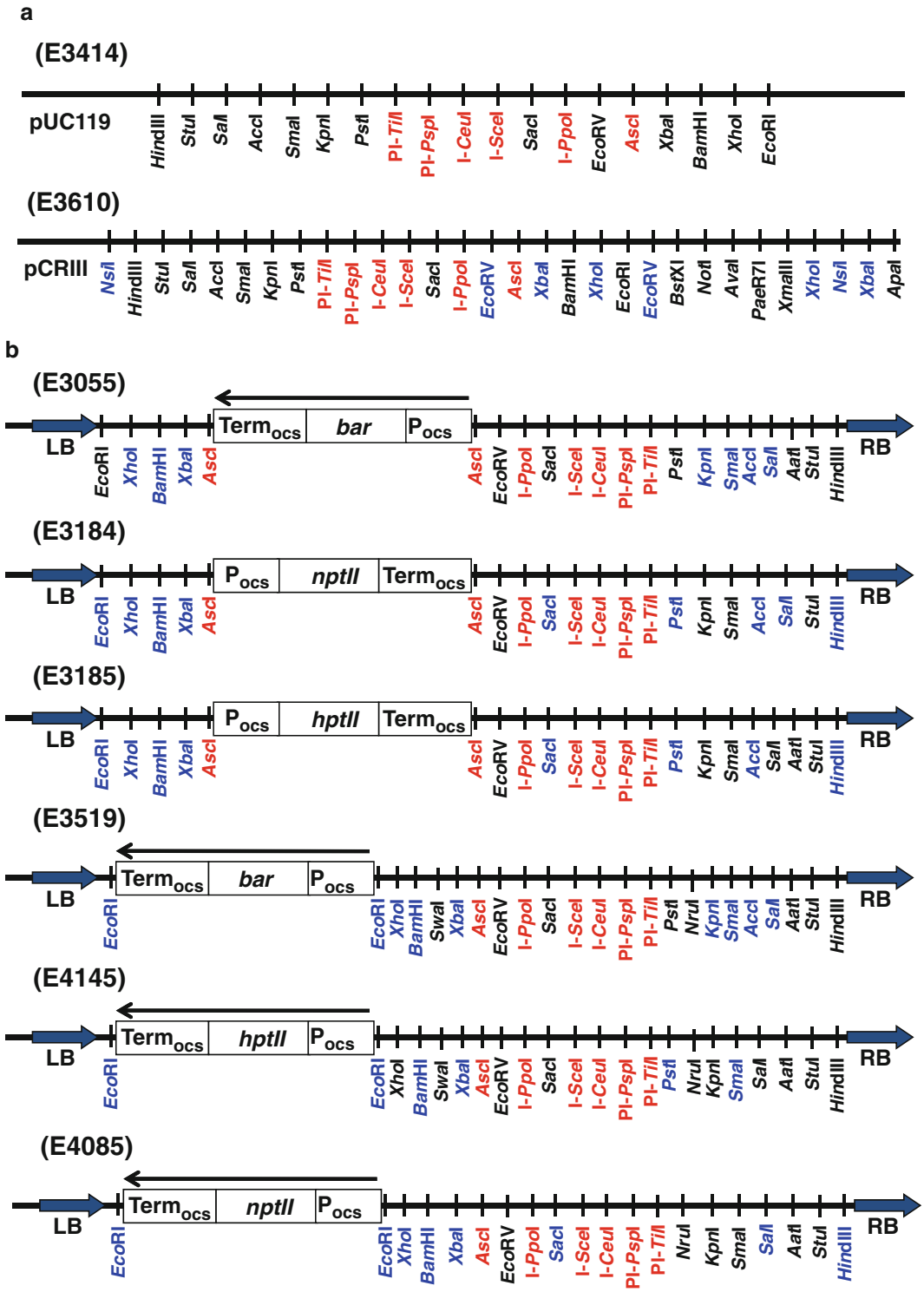

Fig. 3 Restriction/homing endonuclease maps of the "final vectors" used to clone multiple BiFC expression cassettes. (**a**) High copy number vectors based on pUC119 (ampicillin-resistant) and pCRIII (kanamycin-resistant) plasmids. (**b**) T-DNA binary vectors. These binary vectors contain an *aadA* gene conferring spectinomycin

After centrifugation, gently remove the supernatant solution with a sterile pipet.

6. Add 40 ml Protoplast Isolation Solution, cap the tube, and suspend the protoplasts by "rocking" the tube (*see* **Note 15**) gently. Centrifuge the cells at 190 × *g* for 5 min. Again, gently remove the supernatant solution and suspend the protoplasts *gently* in 40 ml of Protoplast Isolation Solution. Cap the tube and centrifuge again 190 × *g* for 5 min. Discard the supernatant solution after centrifugation.
7. Suspend the protoplasts in 45 ml of Protoplast Floating Solution. Cap the tube and centrifuge the cells at 190 × *g* for 10 min. In this solution, the protoplasts will float to the top of the tube, while any non-protoplasted cells will pellet.
8. Gently remove the floating protoplasts with a sterile, cut-end Pipetman P1000 tip and transfer to a sterile 50 ml conical tube.
9. Add 30 ml of W5 Solution and suspend the cells gently. Cap the tube and centrifuge at 190 × *g* for 5 min. The protoplasts will pellet in this solution. Gently remove the supernatant solution.
10. Gently resuspend the protoplasts in 40 ml of W5 Solution. Centrifuge at 190 × *g* for 5 min. Gently remove all *except* 10 ml of the supernatant solution. Gently resuspend the protoplasts in this 10 ml of solution.
11. Make 1:10 and 1:100 dilutions of a small sample of protoplasts in W5 solution, and count the cells using a hemocytometer. Keep protoplasts on ice for at least 30 min before using them.

3.3 PEG-Mediated Transfection of BY-2 Protoplasts

1. Coat 24-well tissue culture plates by adding 0.8 ml of 1 % BSA in each well to avoid protoplast attachment to the bottom of the wells. Leave the plate at room temperature for 30 min.
2. Prepare the 40 % PEG solution.
3. Centrifuge the protoplast suspension at 190 × *g* for 3 min. Remove the supernatant solution and add an appropriate volume of cold MMg solution to adjust the cell density to 1×10^6 cells/ml. Keep the tube on ice.
4. Mix all DNA samples (10 μg of each DNA sample, *see* **Note 16**) in a volume of 10 μl in a 12 × 75 mm polypropylene tube. Gently add 100 μl of protoplasts to the tube and mix well by gently tapping the tube with your fingers.
5. Add 110 μl of 40 % PEG solution to the tube and mix with the cells gently but thoroughly by tapping the tube with your fingers. Leave the transfection reaction at room temperature for 5 min.
6. After 5 min, add 2 ml of W5 solution to the tube and mix thoroughly but gently to make sure the PEG is well mixed

with the W5 solution. Centrifuge the protoplast suspension at 190 × *g* for 3 min.

7. Repeat **step 6** one more time.
8. Remove the BSA solution from the 24-well plate.
9. Remove the supernatant solution from the tubes and add 0.7 ml of incubation solution to each tube. Suspend the cells gently by tapping the tube with fingers. Transfer all cells in each tube into individual wells of the plate. Incubate the plate overnight at room temperature in the dark (*see* **Note 17**).
10. The protoplasts can be used for 24 h after preparation. Just maintain the cells in W5 solution at 4 °C.

3.4 Acquire Fluorescence Images

1. Take out 20 μl of cells from each sample using a cut-end plastic tip and apply on a glass slide.
2. Place a coverslip gently on the slide and make sure there is no bubble trapped under the coverslip (*see* **Note 18**). Attach the coverslip to the slide at the four corners using nail polish.
3. Place the slide on the microscope stage to view fluorescence. Either an epi-fluorescence microscope or a confocal microscope with the proper lens, fluorescent filters, and imaging software can be used (*see* **Note 19**).

3.5 Advanced BiFC and Troubleshooting

1. Multicolor BiFC (*see* **Note 20**, and Fig. 4), BiFC combined with FRET (*see* **Note 21**), bridge-BiFC (*see* **Note 22**), interactions with peptide aptamers (*see* **Note 23**), and screening of cDNA libraries (*see* **Note 24**) are recent adaptations of the protocol.
2. **Notes 25–29** describe several limitations and problems that can arise during BiFC experiments and analysis of interactions.

4 Notes

1. Tzfira's laboratory first described the pSAT system for expressing multiple proteins in plants [28, 29]. The pSAT system consists of numerous "satellite" vectors containing expression cassettes which can be combined into one of several pUC- or T-DNA binary vector-based final vectors. These final vectors contain a "polylinker" containing multiple rare-cutting sites (RCS) described in Goderis et al. [30]. These different rare-cutting restriction or homing endonuclease sites flank the expression cassettes in each of the pSAT vector series (pSAT1-6).

   Table 2 describes the current BiFC vectors from our laboratory. The pSAT vectors are built in modular form (see Fig. 2). Promoters and terminators flank a "standard" multiple cloning

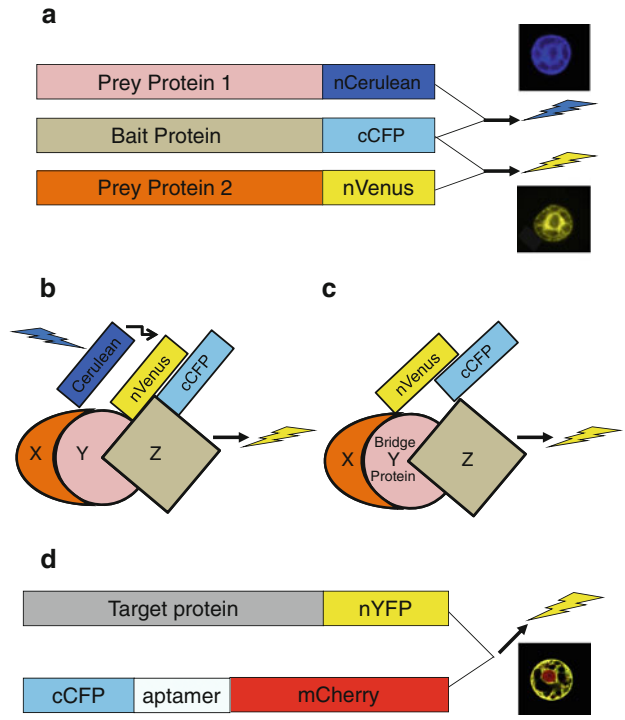

Fig. 4 Schematic diagrams depicting "advanced" uses of BiFC. (**a**) Multicolor BiFC. The "bait" protein is tagged with cCFP, and two different "prey" proteins are individually tagged with either nVenus or nCerulean. Interaction of the nVenus tagged protein with the bait protein may result in *yellow fluorescence*, whereas interaction of the nCerulean tagged protein with the bait protein results in *blue fluorescence*. (**b**) BiFC-FRET to indicate interaction of three proteins. Protein X is tagged with full-length Cerulean, and the two other proteins (Y and Z) are individually tagged with nVenus and cCFP, respectively. Excitation with *blue light* may result in Cerulean fluorescence, which may in turn excite *yellow fluorescence* from interacting proteins tagged with nVenus and cCFP. For this to occur, the three proteins must be closely aligned in a complex. (**c**) Bridge-BiFC. Two proteins, individually tagged with nVenus and cCFP, cannot interact without a "bridging" protein, which simultaneously interacts with both of them, forming a three-protein complex. Only under these conditions may fluorescence complementation occur. (**d**) Interaction of a target "bait" protein, tagged with nYFP, with a peptide aptamer tagged at the N-terminus with cCFP and at the C-terminus with full-length mCherry. If the peptide aptamer and target protein interact, this may permit folding of nYFP with cCFP, generating *yellow fluorescence*. Expression of the aptamer can be monitored by visualizing mCherry *red fluorescence*

site (mcs) composed of numerous six-base recognition restriction endonuclease sites. The promoters and terminators are, in turn, flanked by restriction endonuclease sites, allowing "switching" of these elements with other promoters or terminators. Some of the pSAT vectors additionally incorporate

full-length fluorescent protein genes, either preceding the polylinker (for N-terminal tagging of proteins) or following the polylinker (for C-terminal tagging of proteins). Users can employ these vectors to make fusion proteins and check the subcellular localization of proteins of interest. BiFC pSAT vectors contain fluorescent protein gene fragments for N- or C-terminal tagging of proteins (Fig. 2). These gene fragments encode N-terminal fragments of EYFP, Venus, or Cerulean (nEYFP, nVenus, nCerulean), or C-terminal fragments of EYFP or CFP (cEYFP, cCFP). Venus is a variant of EYFP, generated from multiple amino acid residue mutations of EYFP. This fluorescent protein has rapid and efficient maturation kinetics and is relatively less sensitive to acid and quenching by chloride ion [24] than is EYFP. Therefore, Venus enables the visualization of fluorescent fusion proteins in an acidic environment. Furthermore, the fluorescence intensity of Venus-based BiFC is about ten times higher than that of EYFP-based BiFC [25]. Thus, when using Venus in BiFC assays, less quantity of DNA is needed to ensure fluorescence visualization. On the other hand, when the high intensity fluorescent protein Venus is used in a BiFC assay, the signal-to-noise ratio will be lowered due to possible spontaneous self-assembly of the two split fluorescent protein fragments [31]. Users should be aware of this possibility and may wish to choose a lower intensity variant, such as EYFP, instead.

Although most pSAT-based BiFC vectors utilize a Cauliflower Mosaic Virus (CaMV) double 35S promoter, we have also generated a set of vectors incorporating the nopaline synthase (*nos*) promoter. This weaker promoter mitigates background fluorescence, as described below. In addition, other promoters or terminators, including those of the native gene, can replace the CaMV 35S promoter and terminator using the unique restriction sites (*Age*I and *Nco*I for promoters, *Xba*I and *Not*I for terminators) flanking these regions.

We have also added sequences encoding T7 or myc tags to a subgroup of our pSAT BiFC tagging vectors to facilitate the confirmation of protein expression.

The plasmids described in this chapter have been deposited in the ABRC stock center at The Ohio State University. The authors request that interested parties obtain them from this source.

2. For W5 solution and MMg solution, the user can make various sterile stock solutions first and combine individual components before use based on the proper compositions. These stock solutions include 0.8 M mannitol, 3 M NaCl, 0.2 M KCl, 0.1 M MES, pH 5.7, 1 M $CaCl_2$, and 1 M $MgCl_2$.

3. The pSAT vectors are distinguished by a code in the following order [14, 17]:

   The number (X) of the vector after "pSAT" identifies the rare cutting site flanking the expression cassette (Table 1 and Fig. 2); If "A" follows the vector number, this indicates that the *Nco*I site has been removed from the position preceding the mcs (in vectors used for C-terminal tagging of proteins only; see Fig. 2b).

   The fluorescent protein fragment tag; N indicates that the protein of interest is placed at the N-terminus of the fusion (i.e., the protein is tagged at its C-terminus). C indicates that the protein of interest is placed at the C-terminus of the fusion (i.e., the protein is tagged at its N-terminus).

   The properties of the target protein need to be considered when choosing the optimal BiFC tagging vector.

4. As examples:

   (a) pSAT1-nVenus-N indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the N-terminus of the fusion, tagged at its C-terminus with the N-terminal fragment of Venus (nVenus). A *Nco*I site precedes the mcs; thus, because this site contains an ATG sequence, the user must be careful that this ATG is in-frame with the ATG of the introduced gene.

   (b) pSAT1A-nVenus-N indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the N-terminus of the fusion, tagged at its C-terminus with the N-terminal fragment of Venus. Because there is no *Nco*I site in this vector, the user needs not worry that a "false" ATG will place the protein of interest out of frame.

   (c) pSAT1-nVenus-C indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the C-terminus of the fusion, tagged at its N-terminus with the N-terminal fragment of Venus.

   (d) pSAT1-cCFP-C indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the C-terminus of the fusion, tagged at its N-terminus with the C-terminal fragment of CFP.

5. For each pair of proteins to test for interaction, one must be tagged with a N-terminal fragment of a fluorescent protein, and the other protein must be tagged with a C-terminal fragment of a fluorescent protein.

6. In some situations, existing data may indicate which end of a protein can be tagged and not destroy function. The BiFC tag should thus be placed on this end of the protein. If such data are not available, both ends of the protein should individually

be tagged and tested for interaction with the second protein. In this case, the user should also test whether the tagged fusion protein still possesses its normal function.

7. To generate translational fusions of proteins of interest with fluorescent protein fragments, the user needs to add restriction sites to flank the DNA sequence encoding the open reading frame (ORF) of the protein of interest, then clone the DNA fragment into the mcs of the pSAT BiFC vector.
8. Care must be taken to make sure that the ORF of interest is in-frame with the ORF encoding the fluorescent protein fragment. To tag a protein of interest at its C-terminus (using pSAT-N vectors), the stop codon of the protein of interest must be removed to allow fusion with the ORF encoding the fluorescent protein fragment.
9. When performing BiFC analyses using transient expression in plant cells or protoplasts, one can introduce multiple separate BiFC expression cassettes on different pSAT plasmids, or one can clone all BiFC expression cassettes onto the same vector (the "final vector"). If the user eventually wishes to clone more than one expression cassette into the same final vector, each expression cassette should be flanked by different rare cutting sites. Thus, for example, one cassette should be flanked by *Asc*I sites (pSAT1/1A vectors), one with I-*Sce*I sites (pSAT4/4A vectors), and one with PI-*Psp*I sites (pSAT6 vectors), etc.
10. The user needs to ensure that the first ATG following the promoter region is in-frame with both the gene of interest and the fluorescent protein gene (Fig. 2a, b).
11. Whereas combining all BiFC expression cassettes onto the same vector guarantees that each cell will receive all expression cassettes, this entails additional cloning steps. In our experience, if multiple individual plasmids are co-introduced into protoplasts, there is a >80 % probability that a protoplast competent to take up one plasmid will also take up all additional plasmids.
12. To clone all BiFC expression cassettes into a single plasmid, cDNAs encoding each protein of interest must first be cloned into a pSAT BiFC vector containing an expression cassette surrounded by different rare cutting sites (*see* Fig. 2 and **Note 5**). Each expression cassette is subsequently released from the initial pSAT BiFC vector using the appropriate rare cutting enzyme and ligated into the corresponding site of the final vector [17]. We have constructed several pUC119-derived vectors containing the RCS polylinker (Fig. 3a). These vectors replicate to high copy number in *E. coli*, and are therefore useful for generating large quantities of plasmids for direct DNA delivery into protoplasts or plant cells by electroporation, Ca_2PO_4/PEG-mediated transformation, or particle bombardment.

Alternatively, we have constructed several T-DNA binary vectors containing the RCS polylinker (Fig. 3b). These vectors can be used in *Agrobacterium-mediated* transformation processes, such as the generation of transgenic lines or transient agroinfiltration experiments.

13. When the passage of BY-2 cells becomes a routine, and the cells grow normally, cells should reach the log phase of growth 4–5 days after transfer to fresh medium.
14. Check the cells every hour for the production of protoplasts after placing them in the protoplast enzyme digestion solution. The protoplasts can easily be seen as perfectly spherical cells using an inverted light microscope. BY-2 cells with walls have unusual shapes, but will not be perfectly spherical. Do not "over-digest" the cells. Protoplasts will lose viability if they stay too long in the protoplast enzyme digestion solution.
15. Remember, protoplasts no longer have cell walls. Therefore, they are very fragile and can lyse easily. Extreme caution is necessary during all washing and resuspension steps.
16. Because the transfection efficiency of protoplasts is highly dependent on the quality of plasmid DNA used, the user may consider using CsCl quality DNA or DNA purified using a commercially available plasmid extraction kit (e.g., Qiagen) to guarantee the quality and quantity of DNA for transfection or bombardment into plant cells.
17. The plate can be either wrapped in aluminum foil or kept in a closed chamber without light. Fluorescence signals can be detected as early as 4 h after transfection using fluorescence microcopy. We often finish the transfection in the late afternoon and incubate the cells overnight before viewing the cells.
18. Transfected protoplasts are very fragile, the cells could lyse if the coverslip is forced on top of them. Therefore, gentle handling is necessary. The authors normally place a self-sticking "Hole reinforcing ring" purchased from a stationary supply store on top of slide to form a chamber with depth, then place the protoplasts inside the ring and cover the ring with a coverslip.
19. The authors normally use a Nikon Eclipse 600 epifluorescence microscope or a Nikon A1R confocal microscope to view the samples. For the Nikon Eclipse 600 microscope, the authors use Yellow GFP HYQ41028 as a YFP filter; HcRED#41043 as a RFP filter; 96188 m (Chroma) as a CFP filter. The software Metamorph is used to capture and analyze the fluorescence images. For the Nikon A1R confocal microscope, the authors use either a 20× water/oil lens (Plan Fluor 20×/0.75 Mimm DIC N2) or a 40× water lens (ApoLWD 40×/1.15 W1XS). Nikon Elements ND2 is used for image processing and data management.

20. Recently, several laboratories have developed a number of "advanced" uses for BiFC (Fig. 4). Multicolor BiFC allows one to present simultaneously a given "bait" protein with the choice of several "prey" proteins to determine whether the bait prefers one or the other, or whether the choice of prey directs the subcellular compartment to which the bait–prey protein complex localizes (Fig. 4a) [17, 18, 27, 32]. For example, a given bait protein can be tagged with cCFP, and two prey proteins individually tagged with either nVenus or nCerulean. Because the chromophore of the fluorescent proteins depends on the amino acid sequence of its N-terminus, folding of cCFP with nVenus generates yellow fluorescence, whereas folding with nCerulean generates blue fluorescence. We have used this system to show that *Agrobacterium* VirE2 protein can interact in plants with multiple importin α isoforms. When VirE2 interacts with the isoform AtImpa-4, the complex localizes to the nucleus. However, when VirE2 interacts with all other tested importin α isoforms, the complex remains cytoplasmic or perinuclear [17].
21. BiFC combined with FRET can visualize interaction of three proteins in a complex (Fig. 4b) [33]. In this system, full-length Cerulean or CFP tags one protein, whereas two other proteins contain N- and C-terminal YFP (or its derivatives) tags. Interaction of the two proteins tagged with YFP fragments restores yellow fluorescence, which is visualized by FRET when the third Cerulean/CFP-tagged protein in the complex is excited by blue light. Kwaaitaal et al. [34] recently used BiFC-FRET to identify three proteins in a SNARE complex in barley leaf epidermal cells.
22. Bridge-BiFC can identify three proteins in a complex (Fig. 4c). In this system, expression of two proteins tagged with N- and C-terminal fragments of YFP does not result in fluorescence complementation unless a third, untagged protein is also expressed. The untagged protein serves as a "bridge" to bring together the tagged proteins. Zaltsman et al. [35] used this assay to identify proteins in a SCF complex important for directing proteolytic degradation of target proteins.
23. Our laboratory recently adapted BiFC to identify interactions between target proteins and small peptide aptamers (Fig. 4d) (L.-Y. Lee, S. Park, Y. Wang, H. Iwakawa, Z. Zhang, and S.B. Gelvin, unpublished). We inserted 20 amino acid long peptides between cCFP and mCherry (a highly fluorescent and photostable derivative of DsRed) to make aptamer fusion proteins. Interaction of the peptide aptamer with a target protein tagged with nYFP results in yellow fluorescence and, in some situations, inactivation of target protein function (aptamer "mutagenesis"). Red mCherry fluorescence marks

cells expressing the aptamer, and also indicates the subcellular position of the peptide aptamer. Yellow fluorescence identifies the subcellular location of target protein–aptamer interaction.

24. Our laboratory has also used BiFC to screen a cDNA library with a "bait" protein for protein–protein interactions directly in planta [36]. In this system, proteins encoded by a library of cDNAs are tagged at their N-termini with cYFP. Co-transfection of the cDNA library with a construction expressing the bait protein tagged with nVenus or nYFP resulted in a small number of fluorescent plant cells. The identity of the cDNA(s) encoding interacting protein(s) was established by successive break-down of pools of cDNA clones. Thus, we were able to develop a plant two-hybrid cDNA library screening system.
25. As with any technique, BiFC has limitations:
    (a) The underlying principle of BiFC is that the two portions of the split fluorescent protein must be brought together to fold correctly. Because it is not usually known in what structural conformation two proteins of interest interact, it may be difficult to predict which end of these proteins to tag. Thus, lack of a fluorescence signal does not necessarily indicate that two proteins do not interact. It may merely indicate that they do not interact in a way necessary to bring together the two portions of the split fluorescent protein. Users of BiFC should consider separately tagging both ends of each protein partner.
    (b) Interacting proteins frequently dissociate from each other (i.e., protein complexes can be transient). Folding of the two portions of the split fluorescent protein may irreversibly hold together two proteins, which would normally dissociate. Thus, BiFC can be used to investigate the formation of protein complexes, but cannot easily be used to explore downstream dissociation of these complexes.
    (c) Over-expression of the two peptides of a split fluorescent protein may result in the restoration of fluorescence independent of interaction of the affixed proteins of interest due to the self-assembly of two nonfluorescent fragments from any fluorescent protein. This likely occurs by "mass action." Because BiFC is a relatively sensitive technique, when a fluorescent protein with higher intensity is used, this background noise may be significant. Scientists have frequently observed fluorescence resulting from interaction of a tagged protein with a peptide generated by an "empty vector" construction. Therefore, it is necessary to have good controls. The best control for this situation is to delete or mutate the known interacting domains of the two proteins in question and demonstrate that one cannot obtain a fluorescence BiFC signal using the mutated proteins

for interaction [1, 27]. Unfortunately, many times the investigator does not know the interacting domains in question. Recently, Kodama and Hu [31] described a new variant of nVenus (nVenusI152L). Use of this fragment increases the signal-to-noise ratio of BiFC in animal cells. However, this variant does not similarly increase the signal-to-noise ratio in plants (communication with C-D Hu and results from our laboratory). We have mitigated the problem of background BiFC signal by lowering the expression of at least one of the interacting partners. We have done this by exchanging the strong CaMV double 35S promoter used in our vectors for a weaker promoter such as the nopaline synthase (*nos*) promoter. In addition, use of the less bright fluorescent protein EYFP, rather than Venus, also decreases background fluorescence [36]. Table 2 lists several vectors we have constructed for this purpose.

26. If you do not generate a fluorescence signal, how do you know that all the tagged genes have been introduced into the cells?

    We frequently include a red fluorescent protein (mRFP or mCherry) expression cassette on the same plasmid harboring the nYFP-tagged and cYFP-tagged protein expression cassettes. Generation of a red fluorescence signal indicates that the cells have received the incoming plasmid and expressed the mCherry protein, and can be used to determine the transfection efficiency. In addition, fusion of mCherry to "marker" proteins or organelle targeting sequences can assist in the identification of specific subcellular compartments or organelles. For example, mRFP fused to a nuclear localization signal (NLS) sequence can be utilized to identify nuclei as an alternative to staining cells with DNA-interacting fluorescent dyes such as DAPI. Several of our vectors contain, in addition to the split YFP tag, a T7 or a myc peptide tag. Expression of the fusion proteins can be detected using antibodies directed against these tags.

27. Problems with over-expression: "Forcing" interactions that may not normally occur

    When interpreting BiFC experiments, one must realize that these experiments will identify protein–protein interactions that *CAN* occur, but not necessarily those that *DO* occur. Over-expressing proteins can "force" interactions of proteins that, at best, would normally interact weakly when expressed at their native levels. To mitigate over-expression artifacts, genes encoding the putative interacting proteins can be expressed from relatively weak promoters, such as those from the nopaline synthase (*nos*), octopine synthase (*ocs*), or mannopine synthase (*mas*) genes. In addition, although it may require additional cloning effort, it is best to express the genes from their native promoters.

In addition, it is possible that in their native organism, these proteins would not normally interact because they would not have the opportunity to contact each other due to different temporal or spatial patterns of expression. For example, the proteins may normally be expressed in different tissue or cell types, or at different developmental stages. Alternatively, the proteins may localize to different subcellular compartments and therefore not normally have the opportunity to interact.

28. Problems with over-expression "mis-localizing" the interacting protein pairs

    One of the advantages of BiFC over other methods to detect protein–protein interactions is that the site of interaction can be visualized in living cells. However, it is possible that over-expression of the interacting proteins may "overload" a routing pathway, resulting in mis-localization of the site of interaction (*see* **Note 10**).

29. Lack of generation of a BiFC signal

    Lack of a BiFC fluorescence signal does not necessarily indicate that two candidate proteins do not interact. It is possible that the proteins do interact, but not in such a way as to allow the two complementary fragments of the split fluorophore to fold correctly. BiFC results should be verified by using a different technique, such as co-immunoprecipitation, TAP-tagging, or interaction in yeast, to detect protein–protein interactions.

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Chapter 12

Investigating TLR Signaling Responses in Murine Dendritic Cells Upon Bacterial Infection

Suzana Pinto Salcedo and Lena Alexopoulou

Abstract

Innate immune recognition of microbial components is critical for the onset of an appropriate immune response against invading pathogens, in particular by dendritic cells. Toll-like receptors (TLRs) are key in the detection of a variety of microbial stimuli.

Here we focus on the methodology used to evaluate the role of TLRs in the process of dendritic cell response to bacterial intracellular infections, using bone marrow-derived dendritic cells (BMDCs) as a model system. This protocol describes how to access the level of activation of BMDCs using standard immunology and biochemistry approaches along with examination of infected cells by immunofluorescence microscopy.

Key words TLR, Dendritic cell, Bacteria

1 Introduction

Innate immune recognition of microbial components is critical for the development of an appropriate immune response against invading pathogens. Key contributors to the innate immune recognition of pathogens include the toll-like receptor (TLR)/interleukin 1 receptor (IL-1R) superfamily characterized by the presence of a conserved region designated TIR domain located in the cytosolic face of each TLR. The TIR domain is critical for protein–protein interactions between TLRs with the corresponding TIR-containing adaptors, which through a cascade activate downstream protein kinases [1]. This cascade ultimately leads to activation of specific signaling pathways such as nuclear factor-κB (NF-κB), mitogen activated protein kinases (MAPKs), and interferon regulatory factor (IRF) pathways resulting in production of inflammatory mediators. There are 10 TLRs in humans (TLR1-10) and 12 TLRs in mice (TLR1-9 and TLR11-13) of which TLR1-TLR9 are conserved between the two species. The most relevant TLRs for recognition of bacterial molecules are TLR2, TLR4, TLR5, and TLR9 (Table 1).

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Table 1
Murine TLRs, ligands and TLR-deficient mice

Gene	Main natural TLR ligands	Synthetic TLR agonists	Reference of deficient mice	Available in Jackson laboratory (stock number)
TLR1	Triacylated lipoproteins from gram negative bacteria and mycoplasma	Pam_2CSK_4	[8] [9]	Yes (007020) No
TLR2	Lipoproteins; Lipoteichoic acid (LTA) and peptidoglycan from mycobacteria; zymosan from fungi	Pam_2CSK_4, FSL-I	[10] [11]	No Yes (004650)
TLR3	Viral double-stranded RNA	Polyinosinic-polycytidylic acid (polyI, C)	[12]	Yes (009675)
TLR4	Lipopolysaccharide (LPS) from gram negative bacteria	Monophosphoryl lipid A (MPLA)	[13] [14]	Yes (007227) No
TLR5	Flagellin of bacterial flagella		[15] [16]	Yes (008377) No
TLR6	Diacylated lipoproteins from gram positive bacteria	FSL-I	[17]	Yes
TLR7	Single-stranded RNA	Imidazoquinolines, R848, loxoribine, imiquimod	[18] [19]	Yes (008380) No
TLR8[a]	Unknown	Unknown	[20]	No
TLR9	Unmethylated CpG oligonucleotides (ODN) from bacteria and viruses	Stimulatory CpG ODN	[21] [22]	No
TLR11	*Toxoplasma gondii* profiling		[23]	No
TLR12	*Toxoplasma gondii* profiling		[24]	No
TLR13	Bacterial 23S ribosomal RNA		[25]	No

[a]In humans TLR8 detects viral single-stranded RNA

A number of pathogen-associated molecular patterns (PAMPs) have been described, which activate specific TLRs (Table 1), and extensive studies in mice have attributed key roles of TLRs in murine host responses [2], while clinical studies have revealed the implication of TLR signaling in human host defense [3].

Dendritic cells (DCs) are important sentinels of the immune system monitoring the tissues for microbial threat. Encounter with microbes will initiate a process of activation that enables the DC to respond and elicit an appropriate immune response, by producing specific cytokines and presenting antigens. Many DC populations

have been described in mice and humans, with distinct phenotypic and functional properties [4]. Nonetheless, in vitro systems such as BMDCs are commonly used as a first tool to decipher the interaction with a particular pathogen and the role of specific TLR pathways during infection.

Here we describe a protocol to determine the role of TLR signaling in the activation of DCs upon bacterial infection, with a particular emphasis on intracellular bacteria, using BMDCs from TLR knockout mice, and analysis of the level of activation of BMDCs during infection. Classical readouts for analyzing dendritic cell activation (Subheading 3.3) are cytokine production in culture supernatants or at the RNA level (Subheading 3.3.1), surface expression of activation markers by flow cytometry (FACs) analysis (Subheading 3.3.2), or signaling pathways by western blot (Subheading 3.3.3) and immunofluorescence microscopy (Subheading 3.3.4). We describe the major techniques, critical tips and mention recent technological advances that are enabling more accurate and sensitive measurement of some of these parameters.

2 Materials

2.1 Preparation of BMDCs

1. 6–12 week old mice (*see* **Note 1**).
2. Dissection tools: beaker, 70 % ethanol, forceps, and scissors.
3. DC-Wash Media (DC-WM): RPMI, 5 % fetal calf serum (FCS), 50 μM β-mercaptoethanol.
4. DC-complete Media (DC-cM): Pre-warmed RPMI, 5 % FCS, 50 μM β-mercaptoethanol + Granulocyte-Macrophage Colony Stimulated Factor (GM-CSF) at optimized concentration (*see* **Note 2**). Always add GM-CSF on the day the media will be used.
5. Red cell lysis buffer (eBioscience).
6. 6- and 12-well plates and culture dishes.
7. 15 and 50 mL Falcon tubes.
8. Cell strainers.
9. Refrigerated centrifuge plus rotors appropriate for 15 and 50 mL Falcon tubes.
10. Microbiological safety cabinet.
11. Ice.
12. 10 cm culture plates.
13. 70 % ethanol.
14. Trypan blue.
15. 10 mL syringe attached to a 25 G needle.

16. 10 mL pipettes.
17. Cell strainer.
18. Hemocytometer or cell counter.
19. 37 °C/5 % CO_2 incubator.

2.2 Infection of BMDCs

1. Bacterial culture.
2. 6-, 24-, or 96-well plates.
3. PBS.
4. Paraformaldehyde (PFA) 3.2 % (diluted fresh from a stock of 32 % in PBS).
5. Centrifuge with rotor for tissue culture plates.
6. 0.1 % Triton X-100 in H_2O.
7. Luria Broth (LB) (Tryptone 10 g, Yeast Extract 5 g. NaCl, 10 g/L) Sterilize by autoclaving (121 °C, 20 min).
8. LB agar plates. Add 1.5 % agar to LB broth and sterilize by autoclaving.

2.3 Analysis of Dendritic Cell Activation and TLR Response

1. TLR knockout mice (Tables 1 and 2).
2. TLR ligands (Tables 1 and 3).

2.3.1 ELISA (CBA Flex; Single; Multi)

1. 0.2 μm syringe filter.
2. ELISA or CBA/Flex kits (BD Biosciences).

2.3.2 Flow Cytometry

1. Ice cold PBS with 2 % FCS.
2. Ice cold PBS with 2 % FCS and blocking antibody such as 24G2 hybridoma supernatant.
3. Antibody mix diluted in PBS + 2 % FCS (keep on ice in the dark).

Table 2
Murine TLR-adaptor molecules and deficient mice

Gene	Acts downstream of	Reference of deficient mice	Available in Jackson laboratory (stock number)
MyD88	TLR1-2 and TLR4-TLR13	[26]	No but check the conditional DC-MyD88 (009088)
TRIF	TLR3 and TLR4	[27], [28]	Yes (005037)
TIRAP or Mal	TLR2 and TLR4	[29], [30]	Yes (017629)
TRAM	TLR4	[31]	No

Table 3
Suggestion of TLR agonists

TLR	Agonist
TLR1	Pam_2CSK_4 (Invivogen Pam3CSK4)
TLR2	Pam_2CSK_4 (Invivogen Pam3CSK4)
TLR3	PolyI:C (Invivogen poly (I:C) HMW)
TLR4	LPS from *E.coli* (Invivogen LPS-EB)
TLR5	Flagellin from *S. typhimurium* (Invivogen FLA-ST)
TLR6	FSL-I (Invivogen FSL-I)
TLR7	R848 (Invivogen R848)
TLR9	CpG ODN (Invivogen ODN 1826)

4. If necessary PFA 3.2 % (*see* Subheading 2.2, **item 4**).
5. Flow cytometer.

2.3.3 Western Blot

1. Lysis buffer: 20 mM Tris–HCl pH 7.6, 200 mM $NaCl_2$, 1 mM EDTA, 1 % Triton X-100, and 1/100 protease/phosphatase inhibitor cocktail (*see* **Note 3**).
1. SDS-PAGE gels (commercially available or made following conventional protocols).
2. Immunobilon P membrane (Millipore).
3. ECL System (Amersham GE LifeSciences).
4. Electroblotting machine.

2.3.4 Microscopy

1. Alcian blue coverslips (*see* **Note 4**) or 12 mm coverslips in 24-well plates.
2. Fixative such as 3 % PFA (*see* **Note 5**).
3. PBS with permeabilizing agent if intracellular labeling is required such as 0.1 % saponin and blocking agent (for example 10 % horse serum or 2 % BSA) (*see* **Note 6**).
4. Dark humid chamber (for example, box with lid, with wet Whatman paper and Parafilm covering it).
5. Fine tweezers.
6. Small beakers for washes, two with PBS + 0.1 % saponin, one with PBS and one with ddH_2O.
7. Mounting Media such as ProLong Gold or Mowiol preferentially with antifade.

8. Microscope with epifluorescence objectives (40× or 63× or 100×), immersion oil.
9. Microscope slides and coverslips.
10. Imaging software such as ImageJ or Icy.
11. 37 °C/5 % CO_2 incubator.

3 Methods

3.1 Preparation of BMDCs (See Notes 7 and 8)

1. Prepare DC-Wash Media and place 5 mL in Falcon tubes or 12-well plate mL and place on ice. *See* **Note 9** and Tables 1 and 2 for use of the different available mice.
2. Collect femur and tibias from mice and clean as much tissue as possible first with scissors and then with a paper towel (be careful not to break the bones); place bones in the DC-WM in the Falcon tube or plate.
3. All steps hereafter should be performed in a microbiological safety cabinet. Prepare a 10 cm plate with 10 mL 70 % ethanol, a 10 cm plate with 10 mL DC-WM media for the bones. Also fill a 400 mL beaker with 300 mL 70 % ethanol and immerse a set of clean forceps and scissors (*see* **Note 10**).
4. Place the bones from one mouse in the plate with 70 % ethanol and let stand for 1 min. Then wash by immersing the bones in the plate with DC-WM media to remove the 70 % ethanol.
5. Transfer bones to a 10 cm plate with a drop of DC-WM (enough to wet all the bones).
6. Hold bones with a sterile forceps and cut the ends of the bones with a pair of small (10–12 cm length) sharp scissors.
7. Flush the bone marrow with DC-WM in a 10 cm plate using a 10 mL syringe attached to a 25 G needle (*see* **Note 11**).
8. Pipette bone marrow cells with a 10 mL pipette extensively until you get a homogeneous suspension. Transfer to a Falcon tube.
9. Centrifuge 5–10 min at 450 × *g* at 4 °C.
10. Remove supernatant, disrupt the pellet by tapping the tube, add 2 mL of red cell lysis buffer, pipette up and down a few times to resuspend the pellet, and incubate for 3–4 min at room temperature (*see* **Note 12**).
11. Stop the lysis by adding PBS (five times the volume of the lysis buffer). Centrifuge for 5 min at 450 × *g* at 4 °C.
12. Resuspend cell pellet in DC-WM and pass through a cell strainer into a new 50 mL tube to remove debris that results from lysis of red blood cells and small bone particles.

13. Centrifuge (as in **step 9**), remove the supernatant and resuspend the cell pellet in 5 mL DC-complete media (with GM-CSF) pre-warmed and count cells (for example using an hemocytometer using trypan blue to exclude dead cells or an automatic cell counter).
14. Dilute the cells to a density of 1×10^6 cells per mL and seed 5 mL per well in a 6-well plate or 10 mL per 10 cm culture plates (*see* **Note 13**).
15. Incubate at 37 °C with 5 % CO_2 and replace medium (with GM-CSF) every 2 days. This is done by tipping the plate at a 45° angle and aspirating 4 mL of the media. Add 5 mL fresh media very slowly on the side of the dish (*see* **Note 14**).
16. At day 2 you will see many small clusters of cells loosely attached to the bottom; these are the DCs. After 5 days you will see an increase in the size of the clusters. Cells can be used at day 5 or 6 (*see* **Note 15**).

3.2 Bacterial Infection of BMDCs

1. Cells can be directly infected in the 6-well plates or transferred to 24-well plates with coverslips (*see* **Note 16**). In the first case, one well should be used to collect cells and enumerate them. Precise cell counts are necessary in order to establish the multiplicity of infection (MOI). For all experiments include a negative control and a positive control (*see* **Note 17**).
2. Prepare inoculum by diluting bacteria in complete DC media and place on ice. An MOI of 20:1 for *Brucella* is recommended but this will depend on the pathogen used and should be optimized.
3. Place culture plates with the cells (from Subheading 3.1**, step 16** or Subheading 3.2, **step 1**) on a cold surface to prevent phagocytosis and gently aspirate 80 % the media. Add the inoculum. If cells were previously transferred to a new plate do not aspirate and just add directly the inoculum to the well adjusting the bacterial density to the desired MOI.
4. Centrifuge bacteria onto BMDCs at $400 \times g$ for 5 min at 4 °C and then incubate for 30 min at 37 °C with 5 % CO_2 atmosphere.
5. Gently wash cells twice by adding and removing the media on the side of the well and then incubate for 1 h in medium supplemented with an appropriate antibiotic that will kill extracellular bacteria (*see* **Note 18**).
6. Replace the media with complete DC media supplemented with a reduced concentration of the antibiotic (*see* **Note 19**).
7. Incubate cells at 37 °C with 5 % CO_2 atmosphere up to the necessary time points depending on the pathogen and the readout (*see* **Note 20**).

8. Collect cells and/or supernatants depending on the experiment. Cell collection should be done on ice, with ice-cold PBS, to stop any further maturation, particularly when early time-points are being analyzed. *See* Subheading 3.3, **step 3** on how to collect cells.
9. If necessary to monitor intracellular bacterial survival and/or replication, lyse infected cells with 0.1 % Triton X-100 in H_2O and plate serial dilutions onto appropriate LB agar to enumerated colony forming units.

3.3 Analysis of Dendritic Cell Activation and TLR Response

In each experiment you need: (1) negative control, cells that have been cultured under the same condition as your cells that will be infected but without any bacteria; (2) positive control, cells that have been treated with the specific TLR ligand (Table 3); and (3) experimental samples, cells that have been infected with bacteria of interest. Experiments with BMDCs from wild-type mice and mice that are deficient for a specific TLR or TLR adaptor molecule (Table 2) should be run in parallel.

3.3.1 ELISA (Bead Array; Single; Multi) (See **Note 21**)

1. If necessary to remove pathogens before performing the ELISA (for example for Class 3 pathogens), samples can be filtered through a 0.2 μm syringe filter (*see* **Note 22**).
2. Use an appropriate ELISA kit and follow specific manufacturer's instructions. Alternatively, the determination of the cytokine protein levels in culture supernatant can be done by multiple cytokine bead array kits that allows the measurement of many cytokines by flow cytometry using a small volume of the culture supernatants (~40 μL) following the protocol of the manufacturer. (*see* **Note 23**).

3.3.2 Flow Cytometry

1. Prepare: PBS, PBS + 2 % FCS and place on ice; cool down the centrifuge to 4 °C; Make the appropriate dilution of the antibodies that will be used (*see* **Note 24**).
2. To collect cells for flow cytometry, place plate with infected cells on ice (**step 8** from Subheading 3.2) and extensively pipette cell suspension then transfer to an Eppendorf tube or well (depending on the number of samples). An additional 1 mL ice cold PBS can be added to ensure removal of all cells.
3. Centrifuge 5 min at 4 °C at 800 × *g*.
4. Aspirate supernatant and resuspend cell pellet in 30–50 μL PBS + 2 % FCS, + blocking antibody for Fc receptors (for example 24G2 hybridoma supernatant) at the appropriate dilution and incubate 15 min on ice or at 4 °C (*see* **Notes 25** and **26**).
5. Add 100 μL of PBS + 2 % FCS, centrifuge for 5 min at 800 × *g* at 4 °C.

6. Remove supernatant and resuspend cell pellet in 30–50 μL of antibody mix in PBS + 2 % FCS (or control mix or PBS for the negative control); incubate 20 min on ice protected from light.
7. Add 100 μL of PBS + 2 % FCS, centrifuge for 5 min at 800 × *g* at 4 °C.
8. Remove supernatant and resuspend cell pellet in 200 μL of 3.2 % PFA to fix (*see* **Note 27**). Incubate for 15 min at RT protected from light.
9. Centrifuge cells to remove PFA and resuspend in PBS or, alternatively, add PBS to dilute PFA to 1 % and analyze by flow cytometry.

3.3.3 Western Blot

TLR signaling pathways elicit transcriptional alterations through the activation of nuclear factor-κB (NF-κB), JNK, ERK, and p38 MAPKs, and interferon (IFN) regulatory factors (IRFs), which can be monitored by western blot analysis (*see* **Note 28**).

1. Collect whole-cell protein extracts at different time points from infection plates (prepared in Subheading 3.2, **step 7**). The time points vary depending on the infectivity of the bacteria, as an indication 0, 0.5, 1, 2 and 4 h can be used.
2. If the cells are floating, collect in a 15 mL tube, spin for 5 min at 450 × *g*, discard supernatant. Dissolve cell pellet with 100 μL lysis buffer and transfer in a 1.5 mL tube. If the cells are adherent, remove supernatant, wash cells in the plate with 5 mL PBS, remove PBS add 1 mL fresh PBS and use a rubber cell scrapper to detach the cells. Transfer the cell suspension in 1.5 mL tube, spin for 5 min at 450 × *g*, discard supernatant and dissolve cell pellet with 100 μL lysis buffer.
3. After the addition of the lysis buffer incubate on ice for 10 min.
4. Centrifuge 20 min at 4 °C and at 20,000 × *g*.
5. Collect supernatant that contains the protein fraction.
6. Evaluate the protein concentration using standard techniques such as a Bradford assay.
7. Store samples at −20 °C till use and avoid freeze thawing since proteins can be degraded.
8. Protein extracts (~20 μg) from BMDCs are resolved on SDS-PAGE gels.
9. Transfer to Immunobilon P membrane by electroblotting.
10. Immunobloting is performed with the antibodies of interest and bands are visualized with secondary HRP-conjugated antibodies and the ECL System according to standard protocols.

3.3.4 Immunofluorescence Microscopy

1. Add an aliquot of cell suspension (50–200 μL) to alcian blue coated coverslips, placed in a 24-well plate, and incubate 30 min at 37 °C.
2. Fix cells with 3.2 % paraformaldehyde for 10–15 min (*see* **Notes 5** and **29**).
3. A pre-blocking step of 30 min may be necessary for certain antibodies (*see* **Note 30**).
4. Turn coverslips onto 30 μL of primary antibody mix diluted in 0.1 % saponin with 10 % horse serum in PBS (*see* **Notes 6** and **31**).
5. Incubate for 1 h at RT in a dark humid chamber.
6. Pick coverslip with tweezers and wash (10–20 s) in two beakers sequentially containing PBS with 0.1 % saponin.
7. Invert coverslip onto 30 μL of secondary antibody mix diluted in 0.1 % saponin with 10 % horse serum in PBS. Incubate for 30 min at RT in dark and humid chamber.
8. Pick coverslip with tweezers and wash in two beakers sequentially containing PBS with 0.1 % saponin, one beaker with PBS and one final beaker with water.
9. Mount coverslip in 10 μL mounting media on a glass slide. Allow 2 h for the mounting medium to polymerize before analysis. Slides can be stored at 4 °C when set.
10. Microscopic analysis using an epifluorescence microscope or confocal immunofluorescence microscopes with either ×40, ×63 or ×100 objectives (*see* **Note 32**). For a selection of antibodies *see* **Note 33**.

Use specific plug-ins of image processing software such as ImageJ or Icy (freely available online) for automated counting (*see* **Note 34**).

4 Notes

1. Older mice can also be used; however, a reduced yield of cells may be obtained. Animal experimentation should follow the ethical and legal national regulations.
2. GM-CSF can be commercial or be produced from specific cell lines stably expressing mouse GM-CSF such as J558L. Main stock and aliquots of GM-CSF should be kept at –20 °C. Aliquot in use can be stored at 4 °C for 1 week. Titration needs to be done to select the dilution that gives better yield of DCs (a possible range can be 0.5, 1, and 2 %). This can be done by analysis of CD11c-gated cells labeled for MHC class II, CD40, CD80, and/or CD86 (with and without LPS stimulation) by

flow cytometry. All BMDCs are labeled with an anti-CD11c antibody.

(a). Grow J558L cells in RPMI, 5 % FCS, G418 0.5 mg/mL until obtaining 2–4 large culture flasks.

(b). Harvest cells when the media starts turning yellow by centrifugation 150×*g*, 5 min.

(c). Wash twice in 50 mL tubes using media without G418.

(d). Resupend cell pellet in 10 mL media (RPMI, 5 % FCS).

(e). Place cells in a spinner and add 1.5 mL of media without G418.

(f). After 2 days add 1 L of media (RPMI, 5 % FCS).

(g). Harvest supernatant by pipetting after 7 days.

(h). Titrate each batch before use (*see* **Note 18**).

3. Lysis buffer are also commercially available like the RIPA Buffer.
4. Place coverslips in large beaker with 1 % solution of alcian blue in water. Microwave a few seconds until boiling. Rinse coverslips in water and 70 % ethanol. Dry coverslips. Alcian blue coated coverslips enhance adherence of cells in suspension to the coverslip. Alternatives include poly-L-lysin coated coverslips.
5. Alternative to PFA, AntigenFix (MicromFrance) can be used which is stored at room temperature. Depending on the antibodies to be used alternative fixations may be required such as Methanol at −20 °C for 5 min.
6. Triton X-100 permeabilization may be necessary for specific antibodies. This is done at room temperature by incubating cells for 5 min with 0.1–0.5 % Triton X-100 prior to primary antibody incubation.
7. The media used is critical for a successful preparation of DCs, particularly the FCS and GM-CSF. It is recommended that the FCS from different manufacturers should be tested in advance to select FCS that does not result in activated DCs at the end of the culture [5] GM-CSF should also be titrated to establish the best concentration.
8. Different kinds of DCs can be obtained by using specific culturing protocols [6]. Here we focus on the preparation of GM-CSF differentiated BMDCs, which correspond to monocyte-derived inflammatory DCs observed in vivo during infection. Alternatively, FLT3l can be used that will result in conventional DCs and plasmacytoid DCs. A combination of FLT3l and GM-CSF can be used to enhance preparation of pDCs. Addition of IL-4 to GM-CSF will result in highly activated DCs.

9. TLR deficient mice are viable, fertile, and without any obvious abnormalities and can be used to prepare BMDCs using the method that has been described in Subheading 3.1. Currently TLR deficient mice have been generated for all the murine TLRs (TLR1-9 and TLR11-13) either by gene targeting or ENU mutagenesis and the original papers that describe the generation of the mice, the major ligands (natural and synthetic compounds) that are detected from each TLR and the commercial availability of the mice are presented in Tables 1 and 3; while the mice deficient for the adaptor molecules downstream of TLRs are presented in Table 2. To test whether the bacteria of interest can trigger a TLR response, run in parallel an experiment with BMDCs from wild-type mice and mice that are deficient for a specific TLR or TLR adaptor molecule. In each experiment the wild-type and TLR-deficient mice should be of the same genetic background, the same sex, and approximately the same age. Since usually there is variability between mice it is advisable to use 3–4 mice for each genotype. If this is not possible, then a pilot experiment with 2 mice per genotype can be done to standardize the conditions of the specific bacteria before scaling up to 3–4 mice per group.
10. Instruments should be sterilized at the beginning of the experiment and then dipped in 70 % ethanol between dissection of different mice to avoid cross-contamination.
11. A 6 or 10 cm cell culture dish can be used depending on the number of bones.
12. If other lysis buffers are used follow the manufacturer's instructions. Two homemade alternatives are available;

    Option 1: 1.66 % NH_4Cl final/working concentration (keep at 4 °C; as a 10× stock).

    Option 2: 0.15 M NH_4Cl, 0.001 M K_2Cl at pH 7.2 (keep at 4 °C).

    If lysis buffer 1 is used, resuspend the pellet in 1.8 mL media, add 0.2 mL of 16.6 % (10×) NH_4Cl, pipet up and down, and incubate for 5 min on ice. If option 2 is being used just resuspend cells directly in 1 mL and incubate for 2 min on ice; mix at half time.
13. This concentration may vary depending on your GM-CSF and needs to be optimized. FCS should be decomplemented. Serum can be decomplemented by incubating at 56 °C for 30 min, mixing occasionally, transfer to ice, aliquot, and store at –20 °C.
14. Aspiration can be done until you see the media reach the cluster of cells visible by eye at the center of the inclined plate. Be careful to not remove cells in suspension or clusters of cells.
15. During the differentiation minimize all movements of the plate, even extensive opening and closing of the incubator that

causes vibration will affect the level of maturation. Antibiotics are often added (for example Penicillin–Streptomycin at final concentration of 100 I.U./mL penicillin and 100 μg/mL streptomycin.). We recommend avoiding antibiotics as they will interfere with the bacterial infection.

16. Alternatively, collect cells by gentle pipetting 3–4 times, count cells and seed in fresh plates for example in 24-well plates with 12 mm coverslips or in 96-well plates.
17. Always include a negative control that has undergone all the steps of the experiment (mock infection) to give the background level of maturation and make sure you are starting with immature DCs. Also include a positive control, for example cells stimulated with 1 μg/mL *E. coli* LPS to ensure the DCs are responsive.
18. For example, 100 μg/mL streptomycin or 50 μg/mL gentamycin are used for *Brucella* and 50 μg/mL gentamycin is used for *Salmonella* infections to kill extracellular bacteria.
19. For example, for streptomycin the concentration can be decreased to 20 μg/mL and for gentamycin to 10 μg/mL.
20. For example, if analysis of early events such as NF-κB activation is being performed, cells can be incubated for 1–4 h whereas if cytokine secretion is being measured by ELISA, infection should progress to 12, 24, and in some cases 48 h. Different kinetics may be necessary for different pathogens.
21. Some standard cytokines that can be measured are TNF-α, IL-6, IL-12p40, IL-1β, IL-10, IFN-α, IFN-β. For cytokine detection in culture supernatants dendritic cells are usually stimulated for 16 h, but if needed a time frame of 8–24 h can be used depending on the bacteria of interest. For cytokine detection at the RNA level it is advised to test different time points of 0, 2, 4, 8, 16, 24, 48 h, since different cytokines and bacteria have different kinetics. For activation markers cells are usually infected overnight and some activation markers that can be used are CD86, CD80, MHC class II, or CD40.
22. Samples can be stored at −20 °C for short-term storage or −80 °C for long term storage, but avoid multiple thawing and freezing cycles. If multiple cytokines are to be measured and at different days it is recommended to aliquot the culture supernatants. Thaw samples at room temperature and transfer on ice as soon as they are thawed. Depending on the infectious agent and the cytokine that is measured, dilution of the samples may be required to compare with the standard curve of each assay.
23. Providers of bead array kits: Beadlyte mouse multi-cytokine flex kit, Millipore; Biosource multiplex assay, Invitrogen; Multiplex cytokine bead array, BD Bioscience.

24. Suggestion of antibody mix: CD11c–APC Cy7 (clone N418), CD86–FITC (clone GL-1), CD80–PECy5 (clone 16-10A1), CD40–Alexa 647 (clone HM40-3), MHC II (I-A/I-E)–PE (clone M5/114.15.2). Antibody dilutions need to be determined experimentally as they vary from different companies, batches, and the type of cytometer being used.
25. Phagocytic cells can bind nonspecifically to antibodies unless you block Fc receptors.
26. If you have many samples then the cell suspensions can be transferred in a U bottom 96-well plate; in that case for the removal of the supernatants just invert the plate and give a hard shake just once or, if you the cells are infected, aspirate with a multichannel aspirator adaptor and avoid touching the cell pellet.
27. The concentration of PFA, time of fixation and final concentration to analyze in the flow cytometer needs to be optimized for each pathogen. We recommend using the lowest possible concentration and time that will ensure killing of your bacteria as the PFA may reduce the fluorescence of your antibodies.
28. Engagement of TLRs activates multiple signaling cascades leading to the induction of genes involved in innate immune responses. Binding of ligands followed by dimerization of TLRs recruits TIR domain-containing adapter proteins such as myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP) or MyD88-adaptor-like (Mal) and TRIF-related adaptor molecule (TRAM). Individual TLRs recruit specific combinations of these adapter molecules to elicit specific immune responses tailored to infectious pathogens (Table 2).
29. The time of fixation depends on the pathogen used. Alternative fixations can be used, such as Antigen Fix. If cells were seeded on coverslips at the beginning of the experiment (*see* **Note 15**) then fixation can be carried out directly in the well after aspirating media and washing one time with PBS.
30. Alternative blocking can be used depending on the antibody. For example, 1 % bovine serum albumin (BSA) is very efficient.
31. Volumes of antibody mix and mounting media are given for 12 mm coverslips.
32. Fully activated DCs will present a characteristic change in morphology with appearance of dendrites (Fig. 1a), as well as an increase in MHC class II surface expression and can present collapse of LAMP-1 compartments at the microtubule organizing center (Fig. 1b). In addition, NF-κB can be labeled to follow translocation into the nucleus that occurs during activation or formation of Dendritic Cell Aggresome-Like Induced

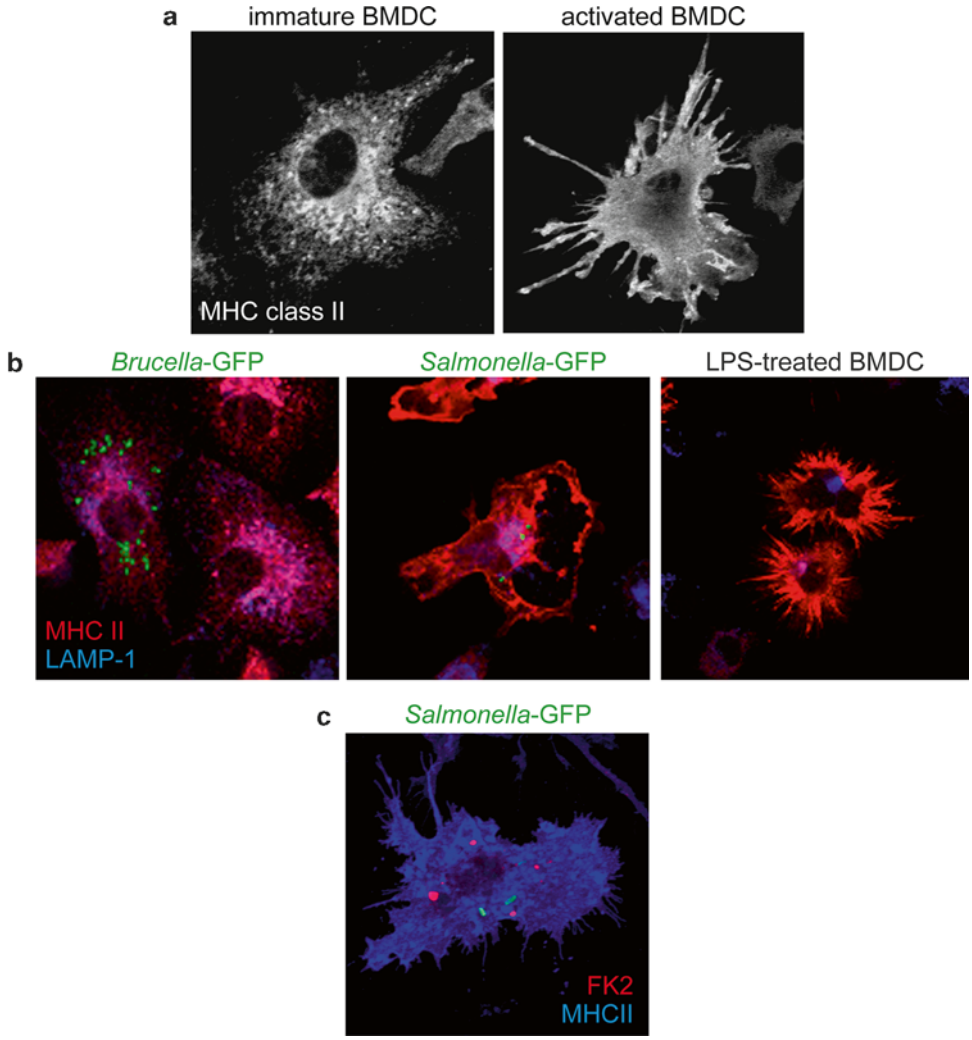

Fig. 1 Confocal fluorescence microscopic analysis of BMDCs. (**a**) Distribution of MHC class II in untreated (immature) and PAM-treated (activated) cells. (**b**) BMDCs infected with GFP-expressing *Brucella abortus* or *Salmonella* Typhimurium for 24 h [32] in comparison with LPS-treated cells. Cells were labeled with LAMP1 (*blue*) and MHC class II (*red*). (**c**) BMDC infected with GFP-expressing *Salmonella* Typhimurium for 24 h and labeled with FK2 antibody to detect DALIS (*red*) and with anti-MHC class II antibody (*blue*) (Color figure online)

Structures (DALIS, Fig. 1c), a transient accumulation of mono- and poly-ubiquitinated proteins characteristic of maturing DCs [7].

33. The following antibodies can be used: Hamster anti-CD11c, clone N418, at 1:100, from Biolegend, anti-MHC II, clone M5/114.15.2, at 1:500, from Biolegend, rat anti-mouse LAMP1, clone ID4B, at 1:500, from Developmental Studies Hybridoma Bank, National Institute of Child Health and Human Development, University of Iowa, anti-p65 NF-κB,

clone C-20, at 1:250, from Santa Cruz (permeabilization should be done prior to labeling with 0.1 % saponin for 10 min at RT followed by 1 h of pre-blocking with 2 % BSA in PBS), mouse-FK2 (DALIS), clone, at 1:1,000, from Biomol.

34. Alternatively, the emerging flow cytometer coupled to imaging systems can be used if available.

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Part III

OMICS and Large Scale Screening

Chapter 13

siRNA Screens Using *Drosophila* Cells to Identify Host Factors Required for Infection

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Abstract

Drosophila melanogaster offers a powerful model system for interrogating interactions between host cells and human bacterial pathogens. *Brucella,* a gram-negative, facultative intracellular bacterium is the causative agent of brucellosis, a zoonotic disease of global consequence. Over the past several decades, pathogen factors that mediate *Brucella* infection have been identified. However, host factors that mediate infection have remained obscure. We have used the power of the *Drosophila* S2 cell system to identify and characterize host factors that support infection by *Brucella melitensis.* Host protein inositol-requiring enzyme 1 (IRE1α), a transmembrane kinase and master regulator of the eukaryotic unfolded protein response, was shown to play an important role in regulating *Brucella* infection, thereby providing the first glimpse of host mechanisms that are subverted by the pathogen to support its intracellular lifestyle. Furthermore, our study also established the *Drosophila* S2 cell as a powerful system for elucidating *Brucella* host factors. Here, we describe a protocol for using the *Drosophila* S2 cell system for studying the *Brucella*–host interaction.

Key words *Brucella*, *Drosophila*, S2 cells, Host factors, Gentamicin protection assay, Immunofluorescence microscopy, RNA interference

1 Introduction

Invertebrate model systems have proven useful for elucidating molecular interactions between bacterial pathogens and host cells [1]. Studies in several such invertebrate systems including the amoebae *Acanthamoeba castellanii* and *Dictyostelium discoideum*, the silkworm *Bombyx mori*, the mosquito *Culex quinquefasciatus*, the nematode *Caenorhabditis elegans*, the greater wax moth *Galleria mollerella*, and the fruit fly *Drosophila melanogaster* have enabled the identification and characterization of novel host mechanisms and virulence factors for several human pathogens, including *Escherichia coli, Pseudomonas aeruginosa*, and *Listeria monocytogenes* [2–4]. Invertebrate systems, most notably *C. elegans* and *D. melanogaster*, have proven useful for large scale, economical,

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high throughput screening studies which cannot be practically performed using mammalian systems [1]. In addition, invertebrate host systems offer the advantage of circumventing ethical concerns that sometimes emerge in designing and implementing experiments with vertebrates. The *D. melanogaster* and *C. elegans* invertebrate model systems also offer small genome sizes, well-studied cellular biology/immunology, conserved genes and cell biological functions, and importantly, availability of powerful genetic and biochemical tools.

D. melanogaster provides a powerful platform for interrogating interactions between host cells and human bacterial pathogens. In addition to the availability of excellent genomic, proteomic, and metabolomic resources for the organism, several aspects of *Drosophila* biology contribute to its attractiveness as a model system for such studies, including the rapid generation time, genetic tractability, easy infection of *Drosophila* tissue culture cells with intracellular bacteria, and the efficiency with which RNA interference (RNAi) technology can be employed for knocking down host gene expression in *Drosophila* cells [5]. Indeed, the ease of RNA interference in *Drosophila* S2 cells has resulted in the elucidation of host factors for diverse pathogens, including E*scherichia coli* [2], *Listeria monocytogenes* [3, 6], *Mycobacterium tuberculosis* [7], *Legionella pneumophila* [1], and *Chlamydia trachomatis* [8, 9]. Importantly, the power of the *Drosophila* S2 cell system has been used to identify and characterize host factors that support infection by the intracellular bacterial pathogen *Brucella melitensis* and thereby, provided the first glimpse of host mechanisms that are subverted by the pathogen to support its intracellular lifestyle [10].

Brucellosis caused by *Brucella*, a gram-negative, facultative intracellular bacterium, is an important zoonotic disease with more than 500,000 new human cases being reported annually worldwide [11, 12]. There are ten recognized species of *Brucella* amongst which, *B. abortus*, *B. melitensis*, and *B. suis* are highly pathogenic to humans [13, 14]. Treatment of human brucellosis includes protracted use of combination antibiotic therapy, which sometimes is associated with complications and relapse of the disease [15]. Several live attenuated vaccines have been approved for animal use and have been extensively deployed to control animal brucellosis [16]. Unfortunately, so far, there is no vaccine approved for human brucellosis [13]. This feature has contributed to the classification of *Brucella* spp. as category B select agents by the US Centers for Disease Control and Prevention [16].

Over the past several decades, pathogen factors that mediate *Brucella* infection have been identified [17–21]; however, the host factors that mediate these processes or that are subverted by the pathogen to secure an intracellular replicative niche have remained less well characterized. Fortunately, recent studies have resulted in the identification of several host factors such as Rho1, Rac1, Cdc42, and Sar1 for *Brucella*, including those that were identified

or characterized in the *Drosophila* screen [22, 23]. In addition, Phosphoinositide 3-kinase (PI3K) activities were shown to support *Brucella* infection [22]. IRE1α, a transmembrane kinase and master regulator of the eukaryotic unfolded protein response, was also shown to regulate host cell susceptibility to infection, thereby implicating the activity of ER resident sensor kinases in regulating this process [10]. Taken together, these studies have indicated that the *Drosophila* S2 cell system provides a useful system for elucidating the *Brucella*–host interaction.

In this book chapter we describe a protocol to study the effects of host protein knockdown on *Brucella* intracellular trafficking and replication.

2 Materials

2.1 Bacterial Strains and Cells

1. Virulent wild type (WT) *Brucella melitensis* strain 16 M and *B. abortus* strain 2308, *and* mutants derived from them are listed in Table 1 (*see* **Note 1**).
2. RAW 264.7 cells, Drosophila S2 cells, J774.A1 cells, Murine Embryonic Fibroblast cells, HeLa cells.

Table 1
***Brucella* strains used in this study**

Species/strain	Characteristics of the strains	Reference
Brucella abortus		
S2308	Wild type strain	[24, 25]
S19	Vaccine strain	[24]
RB51	Vaccine strain	[24]
BA114	S2308virB10::Tn5	[26]
CA180	S2308manB::Tn5	[25, 27]
S2308ΔmanBA	manBA::Km, derived from S2308	[24]
S2308ΔvirB2	ΔvirB2, derived from S2308	[24]
B. melitensis		
16 M	Wild type strain	[24]
16 M-GFP	GFP expressing strain, 16 M-pBBR1MCS-6Y	Weeks et al.[a]
16 MΔmanBA	ΔmanBA, derived from 16 M	[24]
16 MΔvirB2	ΔvirB2, derived from 16 M	[24]
102B2 (BMEI1364)	16 MmucR::Himar1, derived from 16 M	[28]
146D5 (BMEI1178)	16 MmerR::Himar1, derived from 16 M	[28]

[a]Unpublished data

2.2 Media and Antibiotics

1. Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % Fetal Bovine Serum (FBS).
2. Drosophila-SFM medium.
3. Schneider's *Drosophila* medium, containing L-glutamine and sodium bicarbonate and supplemented with 10 % (v/v) FBS.
4. Tryptic soy broth (TSB): 40 g/L in purified water. Sterilize by autoclaving.
5. Tryptic soy agar plates (TSA): 30 g/L in purified water. Sterilize by autoclaving.
6. Farrell's medium: 30 g/L TSA in purified water, 20 mL/L 50 % dextrose, 10 % FBS, Modified *Brucella* Selective supplement (oxoid) (*see* **Note 2**).
7. Gentamicin stock solution. 10 mg/mL.
8. Chloramphenicol stock solution: 25 mg/mL.
9. Kanamycin stock solution: 50 mg/mL.

2.3 Antibodies/Dyes/Reagents

1. ER marker mSpitz-GFP, Golgi marker dGRASP-GFP (*see* **Note 3**).
2. Goat polyclonal anti-*Brucella*, rabbit anti-human M6PR, rabbit anti-human cathepsin D, goat-anti rabbit Sec23, Alexa Fluor 488-conjugated and/or Alexa Fluor 594-conjugated donkey anti-goat/rabbit, phalloidin-Texas red (*see* **Note 3**).
3. 0.2 % (w/v) trypan blue.
4. Effectene Transfection Reagent.

2.4 Microscopy

1. Fluorescence inverted microscope (*see* **Note 4**).
2. Confocal microscope.
3. Coverslips.
4. 24-well flat bottom plates.
5. Fine forceps.
6. Microscope slides.
7. Mounting Medium (VECTASHIELD or ProLong Gold antifade reagent).
8. Nail polish.
9. Imaging and analysis software (*see* **Note 5**).

2.5 Drugs

1. Bafilomycin A1 (BAF). Stock solution in DMSO and used at 200 nM final concentration.
2. Brefeldin A (BFA). Stock solution in DMSO and used at 2.5 μg/mL final concentration.
3. Cytochalasin D [24]. Stock solution in DMSO and used at 2.5 μg/mL final concentration.

4. Myriocin (MR) Stock solution in DMSO and used at 10 μM final concentration.
5. Wortmannin (WM) Stock solution in DMSO and used at100 nM final concentration.

2.6 Solutions and Kits

1. Phosphate buffered saline (PBS).
2. Lysis solution: 0.5 % Tween in water.
3. Peptone saline: 10 g peptone, 8.5 g sodium chloride in 1 L water.
4. Blocking buffer; 1.5 % nonfat dry milk, 0.1 % Triton X-100 in PBS.
5. Effectene Transfection Reagent.
6. Concanavalin A coated coverslips.
7. 3.7 % Paraformaldehyde in PBS, (pH 7.4).
8. In vitro transcription T7 MEGAscript kit.
9. Polymerase Chain Reaction (PCR) master mix.
10. cDNA synthesis kit.

2.7 Equipment

1. CO_2 incubator, 37 °C.
2. 29 °C CO_2 incubator.
3. Shaking incubator.
4. 37 °C incubator.
5. Spectrophotometer.
6. 25 °C incubator for *Drosophila* cells.
7. Centrifuge with rotors that support well plates.
8. Microcentrifuge.
9. Inoculating loop.
10. Hemacytometer.
11. Thermocycler.
12. Gel electrophoresis apparatus.

3 Methods

3.1 Bacterial Cultivation

1. Grow bacteria at 37 °C in TS broth or on TSA plates, supplemented with either kanamycin (Km, 50 μg/mL), or chloramphenicol (Cm, 25 μg/mL) when required.
2. For infection, inoculate 4 mL of TSB with a loop of bacteria taken from a single colony grown on a freshly streaked TSA plate.
3. Cultures are grown with vigorous shaking at 37 °C overnight, or until $OD_{600} \approx 3.0$.

3.2 Cell Culture

1. Cultures of murine macrophage J774.A1 cells, Murine Embryonic Fibroblast (MEF) and HeLa cells are maintained at 37 °C in a 5 % (v/v) CO_2 atmosphere in DMEM supplemented with 10 % (v/v) FBS (*see* **Note 6**).
2. The S2 cells are maintained at 25 °C in Drosophila-SFM medium or in Schneider's Drosophila medium supplemented with 10 % (v/v) FBS (*see* **Note 7**).
3. Cells are seeded in 24-well plates prior to infection (*see* **Notes 8** and **9**).
4. For antibiotic protection assays (*see* Subheading 3.3), 2.5×10^5 cells are seeded in each well.
5. For fluorescence microscopy assays (*see* Subheading 3.6), seed 5×10^4 cells on 12-mm glass coverslips placed on the bottom of 24-well plate before infection.

3.3 Brucella Infection, Drug Treatment, and Gentamycin Protection Assay

1. Host cells (mammalian or S2) are cultured in 24-well plates (*see* **Note 13**) and infected with *Brucella* at a multiplicity of infection (MOI) of 100 CFU per cell.
2. To assess *Brucella* trafficking and replication under drug-mediated depletion of selected proteins in host cells, cells are co-incubated with selected drug (*see* **Note 10**) 1 h prior to and during *Brucella* infection.
3. To synchronize the infection the infected plates are subjected to centrifugation at room temperature for 5 min at $200 \times g$ and incubated at 29 °C (S2 cells) or 37 °C (mammalian cells) under an atmosphere containing 5 % (v/v) CO_2 (*see* **Note 11**).
4. 30 min post-infection, culture medium (*see* Subheading 3.2, **steps 1** and **2**) is aspirated, and the cell monolayer is rinsed 2–3 times with 1 mL PBS (*see* **Note 12**). Fresh medium, supplemented with 40 μg/mL gentamicin (to kill any extracellular bacteria) or gentamicin along with the selected drug is added (*see* **Note 13**).
5. The cells are incubated at 29 °C (S2 cells) or 37 °C (mammalian cells) for another 30 min. Subsequently, the cells are washed to remove the dead bacteria and fresh medium containing 20 μg/mL gentamicin or gentamicin along with the selected drug is added.
6. Incubate the infected cells continuously in media containing antibiotic or antibiotic along with the selected drug for various lengths of time at the appropriate temperature.
7. Various times after infection calculate *Brucella* entry or replication by CFU analysis (*see* **Note 14**), viability of infected host cells (*see* Subheading 3.4), or proceed with immunolabeling (*see* Subheading 3.5). Alternatively, bacterial replication may be monitored using immunofluorescence microscopy assays, as described below (*see* Subheading 3.6).

3.4 Viability Assay of Infected S2 Host Cells

1. S2 cells are coincubated with or without selected drugs 1 h prior to and during *Brucella* infection (*see* Subheading 3.3, **step 2**).
2. Following infection with *Brucella* (MOI = 100), the cultures are centrifuged at 200 × *g* for 5 min to initiate infection and then incubated as described above (*see* Subheading 3.3**, step 4**).
3. Following 30 min of incubation, the medium is replaced with fresh *Drosophila*-SFM medium, supplemented with selected drugs, in addition to 40 μg/mL gentamicin and the infected cells are incubated as described above (*see* Subheading 3.3) for various lengths of time.
4. To quantify the viability of S2 cells, at various time points, vital stain analysis with 0.2 % (w/v) trypan blue is done (*see* **Note 15**).
5. To quantify the viability of S2 cells by image analysis, at various time points, infected cells are transferred onto ConA-coated 12-mm coverslips (*see* **Notes 16** and **17**) in 24-well plates and allowed to adhere for 1 h.
6. The cells are stained with 0.2 % (w/v) trypan blue for 5 min and then fixed during 1 h incubation in 3.7 % (v/v) formaldehyde in PBS.
7. Images are captured with a fluorescence microscope (*see* **Note 4**) for analysis of viability.

3.5 Analysis of Infected Drosophila S2 Cells Expressing Subcellular Markers

To visualize the intracellular trafficking of *Brucella* spp. S2 cells are transfected with the ER marker mSpitz-GFP or the Golgi marker dGRASP-GFP before infection (*see* **Note 3**).

1. S2 cells are grown to ~80 % confluency and then transfected using Effectene Transfection Reagent as per the manufacturer's instructions.
2. For ER visualization experiments, 0.25 μg of each pUAS-mSpitz GFP and pAcpA-Gal4 are used.
3. For Golgi visualization experiments, 0.25 μg of dGRASP-GFP is used in the transfection. Typically, 1.5×10^6 cells are transfected and then grown in 2.2 mL of Schneider's *Drosophila* medium supplemented with 10 % FBS.
4. 3 days post-transfection, cells are replated onto ConA-treated 12-mm glass coverslips placed on the bottom of 24-well microtiter plates (for early time points of less than 8 h). Immunofluorescence microscopy analysis is then performed as described below (*see* Subheading 3.6).
5. For later times points (≥8 h), the transfected cells are reseeded directly in 24-well plates and allowed to adhere for 2 additional hours before infection with *Brucella* (for infection *see* Subheading 3.3).

6. At different post-infection time points, the infected cells are replated onto ConA-coated 12-mm coverslips and allowed to adhere for 1 h.
7. The cell monolayers are then washed three times with PBS, fixed with 3.7 % formaldehyde at room temperature for 1 h, and finally processed for immunofluorescence microscopy (*see* Subheading 3.6).

3.6 Immunofluorescence Microscopy Assay

1. To visualize the intracellular trafficking of *Brucella* spp., by immunofluorescence microscopy, incubate the coverslips with primary antibodies (*see* **Notes 3**, **18**, and **19**) for overnight in blocking buffer (*see* **Note 20**).
2. Wash the coverslips with PBS three times (*see* **Note 8**).
3. Incubate coverslips with 1:1,000 dilution of fluorescent-conjugated secondary antibodies (*see* **Note 21**) for 1 h at room temperature (*see* **Note 22**).
4. Coverslips are subsequently washed with distilled water and mounted in VECTASHIELD media or ProLong Gold Antifade Reagent and visualized with a confocal microscope (*see* **Note 23**).
5. For quantitative analysis, single confocal sections of random fields are acquired, and colocalization of markers scored as positive when nonsaturated signals partially overlap.
6. Images for all immunofluorescence assays for *Brucella* trafficking are acquired with a camera mounted on the microscope (*see* **Note 4**) and are processed with imaging software (*see* **Note 24**).

3.7 Drug Treatments

1. *Drosophila* S2 cells or J774.A1 murine macrophages are incubated at 29 °C or 37 °C, respectively, in 24-well plates for 1 h prior to infection, as described (*see* Subheading 3.3), in media (*see* **Note 25**) supplemented with one of the following drugs at the required concentration; bafilomycin A1 (BAF), brefeldin A (BFA), cytochalasin D [24], myriocin (MR), and wortmannin (WM) (*see* **Note 10**).

3.8 Generation of dsRNAs

1. Primers for generating RNAi that target *Drosophila* Rac1, Rac2, Rho1, Cdc42, Sar1, and PI3Ks are designed using sequence information present in flybase (http://flybase.org/).
2. dsRNAs targeting genes to be knocked down are generated using gene-specific RNAi primers to amplify target sequences from *Drosophila* cDNA mixture with T7 RNA polymerase promoter sequences at the 5′ end [3].
3. For generation of dsRNAs targeting ER-associated or other genes, cDNAs from commercially available *Drosophila* RNAi

Library [Release 1.0-DNA templates (Open Biosystems, Huntsville, AL, USA)] are used directly as templates.

4. 1 or 2 μL (~150 ng) of the PCR products are used to perform in vitro transcription reactions with T7 MEGAscript kit as described by the manufacturer.
5. Aliquots of in vitro transcription products are subjected to quality control via 1 % agarose gel electrophoretic analysis and quantified using a spectrophotometer.

3.9 RNAi-Mediated Gene Knockdown and Assays

1. 1.0×10^6 S2 cells are used to seed the wells in a 12-well plate.
2. Following incubation as described above (*see* Subheading 3.2), dsRNAs (i.e., Rho1, Rac1, Cdc42, Sar1, and PI3Ks) are added to each well to a final concentration of 15 μg/mL.
3. Following 4 days of incubation in the presence of dsRNA, a portion of the culture is removed to check the efficiency of dsRNA mediated gene knockdown by quantitative RT-PCR (Q-PCR).
4. dsRNA-treated S2 cells are transferred to new 24-well plates and allowed to adhere for at least 2 h before infection.
5. The treated cells are infected with *Brucella* as described above (*see* Subheading 3.3).
6. At selected time points, dsRNA-treated and *Brucella* infected cells are lysed and bacterial recovery enumerated or fluorescence microscopy image analysis performed as described above (*see* **Note 14** and Subheading 3.6).
7. The effect of dsRNA mediated gene knockdown on *Brucella* entry and replication in S2 cells is also confirmed by fluorescent microscopic image analysis. S2 cells are used to seed the wells of a 96-well microtiter plate with 5.0×10^4 cells in 200 μl of *Drosophila*-SFM medium.
8. dsRNAs targeting host genes are added to the wells at final concentrations of 15 μg/mL (dsRNAs are added in duplicate in two different plates).
9. dsRNA-treated cells are incubated at 25 °C for 4 days for knockdown of target gene expression.
10. dsRNA-treated cells (2.5×10^4 cells in 100 μl) are transferred into new 96-well plates, infected with *B. melitensis* 16 M-GFP or *B. abortus* 2308-GFP at a multiplicity of infection (MOI) of 50.
11. Following 30 min of infection, fresh media supplemented with 40 μg/mL gentamicin is added to each well and the infected cells are incubated at 29 °C. At 72 hpi, infected cells are replated onto 96-well glass bottom plates coated with ConA, and incubated for 1 h to promote adherence.

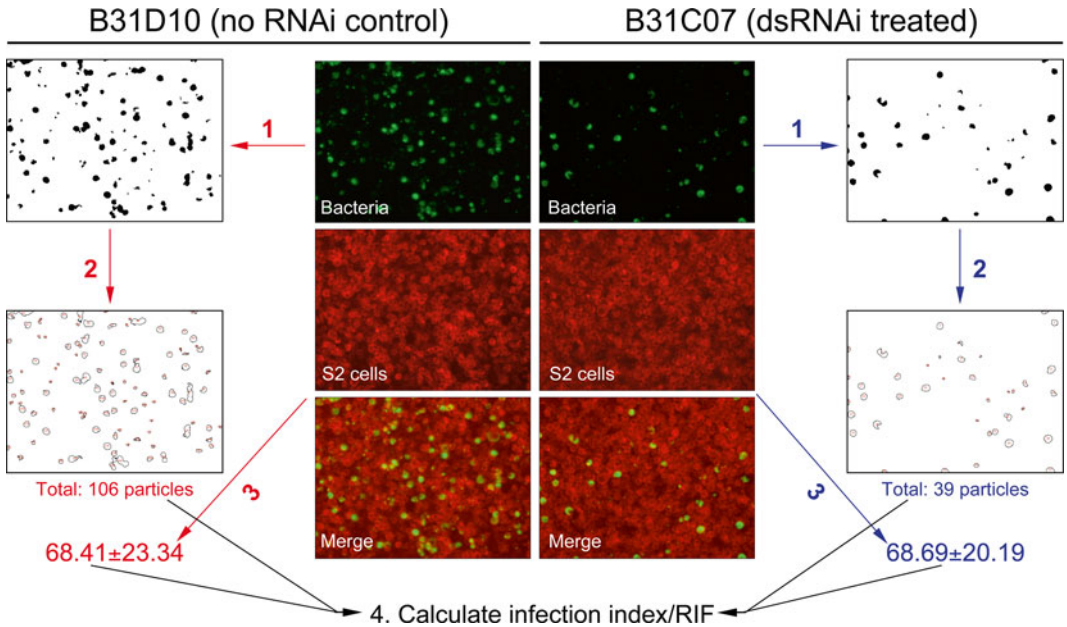

Fig. 1 Schematic representation of image analysis using ImageJ to calculate the relative infection (RIF). *1.* Threshold of bacterial replication in infected cells in an image using the same setting. *2.* Analysis of particles in a threshold image (i.e., the number of infected cells with bacterial replication). *3.* Histogram of the image (cell numbers were adjusted via color density). *4.* Calculation of the infection index and RIF (% of control) of the samples. For example, infection index of sample B31C07 = 39/68.69; RIF of B31C07 = 100 × [39/68.69]/[106/68.41] = 36.12

12. Infected S2 cells are washed three times with PBS, and fixed with 3.7 % (v/v) formaldehyde in PBS at 4 °C overnight, and then stained with phalloidin-Texas red (1:1,000) for 1 h to visualize the host cell actin cytoskeleton.
13. *Brucella* infected S2 cells are viewed with an inverted microscope and multiple 400× images are acquired from each well for image analysis (*see* **Note 5**).
14. Images are analyzed (*see* **Note 5**) and used to determine the relative infection (RIF) [100 × (% of infected dsRNA-treated cells)/(% of infected cells in the untreated control)].
15. The detailed process by which image analysis is performed is shown in Fig. 1.

3.10 Murine Embryonic Fibroblast Cell Infection

1. Seed MEFs (5×10^4cells/well) deficient in two regulatory isoforms of class I_A PI3Ks ($p85\alpha^{-/-}p85\beta^{-/-}$ and $p85\beta^{-/-}$), IRE1α ($IRE1\alpha^{-/-}$) and PERK ($PERK^{-/-}$) and their corresponding WT controls $p85^{+/+}$, $IRE1\alpha^{+/+}$, and $PERK^{+/+}$ MEFs, in 24-well plates (*see* **Note 8**).
2. MEF's are infected as described in Subheading 3.3 with some minor modifications (*see* **Note 25**).

3.11 Statistical Analysis

1. The significance of the data is assessed using ANOVA, and all the analyzed data are normalized with internal controls before analysis (*see* **Note 26**).

4 Notes

1. *B. abortus, B. melitensis, B suis* are classified as select agents requiring registration with Centers for Disease Control and Prevention (CDC) and/or USDA for possession, use, storage, and/or transfer. *Brucella* species belonging to the select agent category are handled in Biosafety Level-3 (BSL-3) laboratories. Individuals working with *Brucella* are required to undergo mandatory training and should meet or exceed the educational requirements for work with select agent before being cleared for work in a BSL-3 setting. The training is repeated annually.
2. *Brucella* is initially streaked from the stock onto a plate containing Farrell's medium (widely used selective medium for *Brucella*). It takes 4–5 days for the bacteria to grow at 37 °C. Subsequently, a single colony from this plate is used to inoculate TSB for use in the experiments. *Brucella* streaked on a Farrell's medium plate can be stored refrigerated for up to 3–4 weeks and then should be discarded and a fresh culture streaked again on Farrell's medium.
3. Anti-*Brucella* antibody is for lighting *Brucella* with secondary antibody conjugated with a fluorescent dye. M6PR (mannose-6- phosphate receptors) is to label late endosome. Cathepsin D is lysosome marker. Sec23 is COPII marker, which will indicate the location of COPII complex (Sar1-Sec23-Sec24). ER marker mSpitz-GFP and the Golgi marker dGRASP-GFP were received as a gift.
4. The fluorescence microscope has different filter cubes which have variety of wavelength for red, green, and blue imaging: TRITC Ex (nm) 557, Em (nm) 576, TXRED Ex (nm) 596, Em (nm) 615, FITC Ex (nm) 490, Em (nm) 525, DAPI Ex (nm) 350, Em (nm) 470.
5. ImageJ software (http://rsb.info.nih.gov/ij/) is used to analyze the images. More than 1,000 S2 cells are counted to obtain the percentage of infection or infection index [(number of infected cells (at least ten brucellae within the cell))/(number of total cells)] in a sample.
6. Fetal bovine serum (FBS) used to supplement the cell culture media should be heat inactivated for 30 min at 56 °C.
7. A new aliquot of S2 cells is thawed every 3–4 weeks (maximum 20 passages). Freshly thawed cells provide consistent infection

rates. Cells are never allowed to overgrow and thus are sub-cultured at 80 % confluency.

8. Cells are counted using a hemacytometer before being seeded onto the glass coverslips. For this load 10 μl of the freshly suspended cells (1:10 diluted in DMEM or PBS) to the hemacytometer and the number of cells per square millimeter is counted (middle 25 squares on the hemacytometer). Cell count (per mL) = number of cells counted in the middle 25 squares X dilution factor X 1×10^4.
9. The host cells used for *Brucella* infection are seeded one day prior to the infection, so that they were 80–90 % confluent at the time of infection.
10. Bafilomycin A1 is a macrolide antibiotic that selectively inhibits vacuolar-type (v-type) H + ATPase. Bafilomycin A1 prevents lysosomal cholesterol trafficking in macrophages and can be used to distinguish different types of ATPases. Brefeldin A is a fungal metabolite and blocks the forward transport between the endoplasmic reticulum and golgi apparatus, Brefeldin A causes an impaired distribution of the membrane proteins. Cytochalasin D is a potent inhibitor of actin polymerization and it disrupts actin microfilaments. Myriocin, a potent inhibitor of serine palmitoyltransferase (SPT), impairs the uptake of transferrin and low-density lipoprotein in mammalian cells. Wortmannin is a very potent, specific, and direct inhibitor of PI3 kinase. The inhibition is irreversible and noncompetitive. Wortmannin does not inhibit PI4 kinase, protein kinase C, or protein tyrosine kinase.
11. For the gentamicin protection assay, centrifugation ($250 \times g$ for 5 min) of the microtiter plates in a bench top centrifuge following infection is crucial to synchronize the infection. Following 30 min of infection, it is also very important to wash the infected host cell monolayers thoroughly three times in PBS (500 μl/well) before incubating it for another 30 min in media containing gentamicin. This washing contributes to removing extracellular bacteria. The gentamicin concentration can be reduced to 20 μg/mL for extended incubation.
12. Washing of the cells (during gentamicin protection assays or immunofluorescence staining) should be performed using gentle agitation to prevent detachment of the S2 cell monolayer.
13. Antibiotic protection assay: Antibiotics such as gentamicin cannot penetrate eukaryotic cells. Infection of eukaryotic cells with a bacteria for a particular time period (30–60 min) followed by incubation of the infected cells in the antibiotic containing medium kills those bacteria that are unable to infect the cells and remain outside in the medium, while sparing those that gain entry inside the cells during the infection process.

The amount of bacteria that were able to infect the cells and were able to replicate further is then determined. At various time points (of interest) post infection, the cells are washed with PBS to remove the dead bacteria and lysed using a detergent (0.5 % Tween-20 in sterile water), thereby, releasing the intracellular bacteria that remained alive and multiplied inside the cells. The lysed cells are plated on solid medium plates and incubated at 37 °C for 24–48 h for the colonies to appear. The colonies on the plates are counted and using the prior knowledge about the number of bacteria used during the initial infection, the number of bacteria that were able to invade or multiply in the cells is determined.

14. CFU analysis: Viable bacteria recovered from the infected cells are enumerated at various times post infection by growth on solid media. The infected cells are washed twice with 1 mL PBS (*see* **Note 27**), and lysed with 0.5 % (w/v) Tween 20 in sterile water. Bacteria released are subjected to 10-fold serial dilution in peptone saline. Ten microliters (μl) of each dilution of the cell lysate is spread on TSA plates and CFU per well determined by enumerating colonies after 3 days incubation at 37 °C. The percent bacterial uptake or invasion is calculated as the number of bacteria recovered 1 h post infection divided by the number of bacteria inoculated into each well. *Brucella* replication efficiency is the # of CFUs at different time points post infection divided by the # of CFUs of *Brucella* entry. Viability assay of drug treated and infected S2 host cell is done to check whether there is any drug induced cell death.
15. Trypan blue is a vital stain that is taken up by the dead cells due to the compromised plasma membrane and hence the cells appear blue under the microscope, while the live cells that exclude the dye due to an intact plasma membrane are not stained. At least 500 S2 cells per sample are counted under the microscope to determine the percentage of viability.
16. Concanavalin A [10] coated coverslips are used to facilitate cell attachment or immobilization during imaging. Coating the coverslips with Con A is a tedious process and therefore to save time we prefer using commercially available Con A coated coverslips.
17. The 12 mm glass coverslips are sterilized by dry autoclaving for 30 min prior to being placed on the bottom of 24-well plate and seeding cells for fluorescence microscopy assays.
18. Prior to immunostaining, coverslips in the 24-well plates are rinsed three times with PBS, and the cell monolayer is permeabilized by incubation for 5 min in 300 μl of 0.1 % (w/v) Triton X-100 in PBS. 300 μl of 1.5 % Nonfat dry milk in PBS (blocking

buffer) is added to each well and incubation at room temperature is continued for 1 h with gentle agitation.

19. For those samples requiring more than one primary antibody, immunostaining is performed sequentially and the coverslips thoroughly washed with PBS between incubations.
20. For immunostaining experiments, primary antibody is diluted 1:200 to 1:250 in blocking buffer (1.5 % nonfat dry milk, 0.1 % Triton X-100 in PBS) and 250 μl is added to each well prior to sealing the plate with Parafilm followed by incubation overnight at 4 °C with agitation.
21. Alexa Fluor 488-conjugated and/or Alexa Fluor 594-conjugated donkey anti-goat/rabbit is used at 1:1,000 dilutions.
22. Following immunostaining with primary antibody, coverslips are rinsed with PBS three times and 250 μl of secondary antibody, diluted 1:1,000 in blocking buffer is added to each well. The plate is covered with aluminum foil and incubated for 1 h at room temperature with gentle agitation.
23. Following immunostaining of mammalian cells, coverslips should be gently rinsed with double distilled water and air-dried for 10–15 min. ProLong Gold anti-fade reagent (3 μl) provides an alternative to VECTASHIELD for reducing photobleaching during imaging. Nail polish is used to seal the coverslips, which can be stored overnight at 4 °C in dark.
24. Image processing software such as Adobe Photoshop can be used.
25. MEF's harboring gene knockdown offer an attractive mammalian cell model system to assess the effect of host protein knockdown on bacterial replication. The MEF's are infected for 60 min followed by incubation in the gentamycin containing media for 60 min, to kill the extracellular bacteria. Replication is determined at 48 hpi. At 48 hpi, the infected host cells are subjected to the appropriate assays, as described above (*see* Subheadings 3.4 and 3.6). Recovery of viable bacteria is determined, as described above (*see* **Note 14**). For fluorescence microscopy and viability assays, seed 5×10^4 cells/well onto 12-mm coverslips in 24-well plates, as described above (*see* Subheadings 3.6 and 3.2).
26. All quantitative data is derived from results obtained in triplicate wells for at least three independent experiments.
27. For bacterial recovery from infected cell monolayer from 24-well plates, it is very important to wash the cells 2–3 times with PBS to wash away the residual antibiotic and dead cells before lysis with 500 μl of lysis solution (0.5 % Tween in water). 100 μl of the resultant lysate is diluted into 900 μl peptone saline or PBS to generate a stock that is used for serial dilution analysis.

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Chapter 14

Purification of Intracellular Bacteria: Isolation of Viable *Brucella abortus* from Host Cells

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Abstract

The pathogenesis of brucellosis depends on the ability of bacteria from the genus *Brucella* to invade and replicate within animal cells. To understand the molecular pathways used by *Brucella* spp. to reach its intracellular niche, robust and reproducible bacteria purification protocols that provide enough material for biochemical and molecular biology studies are essential. Here, we describe a detailed methodology designed to extract and purify viable brucellae from mammalian host cells at different time periods of their intracellular cycle. The yield of proteins and nucleic acids is sufficient to perform immunochemical analysis, genetic studies, transcriptomics, and proteomics among others.

Key words Intracellular bacteria, Brucellosis, Bacterial pathogenesis, Cellular fractionation, Virulence factors

1 Introduction

Members of the genus *Brucella* cause a chronic zoonotic disease named brucellosis, whose main clinical signs are abortion in primary hosts and undulant fever in humans [1]. The pathogenesis of the disease is intimately linked to the bacterial ability to invade, traffic, and finally replicate within endoplasmic reticulum-like compartments of professional and non-professional phagocytes [2, 3]. It has been determined that brucellae mutant strains unable to replicate intracellularly in cell cultures, are also attenuated in animal models and incapable to reproduce the classical signs and symptoms of the disease [4–6]. Thus, in order to understand the brucellae virulence strategies it is mandatory to unravel their intracellular adaptations and the mechanisms involved in the transition from an extracellular environment to an intracellular milieu.

Several molecular determinants have been demonstrated to play a crucial role in the intracellular lifestyle of virulent *Brucella*, such as its lipopolysaccharide, the VirB Type IV Secretion System

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(T4SS), cyclic β-glucans, a flagellar-like apparatus, and a number of components of the central metabolism, all of which are in part regulated by the BvrR–BvrS two component system (TCS) and the VjbR quorum sensing transcriptional regulator [7, 8, 6, 9–11]. Most of the knowledge regarding the role of these systems during the intracellular cycle of the brucellae has been obtained through the generation of mutants in the corresponding genes and the subsequent analysis of downstream effects in terms of loss-of-ability to invade and/or multiply intracellularly.

Other strategies used to understand the intracellular bacterial responses involve molecular reporters. One significant example has been the use of transcriptional and translational fusions of the *virB* operon genes with marker proteins, such as GFP or beta galactosidase. These strategies have indicated that expression of the *virB* operon is induced during the first hours of host cell infection [12, 13]. There are however, very few studies that have isolated viable intracellular bacteria to directly address the changes at the protein or transcriptional level used by the brucellae organisms to adapt to the different phases of the intracellular trafficking and replication [14].

This is not an easy task, since several biological parameters are interplaying. For instance, isolating *Brucella abortus* from infected non-professional phagocytes such as HeLa or Vero cells is precluded, mainly at early times of infection, due to the low number of brucellae invading these cells [2, 15]. In order to partially overcome this issue, *Escherichia coli* Cytotoxic Necrotizing Factor 1 (CNF)-treated HeLa cells or macrophage cell lines have been used as host cells [15]. Although the number of internalized bacteria in macrophages is significantly larger than in other cells, the use of professional phagocytes is not problem-free. Indeed, in macrophages a significant proportion of the internalized bacteria are redirected to lysosomes and destroyed, while only a small proportion escape the host cell-killing action and fuse with compartments of the endoplasmic reticulum [3]. It is thus expected that the bacteria located in degradative compartments would display a different phenotype than the surviving bacteria that successfully reached the endoplasmic reticulum. This is the main reason why a protocol for isolation of viable *B. abortus* has been developed, avoiding the ambiguity of dealing with mixed populations containing dead bacteria that would hamper the phenotypic and molecular analysis of intracellular bacteria [14].

2 Materials

Prepare all solutions and reagents with ultrapure water (resistance = 18 MΩ). Reagents should be ACS grade or superior. All the solutions including sodium dodecyl sulfate (SDS) should be handled with care due to its toxicity. Bacteria of the genus *Brucella* are

classified as select agents (BSL-3) and should be handled accordingly (http://grants.nih.gov/grants/policy/ select_agent/#guidelines). Particular attention should be paid to the adequate disposal of material contaminated with brucellae.

2.1 Cell Culture and Infection

1. HeLa or Raw 264.7 cells, frozen stocks in fetal bovine serum (FBS) supplemented with 10 % dimethyl sulfoxide (DMSO), kept in liquid nitrogen.
2. *B. abortus* 2308, frozen glycerol stocks, kept at –80 °C.
3. Trypticase Soy Broth (TSB): dissolve 30 g of a commercial TSB preparation in 1 L of purified water and sterilize by autoclaving at 121 °C for 15 min. 1 L of TSB contains 17.0 g pancreatic digest of casein, 3.0 g enzymatic digest of soya bean, 5.0 g sodium chloride, 2.5 g di-potassium hydrogen phosphate, and 2.5 g glucose.
4. Sterile 125 mL Erlenmeyer flasks with loose caps.
5. Pyrogen free-FBS. Inactivate complement at 56 °C for 30 min in a water bath.
6. DMEM: Dulbecco's Modified Eagle's Medium.
7. Cell culture medium: DMEM supplemented with antibiotics. Add 50 mL FBS and 5 mL of a penicillin (10,000 μg/mL)/Streptomycin (10,000 U/mL) solution to a 500 mL bottle containing 445 mL of DMEM.
8. Cell infection medium: DMEM, 10 % FBS. Add 50 mL of inactivated FBS to a 500 mL bottle containing 450 mL of DMEM.
9. Extracellular bacteria-killing medium: DMEM, 10 % FBS, 100 μg/mL gentamicin. Add 50 mL of inactivated FBS and 500 μl of a gentamicin solution (100 mg/mL) to 445 mL of DMEM.
10. Cell infection maintenance medium: DMEM, 10 % FBS, 5 μg/mL gentamicin. Add 50 mL of inactivated FBS and 25 μL of a gentamicin solution (100 mg/mL) to a 500 mL bottle containing 450 mL of DMEM.
11. Hank's Balanced Salt Solution (HBS), without calcium and magnesium.
12. Vacuum pump. This equipment should be coupled to a 1 L Erlenmeyer containing 100 mL 5 % chlorine solution to inactivate the aspirated material. A flexible rubber tube coupled to the Erlenmeyer needs to be long enough to be conveniently used in the interior of the laminar hood and a sterile Pasteur pipette attached will be used to aspirate the different media during the infection procedure.
13. Centrifuge. Able to provide 1,000 × *g* centrifugal force and equipped with accessories that allow centrifugation of 6-well culture plates.
14. CO_2 incubator.

15. Heated orbital shaker (37 °C and 100–200 rpm).
16. Spectrophotometer (OD readings at wavelength 400–700 nm).
17. Sterile conical tubes of 50 mL capacity.
18. 6-well culture plates.
19. Neubauer chamber.

2.2 Purification of Intracellular Bacteria

1. 3 mM imidazole–HCl pH 7.4, 250 mL stock solution: Weigh 51 mg of imidazole and dilute in 200 mL of Milli-Q water. Adjust the pH to 7.4 with 1 M HCl. Adjust to 250 mL and store in 30 mL aliquots at −20 °C.
2. Homogenization buffer: 0.3 M sucrose, 3.0 mM imidazole–HCl pH 7.4, 50 mL stock solution: Weigh 5.1 g of sucrose and dissolve in 40 mL of 3.0 mM imidazole–HCl, and then adjust to 50 mL with the same 3.0 mM imidazole–HCl, pH 7.4 solution.
3. 0.8 M sucrose–3.0 mM imidazole–HCl pH 7.4, 10 mL stock solution: Weigh 2.7 g of sucrose and dissolve in 7 mL 3.0 mM imidazole–HCl, pH 7.4 and then adjust to 10 mL with the same 3.0 mM imidazole–HCl, pH 7.4 solution.
4. 1.5 M sucrose–3.0 mM imidazole–HCl pH 7.4 10 mL stock solution: Weigh 5.1 g of sucrose and dissolve in 5 mL 3.0 mM imidazole–HCl, pH 7.4, and then adjust to 10 mL with the same 3.0 mM imidazole–HCl, pH 7.4 solution.
5. 2 M sucrose–3.0 mM imidazole–HCl pH 7.4, 10 mL stock solution: Weigh 6.8 g of sucrose and dissolve in 5 mL 3.0 mM imidazole–HCl, pH 7.4, and then adjust to 10 mL with the same 3.0 mM imidazole–HCl, pH 7.4 solution.
6. 1 M sucrose–0.1 % SDS–3.0 mM imidazole–HCl pH 7.4, 10 mL stock solution: Weigh 3.4 g of sucrose and 10 mg of SDS and dissolve in 6 mL 3.0 mM imidazole–HCl, pH 7.4, and then adjust to 10 mL with the same 3.0 mM imidazole–HCl, pH 7.4 solution.
7. Benzonase solution. Commercial nuclease preparation at 25 U/μL.
8. 1 mL plastic syringes with 25 g × 1 1/2″ needles.
9. Centrifuge. Equipped with swinging bucket rotor able to accommodate 15 mL conical tubes and to provide a centrifugal force of 1,000 × *g*.
10. Ultracentrifuge. Equipped with swinging bucket rotor able to accommodate 11 mL ultracentrifuge tubes and to provide a centrifugal force of 30,000 × *g*.
11. Dounce homogenizer with 10 mL capacity.

12. 15 mL sterile plastic conical tubes.
13. Thin wall 11 mL ultraclear ultracentrifugation tubes.

2.3 Immune Detection of Bacteria

1. Methanol–acetone fixing solution: Mix equal parts of methanol and acetone.
2. Phosphate buffered saline (PBS): Weigh 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4. Dissolve in 800 mL of Milli-Q water. Adjust the pH to 7.4 with HCl and then adjust with Milli-Q water to a final volume of 1 L.
3. Paraformaldehyde: Weigh 4 g of paraformaldehyde and pour in 100 mL of PBS. Add a few drops of NaOH and heat at 60 °C to dissolve.
4. 50 mM ammonium chloride: Weigh 267.4 mg of ammonium chloride and dissolve in 90 mL of PBS, and then adjust to 100 mL with PBS.
5. 10 % horse serum (HS) PBS: Add 10 mL of horse serum (HS) to 90 mL of PBS.
6. Primary and secondary antibodies: Dissolve the primary antibodies (rabbit anti-*Brucella* LPS) and secondary antibodies (anti-Rabbit antibodies conjugated with Texas Red) in 10 % HS in PBS at a previously standardized dilution.
7. Mounting solution: Add 2.4 g of Mowiol 4-88 to 6 g of glycerol and mix. Then add 6 mL of water and leave at room temperature. Add 12 mL of 0.2 M Tris–HCl (pH 8.5) and dissolve by heating at 40 °C. Finally centrifuge at 6,000 × *g* for 20 min. Store in 1 mL aliquots at −20 °C.
8. Coverslips: Round glass coverslips of 12 mm diameter. Autoclave in a 15 mL beaker covered with aluminum foil.
9. Glass slides.
10. Cell counting chamber: Neubauer chamber.
11. Fluorescence microscope: Upright epifluorescent microscope equipped with 60× and 100× objectives. This microscope should be equipped with filters to visualize fluorescein and Texas Red fluorochromes.

2.4 Western Blotting Analysis

1. Lysis buffer: Weigh 2 g of SDS and dissolve in 100 mL of ultrapure water.
2. 10 % pre-cast polyacrylamide gels.
3. Running buffer: Weigh 28.8 g of glycine, 6.04 g of Tris base, and 2 g of SDS. Dissolve in 2 L of water.
4. Laemmli sample buffer: Mix 4 mL of 10 % SDS solution, 2 mL glycerol, 1.2 mL 1 M Tris base pH 6.8, and 2.8 mL of water. Add 2 mg of bromophenol blue.

5. Commercial protein quantification assay.
6. Polyvinyl difluoride membrane (PVDF).
7. Transfer buffer: Weigh 28.8 g of glycine, 6.04 g of Tris base and mix it with 200 mL of methanol and 1.8 mL of water.
8. Protein electrophoresis and blotting equipment.
9. Primary monoclonal antibodies against *Brucella* components: Yst9 monoclonal antibody (mouse) directed against LPS O antigen and monoclonal antibody (mouse) directed against Omp19 protein. Dilute these antibodies at working concentration in PBS containing 0.1 % Tween 20 and 5 % nonfat dry milk.
10. Peroxidase-conjugated anti-mouse antibodies: Dilute this conjugate at working concentration in PBS containing 0.1 % Tween 20 and 5 % nonfat dry milk.
11. Western blotting luminol reagent.
12. Gel-imaging system: equipped with CCD camera suited for chemiluminescence detection.

2.5 Detection of RNA Transcripts

1. Tryptic soy agar plates.
2. 10 % Zwittergent 3-16: Weigh 0.5 g ZWITTERGENT 3-16 and dissolve in 5 mL diethylpyrocarbonate (DEPC)-treated water.
3. TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA): mix 10 mL 1 M Tris–HCl pH 8.0, 2 mL 0.5 M EDTA. Bring total volume to 1 L with ultrapure H_2O. Sterilize by autoclaving.
4. 50 mg/mL lysozyme: weight 50 mg lysozyme and dissolve in 1 mL ultrapure water. Make aliquots according to the required amount per experiment and store at −20 °C.
5. TE buffer, 2 μg/μL lysozyme: mix 96 μL TE buffer with 4 μL 50 mg/mL lysozyme.
6. Commercial RNA extraction kit.
7. Ambion Turbo DNase kit.
8. Commercial PCR kit.
9. Commercial retrotranscription kit.
10. 37 °C incubator.
11. Benchtop centrifuge.
12. Vortex.
13. Spectrophotometer or similar to measure obtained RNA concentration and purity.
14. Equipment for agarose gel electrophoresis.
15. Thermocycler.
16. 10 mL syringe with 21 G ×1″ needle.

3 Methods

3.1 Cell Culture and Preparation of Bacterial Inoculum

1. Cells (HeLa or Raw 264.7) are routinely cultured in 75 cm^2 plastic culture flasks. Detach the cells from the flasks by standard techniques (trypsinization for HeLa cells and mechanical removal using cell scrapers for Raw 264.7 macrophages) and determine cell number using a Neubauer chamber. Seed 1×10^6 cells per well (each well containing 3 mL cell culture medium) in 6-well plastic plates 48 h before the infection (*see* **Note 1**). Prepare at least four 6-well plates per experiment. This number of plates is the minimum required per time point to obtain enough bacteria for further analysis. The day of the experiment the cells should be close to 70–90 % confluence (s*ee* **Note 2**). Approximately 3×10^6 cells per well should be achieved at this point. To increase HeLa cell bacterial infections, a previous treatment with CNF1 is recommended (*see* **Note 3**).
2. Prepare a *B. abortus* 2308 pre-inoculum 48 h before the infection by thawing 500 μL of a frozen stock of brucellae and adding it to 20 mL of TSB in a 125 mL sterile Erlenmeyer flask (*see* **Note 4**). Incubate with agitation at 200 rpm and 37 °C for 18 h (*see* **Note 5**).
3. 18 h before the infection measure the optical density of the bacterial pre-inoculum at 420 nm in a spectrophotometer. Use the optical density data to extrapolate the bacterial concentration [16] and calculate the volume of bacterial culture needed to prepare the pre-inoculum containing 5×10^9 bacteria (*see* **Note 6**). To prepare the bacterial inoculum, add 5×10^9 bacteria to 20 mL of TSB in a 125 mL sterile Erlenmeyer and incubate with agitation at 200 rpm and 37 °C for 18 h.

3.2 Infection

1. Measure the optical density of the inoculum at 420 nm and determine the bacterial concentration in the culture. Then, calculate the volume of cell infection medium required, estimating 1.5 mL per well (for a typical experiment using four 6-well plates, this volume corresponds to 36 mL), and add it to a 50 mL sterile conical tube. Add the volume of the inoculum required for a multiplicity of infection (MOI) corresponding to 2,000 bacteria/cell. Assuming that each well contains 3×10^6 cells, then each well requires 6×10^9 bacteria and thus the concentration of the inoculum would be 4×10^9 CFU/mL (*see* **Note 7**).
2. Aspirate the cell culture medium from each well using a vacuum pump. Gently wash the cells by pouring 1.5 mL of HBS. Repeat the washing step three times for each well.
3. After the last wash, aspirate the HBS and then gently add 1.5 mL of the bacterial inoculum prepared in Subheading 3.2, **step 1**. Then, put the 6-well plates into a plate rotor equipped

with aerosol-tight lids and centrifuge at 1,000 × *g* for 5 min at 4 °C. Finally incubate the plates for 30 min at 37 °C under a 5 % CO_2 atm.

4. Aspirate the infection medium with the vacuum pump and gently wash the cells with 2 mL of HBS as described above (*see* Subheading 3.2, **step 2**). Repeat this step three times for each well. After the final wash, gently add 2.5 mL of extracellular bacteria-removing medium to each well. Incubate for 60 min at 37 °C under a 5 % CO_2 atm. After incubation, aspirate the extracellular bacteria-removing medium with the vacuum pump and gently add 2.5 mL of the cell infection maintenance medium. Incubate for the different time periods required (e.g., 3, 8, 24, and 48 h) at 37 °C in a 5 % CO_2 atm (*see* **Note 8**).

3.3 Extraction and Purification of Intracellular Bacteria

1. Aspirate the cell infection maintenance medium with the vacuum pump and gently wash as indicated above with 2 mL of HBS at room temperature. Repeat this step three times for each well. After the final wash, aspirate the HBS and gently add 1 mL of cell infection medium. Gently detach the cells of the monolayer using a sterile plastic scraper and transfer the cell suspension to a 50 mL sterile plastic tube. Repeat this step for all the 24 wells used in the experiment, combining all the cells in a single tube. After this point, keep the infected cells on ice and perform all the subsequent steps of the extraction and purification protocol at ice cold temperature (~1–3 °C). Centrifuge the cells at 300 × *g* for 10 min and discard the supernatant.
2. Suspend the cell pellet in 1.5 mL of homogenization buffer. Transfer the suspension of cells to a 7 mL Dounce homogenizer and disrupt the cells with strong strokes with the pestle until at least 95 % of the cells have been disrupted as estimated by staining with vital dyes (*see* **Note 9**). After homogenization, transfer the suspension to a 15 mL sterile plastic conical tube. Add benzonase to a final concentration of 25 U/mL and incubate at 37 °C for 30 min. Then gently layer the disrupted cell homogenate onto the top of 1 mL cushion of the 0.8 M sucrose solution in a 15 mL sterile conical tube. Centrifuge in a swinging bucket rotor at 500 × *g* for 5 min at 4 °C. Transfer the homogenate that floats on the 0.8 M sucrose cushion into a sterile 50 mL conical tube, and measure its volume (*see* **Note 10**). Then, add 4 volumes of the 3.0 mM imidazole–HCl pH 7.4 solution (e.g., if 1.5 mL of the homogenate are recovered, add 6 mL of the 3.0 mM imidazole–HCl pH 7.4 solution).
3. Prepare a discontinuous sucrose gradient in a 13.2 mL thin wall ultraclear ultracentrifugation tube by layering the following solutions from bottom to top: 1 mL 1.5 M sucrose, 1 mL 1 M sucrose, 0.1 % SDS, 1 mL 0.8 M sucrose. Gently layer the

diluted homogenate prepared in the previous step on the top of the sucrose gradient. Centrifuge in a swinging bucket rotor at 30,000 × *g* for 25 min at 4 °C. Carefully discard all the supernatant layers and keep the pellet (*see* **Note 11**).

4. Suspend the pellet on 1 mL PBS in a 1.5 mL plastic tube. Centrifuge at 10,000 × *g* for 10 min at 4 °C and discard the supernatant. The pellet contains the extracted and purified intracellular bacteria. At this point the pellet may be frozen at −70 °C. This procedure is recommended when several purification runs are necessary to accumulate enough bacteria for subsequent analysis.

3.4 Immunodetection of Purified Bacteria

1. The purified bacteria can be analyzed by immune fluorescence in order to detect different bacterial components or to determine certain physiological states. For these purposes, resuspend the pellet obtained in Subheading 3.3, **step 4** containing extracted and purified bacteria in 20 µL of PBS at room temperature. Add 5–15 µL of this bacterial suspension to a 12 mm round coverslip placed inside a 24-well plate. Spread the suspension as much as possible. Incubate the preparation at 37 °C until it dries completely. Fix the bacteria to the coverslip by adding 1 mL of methanol–acetone (*see* **Note 12**). Remove the methanol–acetone solution and add 1 mL of ammonium chloride 50 mM for 10 min at room temperature.
2. Separately, take 2 µl of the bacterial suspension, dilute in 18 µl PBS, transfer to a counting chamber (e.g., Neubauer) and estimate the bacterial numbers under a phase contrast microscope using a 100× objective. The sample should be devoid of excess of cell debris and enriched in bacteria above 95 %.
3. Prepare the primary antibody (e.g., rabbit IgG anti-*Brucella* LPS) at the desired concentration in PBS supplemented with 10 % horse serum (HS). Put a 50 µL drop of the antibody solution onto a 2.5 cm^2 Parafilm sheet. Take the coverslip with the fixed bacteria out of the 24-well plate with a forceps and with the help of a tissue take away the excess of the ammonium chloride solution. Put the coverslip upside down on the drop containing the primary antibody. Incubate for 30 min at room temperature.
4. Prepare the fluorochrome-conjugated secondary antibody (e.g., anti-rabbit IgG-FITC) at the desired concentration in PBS, 10 % HS. Put a 50 µL drop of the secondary antibody onto a 2.5 cm^2 Parafilm sheet. With the aid of pointed forceps take the coverslip from the primary antibody and wash the excess of the solution by immersing ten times the preparation on a 50 mL beaker containing 40 mL of PBS. Repeat this step three times on different PBS-containing beakers. Put the coverslip upside

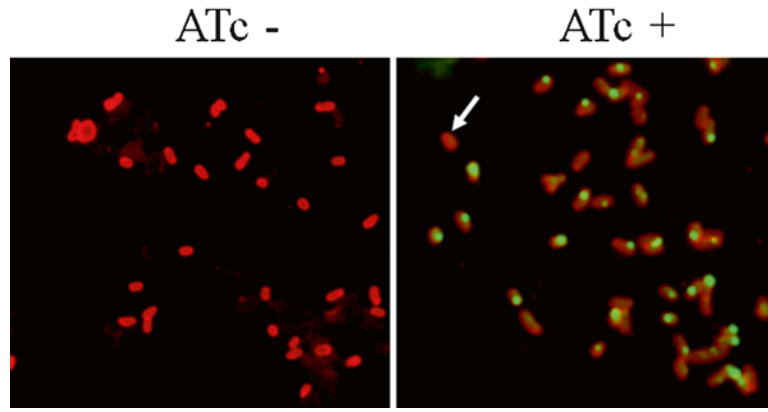

Fig. 1 Intracellular-bacteria-purification protocol renders viable bacteria. HeLa cells were infected with a *B. abortus* 2308 carrying a *gfp* gene under the control of a tetracycline-inducible promoter [21]. After purification, bacteria were readily induced with anhydro-tetracycline (250 nM) for 6 h (ATc+) or left untreated (ATc–). Bacteria were further processed for immunofluorescence using rabbit anti-*Brucella* antibodies (*red*). Notice that live bacteria display both *red* and *green* fluorescence while dead bacteria only shows *red* fluorescence (*arrow*)

down on the drop containing the secondary antibody. Incubate for 30 min at room temperature.

5. On a glass slide put 10 μL of the mounting solution (e.g., Mowiol). With pointed forceps take the coverslip from the secondary antibody and wash the excess of the solution by immersing ten times the preparation on a 50 mL beaker containing 40 mL of PBS. Repeat this step three times on different PBS-containing beakers. With the help of a tissue take away the excess of PBS and put the coverslip upside down on the mounting medium. Analyze the sample with the aid of fluorescence (Fig. 1) and phase contrast microscope in order to detect the structures stained.

3.5 Western Blotting of Purified Bacteria

1. Suspend the purified bacterial pellet in 40 μL of lysis buffer and heat the sample at 95 °C for 20 min. Centrifuge at 10,000 × *g* for 10 min and transfer the supernatant to a 1.5 mL eppendorf tube. Take a 5 μl aliquot to quantify the protein concentration using a detergent-compatible protein quantification kit (*see* **Note 13**).
2. Load between 10 and 20 μg of protein per lane in a SDS-PAGE gel at the desired acrylamide concentrations and separate the proteins at 150 V for 60 min.
3. Transfer the gel to a polyvinyl difluoride membrane (PVDF). Process the membrane by regular immunodetection protocols using the desired antibodies. This protocol allows the detection of several *Brucella* proteins and outer membrane antigens such

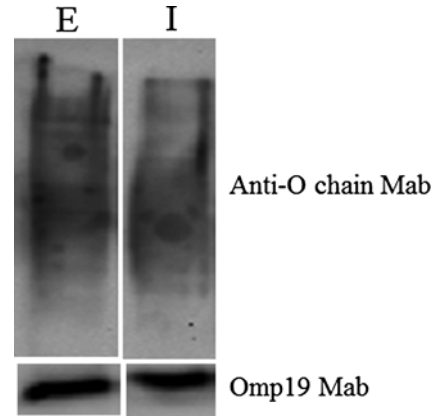

Fig. 2 Antibody detection of molecular determinants from intracellular purified bacteria at the onset of infection. HeLa cells were infected with a *B. abortus* 2308 for 2 h. After this time, intracellular (I) bacteria were purified. Extracellular (E) *B. abortus* 2308 grown in vitro in TSB was used as control. Lysates from intracellular and extracellular bacteria were prepared and total protein quantified. Equal amounts of protein were loaded on 10 % SDS-PAGE and antigens were subsequently transferred to PVDF membranes. The membranes were probed with the indicated primary antibodies and the corresponding conjugates for immunodetection of LPS and Omp19

as Omp19 and LPS (Fig. 2) (*see* **Note 14**). The membrane can be probed as well with antibodies recognizing different proteins from the host cells in order to assess the degree of contamination. Using this procedure it has been determined that the present protocol yields highly purified viable bacteria presenting minor contamination with endoplasmic reticulum proteins and histones [14].

3.6 Detection of RNA Transcripts from Purified Bacteria

1. Determine bacterial concentration of the pellet extracted and purified intracellular bacteria by serial dilution and plate counting. Total amount of bacteria should not exceed a concentration of 10^9 CFU/mL. Resuspend in 100 μL 10 % Zwittergent 3-16 and incubate at 37 °C for 1 h. Add 100 μL TE buffer with 2 μg/μL lysozyme and incubate at 37 °C for 20 min.
2. Extract the RNA using "in column" commercially available kits able to provide high quality RNA. Before loading the column, use a 21 G needle to shear the gDNA. After checking the RNA preparation by agarose gel and measuring concentration and purity, treat one aliquot with Ambion turbo DNAse and determine the concentration again.
3. Perform minus–RT controls by conventional PCR using 15 ng of the RNA preparation. If the controls are negative, proceed with retrotranscription, otherwise perform another round of DNAse treatment.

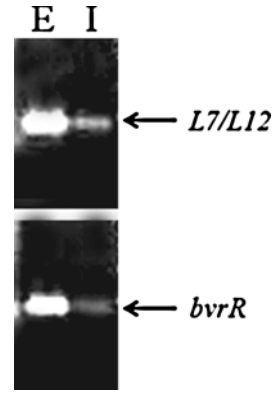

Fig. 3 Detection of mRNA from intracellularly purified bacteria. Total RNA was prepared from extracellular grown bacteria (E) or intracellularly purified bacteria (I). Extracted RNA was subsequently retrotranscribed to cDNA and amplified using specific primers for the indicated genes

4. Retrotranscribe the RNA preparation using commercially available retrotranscriptases, random hexamers, or the primers of interest.
5. Perform PCR using no more than 10 % of the retrotranscribed mix and according to previous standardized conditions (Fig. 3).

4 Notes

1. Before seeding the cells put a 13 mm diameter glass slide in one of the wells. The cells grown and subsequently infected on this slide can be used later on to monitor the efficiency of the infection and intracellular replication by immunofluorescence.
2. The efficiency of bacterial infection is highly dependent on the density of the cell culture. The bacteria initially attach to the junction between the cells and thus at low densities the efficiency of infection is diminished. At very high densities of cells the efficiency of entrance is diminished and, furthermore, the cells will deteriorate rapidly if the purpose of the experiment is to obtain intracellular bacteria after long infection periods (24 or 48 h).
3. This purification protocol was standardized for phagocytic cells (Raw 264.7 macrophages) and non-phagocytic cells (HeLa cells). The latter cell line normally displays a very low brucellae infection rate that rarely surpasses 10 % of infected cells even at very high multiplicity of bacterial infections (MOIs) like the ones indicated in this protocol (2,000 CFU/cell). Since the yield of intracellular material is crucial in order to perform subsequent cell and molecular biology analysis it is

important to increase the initial rate of intracellular bacteria. For this purpose 2 h before infection, HeLa cells can be exposed to 3 ng/mL of purified CNF1 [17]. This treatment will induce a phagocytic behavior on HeLa cells increasing the rate of infection by a factor of 10 through the activation of small GTPases controlling the cytoskeleton and without altering the intracellular trafficking normally followed by the brucellae [15]. The effect of CNF1 on the monolayer can be confirmed using phase-contrast microscopy by the formation of membrane ruffles in the periphery of the cells. Do not preincubate with CNF1 for more than 2 h before infection since this will rather lead to a decreased infection rate [18].

4. To prepare frozen stocks of *Brucella* strike the bacteria in TSA and grow it for 3 days at 37 °C under a 5 % CO_2 atm. Then, inoculate a 125 mL Erlenmeyer containing 20 mL TSB with a dense inoculum from the plate and incubate at 37 °C and 200 rpm for 18 h. At this point, the OD of a 1/10 dilution of the culture measured at 420 nm should be between 0.5 and 0.7. Add sterile glycerol to a final concentration of 10 %, prepare 0.5 mL aliquots on sterile eppendorf tubes, and store a –70 °C.
5. This protocol has been standardized for the fully virulent *B. abortus* 2308 strain [14]. However, the growth curve varies among the different *Brucella* species and strains, and thus, before starting the intracellular purification experiments it is important to precisely determine the corresponding growth curve of the species/strain being analyzed [16]. Once the curve is obtained, run a pilot gentamicin protection cell infection assay using bacterial inoculums at different stages of the growth curve to determine under which conditions the invasion is most successful.
6. Before starting working with *Brucella* is essential that each laboratory standardize a correlation curve between culture OD and bacterial concentration expressed as CFU/mL. For that purpose make a growth curve of the bacterium in 125 mL Erlenmeyer flasks containing 20 mL of TSB and take five aliquots of the culture during different stages of this curve. Measure the OD of each aliquot at 420 nm and determine by serial dilution and plate counting the corresponding bacterial concentration. With these data generate a linear regression curve that allows the simple determination of bacterial concentration from OD readings.
7. Normally for other experiments (e.g., determination of bacterial replication in cells), MOIs 10–20 times lower than those indicated here are used for *B. abortus* 2308. However for isolating intracellular bacteria, especially at early times of infection, much higher MOIs are required. In addition, the MOI to be

utilized during the intracellular-bacteria-purification protocol should be standardized by immunofluorescence in order to reach the maximum efficiency of infection accompanied with the lowest ratio of extracellular/intracellular bacteria. For this purpose pilot experiments should be conducted infecting the cells at different MOIs. Then, in order to determine the intracellular/extracellular bacterial ratio, infected cells should be further processed by differential immunofluorescence using anti-*Brucella* antibodies from two different species [2, 19]. Briefly, infected cells are incubated with the first antibody and the corresponding fluorescently labeled conjugate (e.g., green) before permeabilization. Cells are then fixed, permeabilized, and labeled with the second primary antibody and the corresponding fluorescently labeled conjugate (e.g., red). With this procedure, extracellular bacteria are labeled by both antibodies, whereas intracellular bacteria are only labeled with the second primary antibody. Alternatively, a GFP-*Brucella* may be used in combination with only one anti-Brucella antibody; in this case, fluorescent bacteria replace the use of the second antibody.

8. In a standard replication curve of *B. abortus* in HeLa cells the kinetics should follow a steady bacterial increase following the initial time of infection [19]. In the case of macrophages there is a relatively high initial infection number (time 0) followed by a decrease in bacterial recovery down to 10 % of the original inoculum (time 3–8 h). After these time points there is a subsequent recovery in the bacterial number (24–48 h). Thus, the yield of purified bacteria at early times is considerably lower and more infected 6-well plates might be needed to obtain enough material for subsequent molecular characterization. Particular attention needs to be given to the replication kinetics curve, since activated macrophages (e.g., due to endotoxin contamination) readily kill *Brucella* [20].
9. The efficiency of cell disruption should be monitored microscopically by trypan-blue staining. It is crucial to optimize the release of intracellular bacteria at this point since this has an important impact in the final yield of the purified microorganisms. Other methods for disrupting the cells with higher efficiency are the pressurized-nitrogen chambers or passage through 27-G needles. However, due to the chances of generating aerosols, these procedures should be used with great precaution taking into account biosafety conditions and protocols.
10. When using four 6-well plates for the protocol, the volume usually obtained at this stage is of 1.5 mL. This volume is then reconstituted with 6 mL of the 3.0 mM imidazole–HCl pH 7.4 solution.
11. Particular attention must be paid to the discontinuous gradient formation, avoiding mixing of the different density layers.

For this purpose add each solution slowly through the wall using a long and narrow needle connected to a 1 mL syringe.

12. For some protocols, bacteria may be first fixed in suspension with 3.7 % buffered paraformaldehyde before the drying procedure. This is particularly important when working with GFP-expressing bacteria in order to assess the viability of the isolated microorganisms (Fig. 1). If the bacteria are left to dry first and then fixed, the structure of the GFP will be altered and the fluorescence will be lost.
13. The range of protein concentration varies depending on the cell line and the intracellular time. At early time points (1–4 h) the protein concentration is in the range of 300–600 μg/mL and the total yield of proteins is in the range of 12–24 μg. At later time points (48 h) when the bacteria has reached high intracellular loads the protein concentration is in the range of 1,500–2,000 μg/mL and the total yield of proteins is in the range of 300-400 μg.
14. One of the main purposes of this technique is to compare the response of the bacterium to the intracellular milieu by monitoring changes in the level of proteins, thus, it is crucial to have as control extracellularly grown bacteria. For this purpose take a 1 mL aliquot of the *Brucella* strain used in the experiment grown on TSB and pellet the bacteria by centrifugation. Suspend the pellet in 200 μl of lysis buffer and heat the samples at 95 °C for 20 min to release the bacterial proteins. Centrifuge the suspension at 10,000 × *g* for 10 min and collect the supernatant. Quantify the proteins in the supernantant and load in the SDS-PAGE gel the same amount of protein used for the intracellularly purified bacteria.

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Chapter 15

RNA Sequencing of FACS-Sorted Immune Cell Populations from Zebrafish Infection Models to Identify Cell Specific Responses to Intracellular Pathogens

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Abstract

The zebrafish (*Danio rerio*) is increasingly used as a model for studying infectious diseases. This nonmammalian vertebrate host, which is transparent at the early life stages, is especially attractive for live imaging of interactions between pathogens and host cells. A number of useful fluorescent reporter lines have recently been developed and significant advances in RNA sequencing technology have been made, which now make it possible to apply the zebrafish model for investigating changes in transcriptional activity of specific immune cell types during the course of an infection process.

Here we describe how to sequence RNA extracted from fluorescently labeled macrophages obtained by cell-sorting of 5-day-old zebrafish larvae of the transgenic *Tg(mpeg1:Gal4-VP16);Tg(UAS-E1b:Kaede)* line. This technique showed reproducible results and allowed to detect specific expression of macrophage markers in the mpeg1 positive cell population, whereas no markers specific for neutrophils or lymphoid cells were detected. This protocol has been also successfully extended to other immune cell types as well as cells infected by *Mycobacterium marinum*.

Key words RNA sequencing, FACS, Immune cells, Zebrafish larvae dissociation, Transcriptome analysis

1 Introduction

Infection is associated with complex changes in gene expression patterns of both host and pathogen [1]. An insight into these transcriptional programs can help in identifying new virulence determinants and mechanisms of host defense. The development of genome-wide RNA sequencing (RNAseq) over the last 5 years has revolutionized our approach of transcriptomics [2]. RNAseq consists of a massively parallel sequencing of cDNA obtained from a RNA sample. The millions of sequences obtained (called reads) are then mapped onto a reference sequence in order to assess the presence and the expression level of a transcript in the sample.

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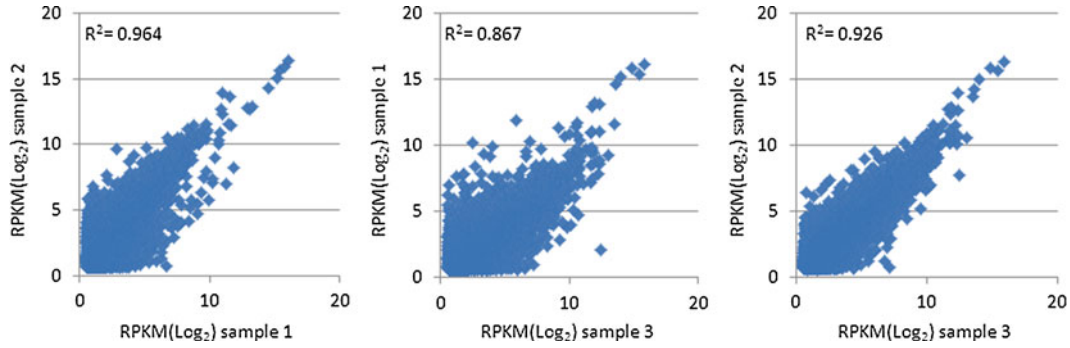

Fig. 1 Reproducibility between three biological replicates. The square of the Pearson correlation coefficient (R^2) was calculated between each replicate sample obtained from fluorescence positive cells based on RPKM (read count per kilobase per million mapped reads) values of genes detected in both replicates (i.e., RPKM >0.5 in both samples). A scatter plot is used to represent gene counts (i.e., Log_2 RPKM values) for replicate samples. Gene count reproducibility was remarkably high considering the numerous steps required to obtain RNAseq libraries from FACS sorted zebrafish larvae. Nevertheless, variation between samples (for example samples 1 and 3 show lower reproducibility than the other combinations) indicates that at least three replicates are required to obtain good statistical results

cDNA read lengths of over a hundred nucleotides can now routinely be obtained with the use of paired-end technology to link the ends of short cDNA fragments [3]. Thus, RNAseq has been described as a powerful method to characterize transcriptional landscapes and discover novel transcripts or alternative splice forms [4]. RNAseq has also proved to be an accurate method for quantitative analysis of differential gene expression [5]. With the development of efficient cDNA synthesis and library preparation protocols, it is now possible to determine the transcriptome of very small populations of cells or even of single cells [6–9].

The zebrafish has recently emerged as a nonmammalian vertebrate model to study host–pathogen interactions, providing many versatile tools for genetics and intravital imaging [10–13]. Zebrafish infection models have been developed for a number of intracellular pathogens, such as *Burkholderia*, *Listeria*, *Mycobacterium*, *Salmonella*, and *Staphylococcus* species [14–18]. Tag-based and full mRNA sequencing analyses have already been used to study the transcriptome of adult zebrafish or embryos in response to pathogen challenge [19–23]. However, until now these studies were limited to determining the immune response at whole organism or organ level. The development of fluorescent reporter lines for different immune cell types [24–28], together with the latest advances in RNAseq technologies, has now made the sequencing of specific immune cell populations in zebrafish feasible.

Here we describe a protocol to sequence the transcriptome of macrophages obtained by cell-sorting of 5 day-old *Tg(mpeg1:Gal4-VP16);Tg(UAS-E1b:Kaede)* transgenic zebrafish larvae [24]. With this protocol, we succeeded in obtaining three reproducible replicates of the fluorescence positive macrophage transcriptome (Fig. 1).

Differential analysis of gene expression between fluorescence positive and fluorescence negative cells resulted in the detection of several known macrophage markers (e.g., *mpeg1*, *mhc2dab*, *mfap4*, *csf1ra*, *marco*, *irf8*), general myeloid markers (e.g., *spi1*), and pan-leukocytic markers (e.g., *coro1a*, *ptprc*, *ptpn6*), whereas no markers specific for neutrophils (e.g., *mpx*) or lymphoid cells (e.g., *lck*, *rag1*, *rag2*) were detected in the enriched pool of fluorescence positive cells.

With this protocol, we have also succeeded in determining the transcriptome of neutrophils and pre-lymphoid cells obtained by cell-sorting of respectively 5 day-old Tg(*mpx:eGFP*) [27] and Tg(*lck:eGFP*) transgenic larvae [26] (data not shown). Furthermore, this protocol has successfully been used to sort and sequence the transcriptome of infected cells obtained from AB/TL larvae infected with *Mycobacterium marinum* expressing a mCherry fluorescence marker (unpublished data) and it is currently being used in our laboratory to investigate the transcriptional reprogramming of macrophages during different stages of *M. marinum* infection. The next challenge in RNAseq analyses for host–pathogen interaction studies is the sequencing of both host cell and pathogen transcriptomes simultaneously. The development of the third generation sequencing platforms could make this so-called dual RNAseq feasible within the next year [1]. In this chapter we describe how to dissociate larvae by trypsin treatment and sort fluorescently labeled cells from the resulting single cell suspension by FACS. Subsequently, we explain how RNA extracted from these sorted cells can be used to prepare libraries for RNAseq. Furthermore, we describe the main steps required to analyze our RNAseq results in order to show the reproducibility and relevance of the data. However, the primary aim of this protocol is not to describe a step-by-step method to analyze RNAseq results. Several papers have described how to align and map RNAseq results with different software [29, 30]. Other papers describe or compare several ways to perform a differential analysis to compare gene expression levels across different samples [31].

2 Materials

2.1 Cell Dissociation and FACS Sorting Components

1. Embryos from the zebrafish AB/TL control line 5 days post-fertilization (*see* **Note 1**).
2. Embryos from the transgenic zebrafish reporter line *Tg(mpeg1:Gal4-VP16)^{gl24};Tg(UAS-E1b:Kaede)^{s1999t}* 5 days post-fertilization [24].
3. Incubator (28.5 °C).
4. Egg water: "Instant Ocean" Sea Salts 60 μg/mL. 0.2 mM Phenylthiourea (PTU) (*see* **Note 2**).
5. Calcium-free Ringer solution: 5 mM HEPES (pH 7.2), 2.9 mM KCl, 116 mM NaCl.

6. Dissociation solution: Trypsin 0.25 % supplemented with 1 mM EDTA (Gibco®) (*see* **Note 3**).
7. Fetal calf serum (FCS) 100 % inactivated by heating for 30 min at 56 °C in a water bath with mixing.
8. $CaCl_2$ 0.8 M.
9. Dulbecco's Phosphate Buffered Saline (DPBS) 1×.
10. Resuspension solution: Leibovitz's L-15 medium + L-Glutamine without Phenol Red, FCS 10 %, 0.8 mM $CaCl_2$, penicillin 50 U/μL, streptomycin 0.05 mg/mL.
11. Sterile disposable 50 μm filters adaptable on Falcon tubes.
12. BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) with the BD FACSDiva software (version 6.1.3).
13. Cell collection solution: Leibovitz's L-15 medium + L-Glutamine without Phenol Red, FCS 10 %, zebrafish embryo extract 10 % (*see* **Note 4**), 0.8 mM $CaCl_2$, penicillin 50 U/μL, streptomycin 0.05 mg/mL.
14. 35 mm culture dishes.

2.2 RNA Extraction and Library Preparation Components

1. RNAqueous®-Micro Kit (Ambion®).
2. 100 % ethanol, ACS grade or better.
3. RNase-free low retention microcentrifuge tubes.
4. RNase-free filtered pipette tips.
5. Heating blocks at 75 and 37 °C.
6. Refrigerated microcentrifuge capable of at least 13,600 × g.
7. Agilent Bioanalyzer 2100, RNA 6000 Pico kit, DNA 1000 kit, and High sensitivity DNA kit (Agilent, Santa Clara).
8. Centrifugal Evaporator.
9. Clontech SMARTer™ Ultra Low RNA Kit for Illumina Sequencing (Clontech).
10. Agencourt AMPure XP (Beckman Coulter).
11. Magnetic rack for 1.5 mL microcentrifuge tubes.
12. PCR machine, and qPCR machine.
13. Covaris S220 Focused-ultrasonicator.
14. Illumina Truseq DNA Sample Preparation Kit v2 (Illumina Inc., San Diego, USA).
15. KAPA Library Quantification Kit (KAPA Biosystems).
16. Sequencing facility equipped with a HiSeq 2000 (Illumina Inc., San Diego, USA).
17. A set of software for RNAseq read analysis (*see* **Note 5**).

3 Methods

3.1 Cell Dissociation and FACS Sorting

1. Collect 150–200 5-day-old larvae grown in egg water in an incubator set up at 28.5 °C and transfer them into a 35 mm culture dish (*see* **Note 6**).
2. Rinse the larvae in 3 mL of calcium-free Ringer solution for 15 min.
3. Carefully remove as much as possible calcium-free Ringer solution and add 2–3 mL of dissociation solution pre-warmed at 28.5 °C.
4. Incubate for 90 min at 28.5 °C in an incubator. During incubation, grind up the larvae by pipetting up and down with a 1 mL tip for 10 min (*see* **Notes 7–9**).
5. Stop the reaction by adding $CaCl_2$ to a final concentration of 1 mM and fetal calf serum to 10 % (*see* **Note 10**).
6. Transfer cells into a microcentrifuge tube and centrifuge for 3 min at 800 × g (*see* **Note 11**).
7. Rinse the cells in 1 mL of DPBS and centrifuge again for 3 min at 800 × g.
8. Resuspend the cells in 1–1.5 mL of resuspension solution to obtain a concentration of 10^7 cells/mL (*see* **Note 12**).
9. Place a sterile disposable 50 μm filter on a 15 mL tube and load the cells onto it.
10. When all the liquid has passed through the filter, put the 15 mL cell-containing tube on ice and immediately proceed to cell sorting (*see* **Note 13**).
11. Subject the cell suspension to FACS for 20–30 min at 4 °C and collect the different cell fractions in microcentrifuge tubes containing 200 μL of cell collection solution (*see* **Notes 14** and **15**).
12. After cell sorting, the cells are kept on ice. Proceed to RNA extraction as soon as possible (*see* **Note 16**).

3.2 RNA Extraction, Library Preparation and RNA Sequencing

1. Pellet the cells by centrifugation at 13,000 × *g* for 4 min and proceed to RNA extraction.
2. For RNA extraction, use the RNAqueous®-Micro Kit and proceed according to the manufacturer's protocol.
3. At the end of the procedure, RNA extracted from nonfluorescent cells is resuspended in a final volume of 10 μL.
4. Before cDNA synthesis, remove DNA contaminants by DNase treatment using the DNase provided in the RNAqueous®-Micro Kit.

5. After DNase treatment, transfer RNA into low retention microcentrifuge tubes and store at −80 °C (*see* **Note 17**).
6. Before cDNA synthesis, measure RNA quantity and quality with an Agilent Bioanalyzer 2100 and RNA 6000 Pico kit (*see* **Note 18**).
7. If the RNA concentration is lower than 1,000 pg/μL, concentrate to a final volume of 1 μL using an Eppendorf Vacufuge set at 30 °C for approximately 10 min (*see* **Note 19**).
8. For each RNA sample, synthesize cDNA using the Clontech SMARTer Ultra Low RNA Kit for Illumina Sequencing according to the manufacturer's protocol (*see* **Note 20**).
9. Purify cDNA with AMPure XP beads according to the SMARTer Kit manual.
10. Verify quality and quantity of the cDNA syntheses by running 1 μL on an Agilent Bioanalyzer 2100 using the High sensitivity DNA kit (*see* **Note 21**).
11. Shear the amplified cDNA with a Covaris S220 system using microTUBE's and the settings recommended in the SMARTer Kit manual.
12. For each cDNA sample carry out library preparation with the Illumina TruSeq DNA Sample Preparation Kit v2 according to the manufacturer's protocol (*see* **Note 22**).
13. Verify library quality by running 1 μL of the libraries on an Agilent Bioanalyzer using the DNA 1000 kit (*see* **Note 23**).
14. Quantify the number of amplifiable molecules in the libraries using the KAPA Library Quantification Kit (KAPA Biosystems) (*see* **Note 24**).
15. Sequence the libraries on an Illumina HiSeq 2000 to obtain the desired number of paired end reads with a read length of 50 nucleotides (*see* **Notes 25** and **26**).

3.3 Data Analysis and Quality Assessment

1. Perform a quality trimming of the raw sequencing reads obtained from the CASAVA pipeline (Illumina Inc.) (*see* **Note 27**).
2. Align and map the uniquely mapping reads on the reference (*see* **Note 28**).
3. Check the reproducibility of biological replicates by calculating the square of the Pearson correlation coefficient (R^2) between all the counts or RPKM (read count per kilobase per million mapped reads) values from genes detected in both replicates (*see* **Notes 29** and **30**).
4. Perform differential expression analysis to detect significantly upregulated and downregulated genes in the fluorescence positive cells compared with the negative background (*see* **Note 31**).

4 Notes

1. Working with embryos and early larval stages does not require animal experimentation authorization. However, manipulation of larvae that have reached the free feeding stage and husbandry of adult fish require proper animal experimentation authorization according to standard regulations in each country.
2. Pigmentation of the larvae does not interfere with fluorescent-based cell sorting. If required, 0.2 mM Phenylthiourea (PTU) can be added to the egg water in order to prevent melanization and allow screening of transgenic larvae. However, one should notice that addition of PTU in egg water can interfere with biological functions [32, 33].
3. Trypsin reagents form different suppliers were tested for dissociation of zebrafish larvae. We obtained the best dissociation efficiency and cell survival with Trypsin 0.25 % supplemented with 1 mM EDTA from Gibco®.
4. Stock of zebrafish embryo extract obtained from 200 AB/TL larvae was prepared according to the protocol from the zebrafish book [34].
5. Various software programs, charged or free, are available. We have used Illumina HCS version 1.15.1 for image analysis and base calling, CLCbio Assembly Cell v4.0.6 for quality trimming of sequence reads and mapping of filtered reads to Ensembl transcripts, and the DEseq package (version 1.8.3; [35]) available in Bioconductor (version 2.10) for analysis of differential gene expression between fluorescence positive and negative cells. Computer analyses will not be explained in detail in this protocol because it requires a complete bioinformatic protocol to explain each step of the analysis.
6. The number of larvae one needs to collect depends on the number of fluorescent cells expected to be collected after FACS sorting. With the *Tg(mpeg1:Gal4-VP16);Tg(UAS-E1b:Kaede)* line, 0.1 % of the cells are fluorescence positive and using 200 fish allows to obtain an average of 6,000 cells.
7. Incubation time depends on the number of larvae and also their age. An incubation time of 90 min at 28.5 °C works well with 150–200 larvae collected 5 or 6 days post-fertilization. If more than 200 embryos need to be dissociated simultaneously, sample should be separated into two different culture dishes filled up with 2 mL Trypsin 0.25 % each.
8. A dissociation time too short leads to a decrease in the number of cells obtained after FACS sorting. A dissociation time too long is harmful for the cells. In order to be sure to have a perfectly homogenous mixture of single cells, it is particularly

important to disintegrate cell aggregates that may form during incubation at 28.5 °C.

9. The influence of trypsin treatment on the transcriptome is difficult to assess. In order to minimize artefactual signal, dissociation of all the samples should be performed under the same conditions (number of larvae, dissociation time, trypsin reagent batch …).
10. Stop the reaction when the solution appears as fluid as water. One can also assess the dissociation by observing a mainly single cell suspension under a microscope.
11. One should observe a pellet of cells with a silvery color. If there are too many cells, some of them will remain in the supernatant. In this case, centrifuge the supernatant again and collect the remaining cells.
12. A cell count has been performed during the first experiments. Based on these results we found that resuspending dissociated cells from 200 5-day-old larvae in 1 mL allows obtaining the desired concentration of 10^7 cells/mL.
13. This step allows collecting only dissociated single cells. If the cells are not well dissociated, the filter could become blocked. If this happens, shaking the filter carefully may help the liquid pass through the filter. If this does not work, load the remaining liquid on a new filter.
14. We have used a FACSAria III (BD Biosciences) with the BD FACSDiva software (version 6.1.3). To sort Kaede green positive cells a Coherent Sapphire solid-state 488 nm laser with 15.4 mW power was used. Laser settings applied were 505 LP, 530/30 BP. In order to set up sorting gates, we have previously sorted single cell suspensions from 5 dpf AB/TL control larvae obtained with the same protocol. Gates are set up in order to exclude all autofluorescent cells. Sorting of additional cell suspensions showed that no more than 10–30 false positive cells are sorted per 150 AB/TL larvae with these predefined gates. In contrast, we were routinely able to obtain more than 6,000 positive cells from 150 *Tg(mpeg1:Gal4-VP16);Tg(UAS-E1b:Kaede) larvae*.
15. As many cells as possible are collected for the fluorescent populations and a maximum of 500,000 cells for the negative fraction. Cell collection solution allows cell survival [36]. However, FACS should not be performed for more than 30 min per sample in order to avoid damaging the cells.
16. The purity of sorted cells can be assessed by fluorescence microscopy and by performing cytospin preparations followed by Giemsa staining of cells. For cytospin analysis, cells should be maintained in culture for a few hours after FACS because

cells adopt a round-shaped morphology after trypsin treatment making it impossible to differentiate the immune cell types.

17. Transferring RNA into low retention microcentrifuge tubes is useful to avoid RNA loss by binding to plastic tubes. This is particularly relevant for low concentrated RNA kept for several weeks at −80 °C.
18. RNA quality is reflected by the RNA Integrity Number (RIN) provided at the end of the run on the bioanalyzer. RNA samples with a RIN comprised between 7 and 10 are usually requested by sequencing platforms. Low RNA concentrations (<200 pg/μL) can sometimes not be estimated with the bioanalyzer and therefore no RIN is associated with these samples. Nevertheless, if the peaks corresponding to ribosomal RNA are detected, these samples can be used to obtain a good quality library as shown below (Fig. 2).
19. Sometimes a concentration step may be harmful for RNA. If a degradation of RNA is noticed during this step, adding RNase inhibitors has been described as an efficient way to reduce RNA degradation [37].
20. A protocol has been recently described to perform cDNA synthesis with the Clontech SMARTer Ultra Low RNA Kit for Illumina Sequencing followed by library sequencing with one cell only [6, 8]. With this protocol, a unique cell is lysed directly in cDNA synthesis buffer. It seems likely than this protocol could be further adapted for a small number of cells obtained by FACS or microdissection.
21. A good amplification product should produce an electropherogram with an increase in fluorescence intensity comprised between 400 and 10,000 bp, with a maximum intensity at 1,000–2,000 bp (Fig. 2a, b). A signal detected between 0 and 250 bp indicates the presence of poly-A stretches. If poly-A stretches are present but in minority compared to the amount of amplified cDNA, the sample can be used for library synthesis. Absence of fluorescence between 400 and 10,000 bp indicates that synthesis of cDNA has failed, whereas the presence of several discrete peaks suggests a contamination of the sample. If the synthesis of cDNA fails, carefully check that no degradation of RNA has occurred when concentrating the RNA. For more information one can refer to Clontech SMARTer™ Ultra Low RNA Kit for Illumina Sequencing handbook.
22. Compared to the manufacturer's protocol, only two modifications have been made. In the adapter ligation step the adapters were diluted 20-fold, and in the amplification step fifteen cycles were used instead of ten. These modifications have been found to increase efficiency of cDNA synthesis.

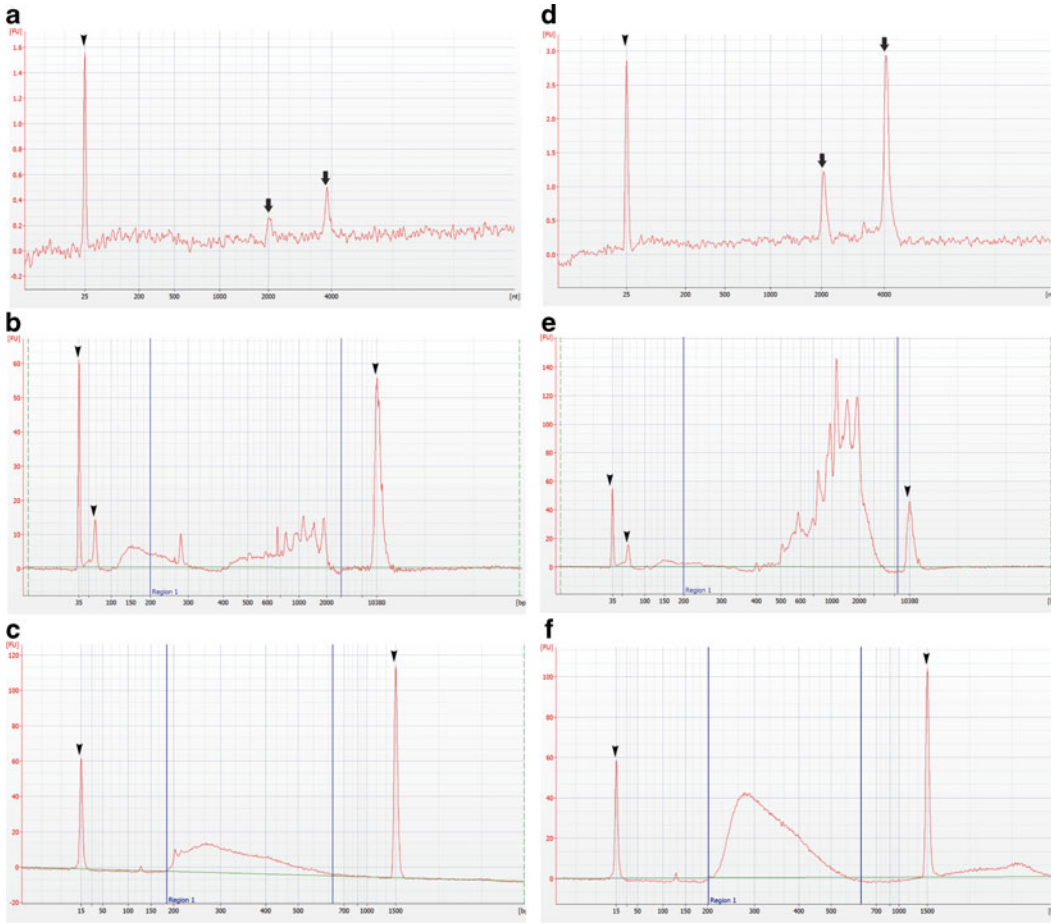

Fig. 2 Electropherograms of extracted RNA, amplified cDNA, and final libraries. Each step of the library preparation is checked by running 1 μL on an Agilent Bioanalyzer 2100. *X*-axis represents RNA or DNA fragment size in nucleotides (nt) or base pair (bp), respectively. *Y*-axis represents intensity of fluorescence detected (FU). Representative results are shown for two RNA samples obtained from *Tg(mpeg1:Gal4-VP16);Tg(UAS-E1b:Kaede)* positive cell fractions. (**a**–**c**) Results obtained during preparation of sample 1. (**d**–**f**) Results obtained during preparation of sample 2. (**a**, **d**) Electropherograms of RNA obtained after extraction from FACS sorted cells with the RNAqueous®-Micro Kit after DNase treatment. (**b**, **e**) Electropherograms of amplified cDNA obtained with Clontech SMARTer™ Ultra Low RNA Kit for Illumina Sequencing after 15 amplification cycles. (**c**, **f**) Electropherograms of libraries obtained after library preparation from the corresponding cDNA using the Illumina TruSeq DNA Sample Preparation Kit v2. RNA concentrations for samples 1 and 2 are 47 and 144 pg/μL, respectively. In both cases the presence of ribosomal RNA peaks in the electropherograms (*arrows* in **a**, **d**) indicated the integrity of the RNA sample, but a RIN value failed to be calculated. These low amounts of RNA from fluorescence positive cells gave rise to cDNA preparations with a broad size distribution (areas in between the *blue lines* in **b** and **e**) and Illumina TruSeq libraries (areas in between the *blue lines* in **c** and **f**) that were successfully applied to RNAseq with a HiSeq 2000 system. With these libraries, we obtained 10 million reads per sample, which was sufficient to detect an average of 11,000 expressed genes (with RPKM values $\geq$0.5) in fluorescence positive cells on a total of 27,882 genes present in our reference. Peaks indicated by *arrowheads* at the start (**a**–**f**) and end (**b**, **c**, **e**, **f**) of the electropherograms are size markers used for calibration of the Bioanalyzer

23. A good library preparation gives a fluorescence absorption distributed between 200 and 600 bp with a maximum intensity around 200–300 bp. Absence of fluorescence between 200 and 600 bp indicates a failure in library preparation whereas a peak of fluorescence shifted either to the low or high DNA size reveals too much shearing or not enough shearing of cDNA, respectively.
24. Library quantification is primordial to load the optimal amount of DNA into the sequencing flow cell and thus achieving an optimal sequencing result.
25. Sequencing 10 million paired end reads per sample was sufficient to detect differential expression of macrophage markers (e.g., *mpeg1*, *mhc2dab*, *mfap4*, *csf1ra*, *marco*, *irf8*) between fluorescence positive and fluorescence negative cells. However, more reads (100 million) are recommended to detect rare transcripts and to analyze alternative transcription start sites, splicing or polyadenylation.
26. Library preparation and sequencing (**steps 6–15**) are often carried out by sequencing facilities. However, library preparation from RNA extracted from FACS sorted cells differs from regular protocols and should be carefully discussed with the sequencing facility.
27. For this analysis we used the quality trim option available in CLC Assembly Cell v4.0.6 beta (CLC bio, Denmark) with standard settings. Alternatively, one can use the Filter FASTQ option freely available in the Galaxy pipeline [38].
28. To align and map reads we used CLC Assembly Cell 4.0.6 beta (CLC bio, Denmark). Reads were mapped to Ensembl transcripts (Zv9_63) using the clc_ref_assemble_short module. Transcripts were then accumulated to their corresponding Ensembl gene using the assembly_table module. Reads belonging to the same gene were finally summed together using a custom perl script. Alternatively one can use other aligners such as Bowtie [39] or TopHat [40] and the genomic databases available on Ensembl.
29. The square of the Pearson coefficient (R^2) reflecting the linear correlation between the RPKM of two samples is used to assess the reproducibility of these two independent experiments. Our results gave a R^2 higher than 0.9 for the reads obtained from the fluorescence negative cells. For the fluorescence positive cells, of which the number of cells obtained after FACS is low, R^2 values between 0.867 and 0.964 were obtained (Fig. 1). Thus, our results show good correlation, but should be reproduced at least in three replicates in order to obtain significant results with the differential expression analysis.

30. We noticed that incorporating nonunique mapped reads in our analysis dramatically decreased the Pearson correlation coefficient between our replicates. Thus, we strongly recommend working with unique mapped reads.
31. To perform statistical analyses we used the R package DESeq [35] and selected genes upregulated or downregulated more than twofold with an adjusted *p*-value smaller than 0.1. Alternatively, differential analyses can be performed with other R packages such as EdgeR [41] or Bayseq [42].

Acknowledgments

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Chapter 16

Taking the Shortcut for High-Throughput Shotgun Proteomic Analysis of Bacteria

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Abstract

Currently, proteomic tools are able to establish a complete list of the most abundant proteins present in a sample, providing the opportunity to study at high resolution the physiology of any bacteria for which the genome sequence is available. For a comprehensive list, proteins should be first resolved into fractions that are then proteolyzed by trypsin. The resulting peptide mixtures are analyzed by a high-throughput tandem mass spectrometer that records thousands of MS/MS spectra for each fraction. These spectra are then assigned to peptides, which are used as evidence of the existence of proteins. In addition to generating a list of protein identifications, this shortcut to proteomics uses the number of spectra recorded for each protein to quantify the observations. Here, we describe one of the most simple sample preparation methods for high-throughput proteomics of bacteria, as well as the subsequent data processing to extract quantitative information based on the spectral count approach.

Key words High-throughput proteomics, Quantitative proteomics, Bacterial proteome, Spectral count, Tandem mass spectrometry

1 Introduction

Proteomic analyses are an integral part of microbiology, giving unparalleled insights into microbial physiology, metabolic pathways and their regulation, posttranslational modifications, and pathogenic bacteria–host interactions [1–4]. Such analyses are especially useful in discovering biomarkers for pathogenesis and determining novel mechanisms of antibiotic resistance [2]. Using a combination of electrophoresis, chromatography, and mass spectrometry, it is possible to enumerate hundreds of the most abundant proteins in a bacterial culture. To accomplish this feat, cells are lysed and their contents are either analyzed directly by mass spectrometry or first resolved using gel electrophoresis in denaturing conditions. In both cases, the purified proteins are digested using trypsin into predictable peptide fragments prior to mass

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spectrometric analysis. The peptides are separated on the basis of hydrophobicity using a reverse phase chromatographic column, and their molecular weight is estimated by mass spectrometry. The peptides are further fragmented in the mass spectrometer, and the molecular weight of the fragments is then established by tandem mass spectrometry. The chromatographic retention time, peptide mass, and MS/MS spectra are then used to identify the peptide sequences from all the possible theoretical peptides in a database. The peptides can then be mapped back to proteins. Computation of the spectral count associated with each peptide [5], normalized by the length of the protein [6] or by its molecular weight [7], allows the comparison of relative abundances of proteins between samples. This shortcut does not apply to compare a given protein to another within a sample because each of their peptides has its own ionization potential. However, this technique appears quite robust when considering large groups of proteins.

Many variations in sample preparation are possible, all with their respective advantages and disadvantages. The method outlined here is, in our experience, the minimum amount of processing to produce the maximum amount of useful data. In addition to sample preparation and detection, quantitation can also be accomplished in a variety of ways. The label-free method called "spectral counting" performs well in comparison with other semiquantitative methods [8] relying on chemical or isotopic labeling, without the added time or cost. This "shortcut" to shotgun proteomics is therefore an efficient method for high-throughput analysis of bacterial proteomes when extracting the main differences between samples is required.

Using the method detailed below, we have explored bacterial proteomes [7, 9, 10], exoproteomes from diverse sources [11–13], and nucleoid proteins [14]. We routinely observe thousands of peptides per run when using a state-of-the-art tandem mass spectrometer, identifying hundreds of proteins. We have confidently ($p < 0.05$–0.01) detected changes in protein abundance as low as 1.5–2.0-fold within a reasonable range of false discovery rates.

2 Materials

Prepare all solutions using ultrapure water. Reagents used for mass spectrometry must be of analytical grade. We recommend working with small aliquots and preparing fresh reagents regularly to avoid contamination.

2.1 Harvesting Bacterial Cultures

1. Bacterial culture.
2. Centrifuge. Typically, any centrifuge for eppendorf tubes operated at high speed will be appropriate, but if large culture

volumes need to be harvested (low density cells), the most appropriate centrifuge for the tube size should be used.

3. Wash buffer: 50 mM Tris–HCl. Store at 4 °C.
4. Lithium dodecyl sulfate (LDS) buffer 4×: 40 % glycerol, 4 % LDS, 4 % Ficoll-400, 0.8 M triethanolamine-Cl pH 7.6, 0.025 % phenol red, 0.025 % Coomassie G250, 2 mM EDTA disodium.
5. Balance.
6. Ultrasonic probe.

2.2 SDS PAGE

1. Protein gel sample buffer: Dilute 1 mL of LDS sample buffer (4×) in 2.8 mL of water. Add 200 μL of β-mercaptoethanol. Store aliquots at −20 °C. Allow to reach room temperature before use.
2. Polyacrylamide gels: e.g., 4–12 % Bis–Tris gradient 10-well gels. Store at 4 °C.
3. Electrophoresis system.
4. PAGE running buffer: Dilute 100 mL of 20× MES in 1.9 L of water. Store at 4 °C.
5. Coomassie Blue stain.
6. Heating block.
7. Loading tips or syringe.
8. Ultrapure water to rinse gel.

2.3 Reduction, Alkylation, and Digestion of Proteins

1. Clean scalpel or razor blade.
2. Orbital Shaker.
3. Destain solution: Mix 10 mL of methanol with 10 mL of 50 mM ammonium bicarbonate.
4. Dehydration solution: Mix 10 mL of acetonitrile with 10 mL of 50 mM ammonium bicarbonate (*see* **Note 1**).
5. Pure acetonitrile.
6. SpeedVac.
7. Deionized water.
8. Reduction solution: Weigh 38.5 mg of dithiothreitol in a 15-mL centrifuge tube. Add 10 mL of 50 mM ammonium bicarbonate.
9. Alkylation solution: Weigh 102 mg of iodoacetamide in a 15-mL centrifuge tube. Add 10 mL of 50 mM ammonium bicarbonate. Store in the dark.
10. Enzyme solution: Reconstitute lyophilized sequencing-grade trypsin to a final concentration of 0.1 μg/μL in0.01 % trifluoroacetic acid. Reconstituted enzyme can be aliquoted and

stored at −20 °C for several months or at 4 °C for up to 1 week (*see* **Note 2**).

11. Trypsin enhancer solution: Reconstitute 1 mg lyophilized ProteaseMax (Promega) with 100 μL of 50 mM ammonium bicarbonate (*see* **Note 3**). The resulting solution contains 1 % ProteaseMax and should be aliquoted and stored at −20 °C.
12. Digestion solution: 16 μL of 50 mM ammonium bicarbonate, 2 μL of 0.1 % ProteaseMax, and 2 μL of 0.1 μg/μL trypsin (20 μL total volume) per gel piece. Keep on ice until use.
13. Ice.
14. Heating block—37 ° C, 56 ° C.
15. Trifluoroacetic acid: 5 % stock solution in water. Prepare 0.5 and 0.1 % solutions in water.

2.4 nanoLC-MS/MS

1. Mass spectrometer: LTQ Orbitrap XL (ThermoFisher) coupled to an UltiMate 3000 LC system (Dionex).
2. Reverse-phase Acclaim PepMap100 C18 μ-precolumn (5 μm, 100 Å, 300 μm i.d. × 5 mm, Dionex-ThermoFisher).
3. Nanoscale Acclaim PepMap100 C18 capillary column (3 μm, 100 Å, 75 μm i.d. × 15 cm, Dionex).
4. LC solvents: 0.1 % formic acid (A) and 0.1 % formic acid, 80 % acetonitrile (B) in water.

2.5 Identification

1. Mascot Daemon software (version 2.3.2, Matrix Science).
2. IRMa 1.30.4 software.

2.6 Quantitation

1. Microsoft Excel and PatternLab v2.1.1.12 software.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Be sure to take adequate precautions (use gloves, clean surfaces with ethanol) to avoid keratin contamination.

3.1 Harvesting Bacterial Cultures

1. Centrifuge for 5–15 min at 2,000–6,000 × *g* at 4 °C in a pre-weighed centrifuge tube (*see* **Note 4**).
2. Wash as briefly as possible 1–2 times with cold Tris–HCl. Remove residual washing liquid after a brief centrifugation. Weigh the wet cell pellet (*see* **Note 5**).
3. Resuspend directly in 200 μL of 1× LDS buffer (*see* **Note 6**).
4. Sonicate briefly (approximately 1 min at 30 % amplitude).

3.2 SDS-PAGE

1. Dilute the sample 1:10 in 1× LDS buffer (*see* **Note 7**).
2. Heat at 95 °C for 5 min in a heating block.

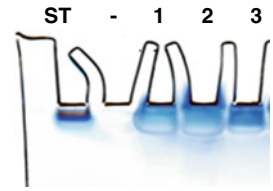

Fig. 1 SDS-PAGE gel for a one-shot shotgun proteomic analysis. A standard (here: SeeBlue Plus2 marker from Invitrogen, ST) and three samples (1, 2, and 3, respectively) have been loaded and run for a few minutes. Note that the pre-stained protein markers are not fully resolved by this procedure. The proteins included in the gel are stained with SimplyBlue SafeStain and will be excised as a single polyacrylamide band for in-gel trypsin proteolysis. The wells and the resolving gel are clearly delineated

3. Load 20 µL onto a polyacrylamide gel (*see* **Note 8**).
4. Run at 200 V for approximately 5 min or until all of the sample has entered into the gel but has not progressed more than 10 mm (*see* Fig. 1).
5. Rinse the gel three times with water.
6. Stain with Coomassie Blue for 30–60 min.
7. Rinse well with water (*see* **Note 9**).

3.3 Reduction, Alkylation, and Digestion

1. Excise bands of interest using a clean scalpel or razor blade and transfer to eppendorf tube (*see* **Note 10**).
2. Destain with 200 µL of methanol:ammonium bicarbonate, shake for 1 min at 500 rpm, and discard the fluid. Repeat this step once (*see* **Note 11**).
3. Dehydrate with 200 µL of acetonitrile–ammonium bicarbonate, shake for 5 min at 600 rpm, and discard the fluid.
4. Dehydrate with 200 µL of pure acetonitrile, shake for 1 min at 600 rpm, and discard the fluid.
5. Dry in a SpeedVac for 2–5 min.
6. Rehydrate the gel piece(s) with 100 µL of reduction solution; incubate for 20 min at 56 °C, shaking at 500 rpm. Discard the fluid.
7. Add 100 µL of alkylation solution; incubate for 20 min at room temperature in the dark. Discard the fluid.
8. Wash with 400 µL of deionized water; shake for 1 min at 600 rpm; discard the fluid. Repeat this step once (*see* **Note 12**).
9. Dehydrate with 200 µL of acetonitrile–ammonium bicarbonate, shake for 5 min 600 rpm, and discard the fluid.
10. Dehydrate with 200 µL of pure acetonitrile, shake for 1 min at 600 rpm, and discard the fluid.
11. Dry in a SpeedVac for 2–5 min.

12. Rehydrate the gel piece(s) with 20 μL of enzyme solution, and incubate for 20 min on ice. Remove excess liquid.
13. Add 50 μL of 0.01 % ProteaseMax; shake briefly.
14. Incubate for 4 h at 37 °C (*see* **Note 13**).
15. Transfer the solution to a clean tube (or well; *see* **Note 10**) and add 5 μL of 5 % trifluoroacetic acid. If the recovered volume is less than 50 μL, which is often the case for larger gel pieces, add 0.1 % trifluoroacetic acid equivalent to the lost volume, shake for 5 min at 500 rpm, and pool the solution with the previously recovered volume (*see* **Note 14**).

3.4 nanoLC-MS/MS

Settings and conditions are described for the LTQ Orbitrap XL (ThermoFisher) coupled to an UltiMate 3000 LC system (Dionex) equipped with a reverse-phase Acclaim PepMap100 C18 μ-precolumn (5 μm, 100 Å, 300 μm i.d.×5 mm, Dionex-ThermoFisher) followed by a nanoscale Acclaim PepMap100 C18 capillary column (3 μm, 100 Å, 75 μm i.d.×15 cm, Dionex).

1. Load 1–10 μL (maximum volume allowed by the system) of the acidified peptide mixture and resolve over a 30 min linear gradient from 5 to 60 % solvent B using a flow rate of 0.3 μL/min. Adjust the loading volume as a function of the total current measured by the mass spectrometer to avoid saturating the detector.
2. Collect full-scan mass spectra over the 300–1,800 *m/z* range and MS/MS on the three most abundant precursor ions (minimum signal required set at 15,000, possible charge states: 2+ and 3+), with dynamic exclusion of previously-selected ions (exclusion duration of 60 s) (*see* **Note 15**).

3.5 Identification

1. Generate peak lists using the Mascot Daemon software (version 2.3.02, Matrix Science). Data import filter options should be as follows: 400 (minimum mass), 5,000 (maximum mass), 0 (grouping tolerance), 0 (intermediate scans), 10 (minimum peaks), 2 (extract MSn), and 1,000 (threshold) (*see* **Note 16**).
2. Search MS/MS spectra against an appropriate database using the following parameters: 2 (maximum number of missed cleavages), 5 ppm (mass tolerance for the parent ion), 0.5 Da (mass tolerance for the product ions), carbamidomethylated cysteine residues (fixed modification), oxidized methionine residues (variable modification) (*see* **Note 17**).
3. Compile Mascot results using IRMa 1.30.4 [15] and the following criteria: 0.05 (*p* value to report protein score cutoff), 10 (peptide score cutoff), 0.5 (sub sets threshold); score and rank filter criteria: 0.01 (score threshold by query identity *p* value), 1 (rank). Discard all hits without any significant peptides, and merge any hits with their respective super-hits.

3.6 Quantitation

IRMa exports the peptide and protein identifications from Mascot as an Excel spreadsheet. Also included in this spreadsheet are the spectral count values for each protein. To compare spectral count values for proteins between LC-MS/MS runs, it is first necessary to assign all MS/MS spectra to a unique peptide and then to normalize the spectral count values to the total number of spectra observed in that run. This action and any subsequent statistical analysis can be performed with a variety of software. The instructions included here are for manipulation in Microsoft Excel and comparison in PatternLab v2.1.1.12 [16, 17] (*see* **Note 18**). At least three replicates per measurement are necessary for statistical evaluation with a student's *t*-test.

1. Use the Advanced Filter function to remove replicates of any peptide sequences identified multiple times (parsimony rule). The spectral count can now be revised to only count each MS/MS spectrum once, as well as the number of unique peptides per protein.
2. In Excel, sort proteins first by peptide number, then by protein score. To apply the "at least two peptides per protein" identification rule, remove the proteins identified with only one peptide.
3. To evaluate data in PatternLab, it first has to be formatted appropriately. For the most up-to-date information on file formatting, see "http://pcarvalho.com/patternlab/downloads/exampleData/."
4. In PatternLab, choose T-fold from the Select menu. Import the appropriately formatted data as a SparseMatrix and an Index Table. Normalize the values by the Total Signal. Set the minimum number of replicates to two or three for all classes, and parse.
5. Set the *p*ValueLowerClip for the selection of the differentially detected proteins on the basis of the biological question and the dataset, typically 0.05 (*see* **Note 19**).
6. Alternatively, set the false discovery rate. The BH *q*-value default parameter is 0.05. An optimization of the F-stringency factor is proposed; this function allows the evaluation of *p*-value and Fold Change thresholds.
7. Export the results into Excel for Log2 calculation of fold changes and data presentation as exemplified in Fig. 2.

4 Notes

1. Acetonitrile needs to be stored in an appropriate flammable cabinet and should be handled while wearing gloves.
2. Passing reconstituted trypsin through multiple freeze–thaw cycles is not recommended. Solutions containing enzyme should be kept cold at all times.

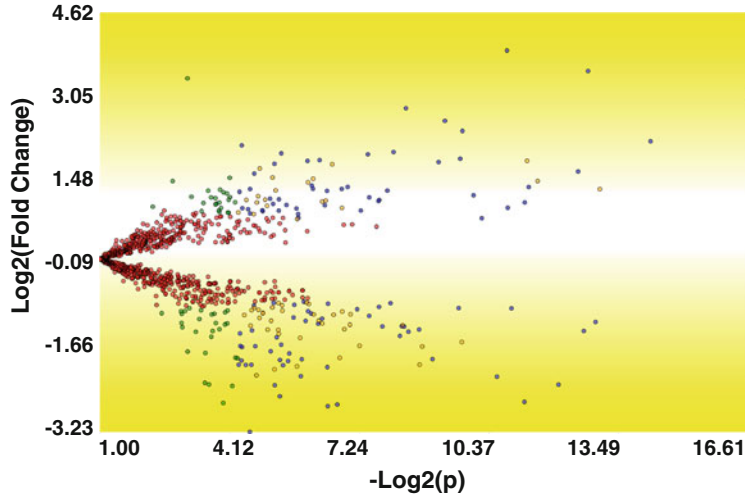

Fig. 2 PatternLab representation of a proteomic dataset: Fold-change as a function of the *p* value. A total of 1,065 proteins were detected with at least two peptides and their spectral counts extracted from six proteomic analyses (three biological replicates from two different physiological conditions). Four statistical groups (F-stringency: 0.06; False Discovery Rate: 0.05) have been represented with different colors: *Blue dots*, identifications satisfying both the automatic fold and statistical criteria; *Green dots*, identifications satisfying the fold criterion but not the criteria for statistical significance (should not be further considered); *Red dots*, identifications that did not meet the fold change criterion (should not be further considered); *Orange dots*, identifications that were filtered out by the L-stringency (further experimentation is needed to verify that they are indeed differentially detected) (Color figure online)

3. ProteaseMax is a specific reagent made by Promega. Other detergents could be used, but this protocol was designed using ProteaseMax, and others may not work exactly the same. The ratio of detergent to digest solution is an important parameter for digestion efficiency and later LC separation, and it may not be the same for other products.
4. The minimum culture volume should correspond to a 2–5 mg cell pellet (wet weight); 20–50 mg is preferred. During centrifugation, the cells undergo cold shock and oxygen deprivation, which can cause modifications to the proteome. Harvesting steps should therefore be kept as short as possible.
5. At this point, cell pellets can be frozen for later analysis.
6. Optical density measurements can be useful in determining the optimal sampling time and volume to obtain adequate biomass (*see* **Note 4**). Growth curves and optical density measurements should be performed for each type of bacteria to be sampled as kinetics and optical properties will vary. If less biomass is obtained, omit the 1:10 dilution prior to SDS PAGE.

7. For less concentrated samples (2–5 mg cell pellet), omit this dilution step.
8. We use 4–12 % Bis–Tris gels. However, for such short run times, the actual composition of the gel is not important.
9. At this point, the gel can be stored in 20 % sodium chloride in water at 4 °C for long term storage. Gels stored this way are stable for several months.
10. Larger gel sections can be further cut into pieces approximately 4 mm^3 to increase the surface area in contact with solution. Also, depending on the number of samples, it may be advantageous to place gel pieces either in microcentrifuge tubes or in 96-well plates.
11. Some remaining color can be tolerated.
12. If using 96-well plates, wash with 200 µL of water.
13. For 96-well plates, place the plate in a plastic bag and seal to prevent evaporation during incubation.
14. Peptide solutions that will not be analyzed immediately should be frozen and stored at –20 °C or colder [18]. For optimal long-term storage, peptide solutions should be lyophilized in low-adsorption tubes which prevent peptides to stick to the tube walls [19].
15. Dynamic exclusion parameters should be adjusted depending on the amount and diversity of peptides expected in the sample. Polydimethylcyclosiloxane ions generated in the electrospray process from ambient air (protonated $[(CH_3)_2SiO)]_6$, *m/z* at 445.12002) can be used for internal recalibration. Blanks should be run at least before and after the analysis. Depending on the richness of the samples, it may also be necessary to run blanks between individual samples to avoid any carryover of the most abundant peptides. The reproducibility of the analytical method should be assessed regularly with known control peptide mixtures.
16. ThermoFisher supplies the "extract_msn.exe data" import filter as part of its Xcalibur FT package (version 2.0.7). The exact mechanism for transferring raw data will depend on the system used.
17. Customized databases can be generated by exporting the appropriate references from the National Center for Biotechnology Information database. Alternatively, the NCBI or SwissProt databases, or subsets thereof, can be searched. Databases can also be constructed from in-house sequencing results; however, the appropriate parse rules will need to be determined empirically. Mass tolerances can be adjusted based on system performance. Modifications should be appropriate to the sample, i.e., if phosphorylated, acetylated, or glycosylated

proteins are expected to be observed, be sure to include these options as a variable modification.

18. For ease of manipulation of multiple data sets, actions in Excel can be recorded as a macro.
19. If the list of proteins to compare is large, i.e., 1,500, a *p*-value of 0.05 is stringent but may result in as many as 75 false-positives. Although 100 proteins might be differentially detected between the datasets, approximately 5 could be false-positives. In discovery mode, a *p*-value between 0.05 and 0.10 could be of interest to select more candidates, which should be further analyzed by other means. In confirmation mode, a more stringent *p*-value is recommended. Evaluation of the false discovery rate in multiple hypothesis testing is done automatically by calculating the Benjamini–Hochberg (BH) *q*-factor [20].

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Chapter 17

Comparative Genomic Analysis at the PATRIC, A Bioinformatic Resource Center

Alice R. Wattam, Joseph L. Gabbard, Maulik Shukla, and Bruno W. Sobral

Abstract

The Pathosystems Resource Integration Center (PATRIC) is a genomics-centric relational database and bioinformatics resource designed to assist scientists in infectious-disease research. This method paper provides detailed instructions on using this resource to finding data specific to genomes, saving it in a personalized workspace and using a variety of interactive tools to analyze that data. While PATRIC contains many diverse tools and functionalities to explore both genome-scale and gene expression data, the main focus of this chapter is on comparative analysis of bacterial genomes.

Key words Comparative genomics, Metabolomics, Metadata, Phylogeny, Genome analysis tools

1 Introduction

The National Institute of Allergy and Infectious Diseases (NIAID) established the BioInformatics Resource Centers (BRCs) to provide genomics-centric resources for priority microbial pathogens [1]. One of these BRCs, the *Path*osystems *R*esource *I*ntegration *C*enter (PATRIC, www.patricbrc.org) [2] is a Web-based information system designed to support basic and applied biomedical research on bacterial infectious diseases. PATRIC provides integrated genome-scale data, metadata, and analysis tools for all publically available bacterial genomes with special focus on NIAID Category A-C bacterial pathogens. As of December 2012, PATRIC has released the genomic data for total of 7,622 bacterial genomes. To provide consistency in comparative genomic analysis, all bacterial genomes in PATRIC are annotated in a standardized manner using the RAST (Rapid Annotation using Subsystem Technology) system [3]. RAST predicts genes, assigns gene functions, and reconstructs metabolic pathways. It is powered by a robust assembly of subsystems that have been curated based on evaluation of hundreds of prokaryotic genomes and the clustering

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of common protein families encoded within these genomes, called FIGfams [4]. PATRIC also provides a free genome annotation service through RAST to allow users to annotate their own genomes (http://www.patricbrc.org/portal/portal/patric/RAST). Genome metadata parsed from genome project data and curated from other sources supports searching for and locating genomes of interest based on various combinations of more than 60 different metadata fields (e.g., country of isolation, host, disease, collection date, etc.).

In addition to the RAST annotations, PATRIC includes other reference annotations and an Identifier (ID) Mapping tool, which allows users to quickly map between PATRIC identifiers and identifiers used by other database resources. PATRIC's Protein Family Sorter allows researchers to compare protein families across closely related or diverse groups of genomes, visualize them using an interactive heatmap, a specialized visualization tool that provides an overview of the distribution of protein families across a selected set of genomes. PATRIC also provides researchers with the ability to generate multiple sequence alignments and phylogenetic trees for individual families. The Comparative Pathway Tool allows comparison of consistently annotated metabolic pathways across closely related or diverse groups of genomes and visualize them using interactive KEGG maps and heatmap viewer (providing an overview of the distribution of the set of enzyme commission (EC) numbers within a selected pathway across a set of genomes).

Web sites and databases that provide access and analysis capabilities to thousands of genome sequences and the annotations associated with them are inherently complex and often difficult for researchers to navigate, especially if they are not computationally skilled. PATRIC is designed primarily to allow experimental biologists to view and analyze genome-scale data in an easy and efficient manner. In addition, PATRIC also provides batch data download tools to help bioinformaticians. The PATRIC homepage shows data analysis workflows that demonstrate how biologists can use multiple data types and analysis tools available at PATRIC to solve complex research problems. In addition, PATRIC also offers direct assistance to get naïve researchers over that first hurdle and make them comfortable with a complex Web site. This review is designed to provide that information. Here four different workflows that biologists commonly request are presented: (1) find one or more genes of interest, (2) find one or more genomes of interest, (3) compare protein families across closely related genomes using the Protein Family Sorter, and (4) compare metabolic pathways across hundreds of genomes using Comparative Pathway Tool.

2 Materials

To set the context for the four workflows, we first describe the PATRIC home page (Fig. 1) and specifically point out relevant components. As typical in many Web designs, PATRIC supports site-wide searching (a.k.a. Google-like searching) through a simple text box located in the top left of every PATRIC page. This PATRIC Search allows users to quickly find genes or genomes of interest using any number of keywords including gene symbol, RefSeq ID, genome name, and metadata associated with collection and isolation (*see* Subheadings 3.1.1 and 3.2.2, respectively). At the top of every PATRIC page there is a main navigation bar providing access to the breadth of PATRIC's resources including 22 watch list genera (organisms) and the collection of searches and tools.

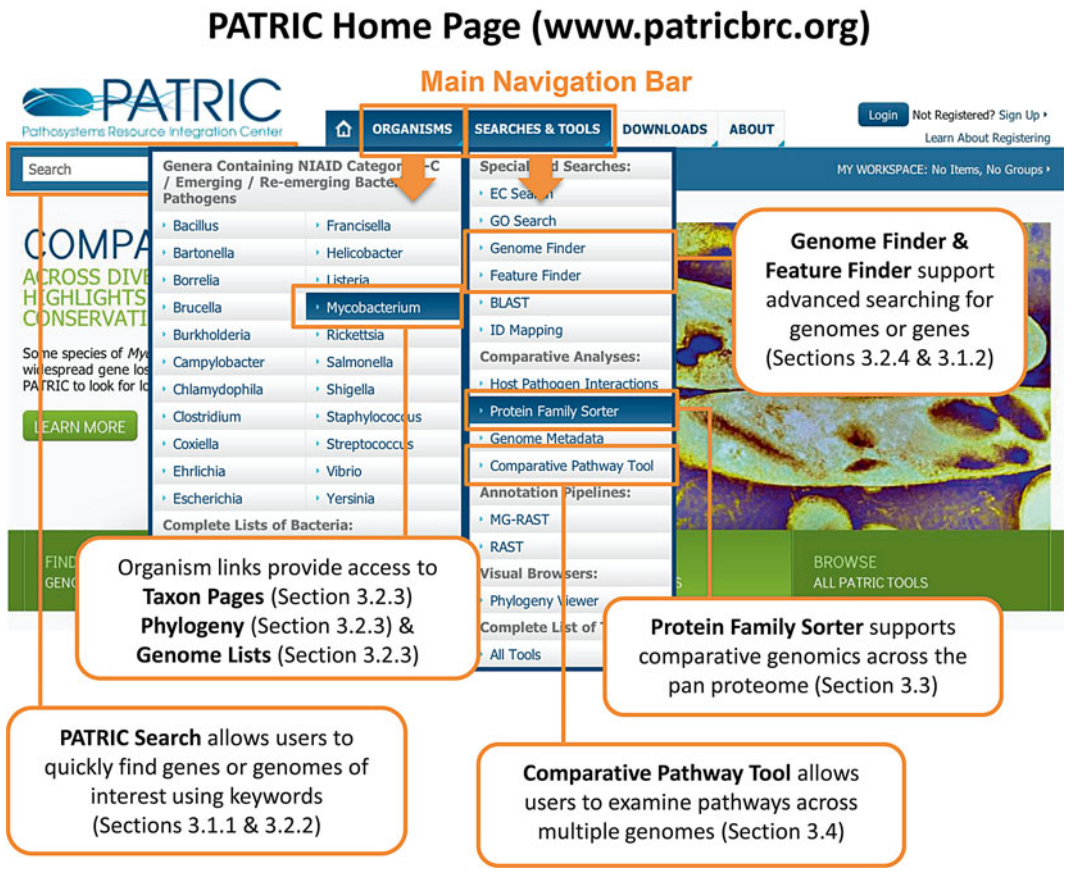

Fig. 1 The PATRIC home page allows users to (1) employ the PATRIC search to quickly find genes or genomes of interest using any number of keywords including gene symbol, RefSeq id, genome name, and metadata associated with collection and isolation; (2) navigate the site via the "Organisms" menu, accessing taxon pages, order-level phylogenetic trees, genome lists and more specific to organisms (and higher level taxa) of interest; (3) navigate the site via the "Searches and Tools" menu to initiate workflows without limiting analysis to a specific phylum, class, order, etc.

As explained in subsequent sections, many of PATRIC's comparative analysis tools can be accessed in multiple ways, depending upon whether users are approaching the problem through an organism-specific lens (e.g., comparing *Mycobacterium* proteins or genomes), or through an organism-agnostic lens (e.g., comparing genes with certain characteristics across all bacteria). Navigating the site via the "Organisms" menu, one can access taxon pages (*see* Subheading 3.2.3), order-level phylogenetic trees (*see* Subheading 3.2.3) and genome lists (*see* Subheading 3.2.3) specific to organisms (and higher level taxa) of interest. Alternatively, navigating the site via the "Searches and Tools" menu, one can quickly initiate the four workflows described below without limiting oneself to a specific phylum, class, order, and so forth. The four workflows employ the tools available via the "Searches and Tools" menu including, Feature Finder (*see* Subheading 3.1.2), Genome Finder (*see* Subheading 3.2.4), Protein Family Sorter (*see* Subheading 3.3), and Comparative Pathway Tool (*see* Subheading 3.4), respectively.

It is important to note, that all of PATRIC's bacterial data is organized by NCBI taxonomy. As such, there is an inherently hierarchical ordering of data that spans the continuum from all bacteria, to phylum, to class, to order, to genus, to species down to individual genomic features (e.g., proteins). At every level between super kingdom (i.e., all bacteria) and species, PATRIC provides a taxon page; below that PATRIC provides similarly endowed genome-level pages and feature-level pages. Each taxon page summarizes all genomes and features contained within that taxon (Fig. 2). For example, the "All Bacteria" taxon page includes *all* PATRIC genomes and features, while the "*Mycobacterium*" taxon page includes only those genomes and features contained within that genus.

At the top of each taxon page is a navigation breadcrumb that depicts both the current taxon along with appropriate higher-level taxa (i.e., the lineage). All components of the breadcrumb are clickable, affording quick navigation up and down the hierarchical taxonomy structure. Directly beneath the breadcrumb is a set of data-specific tabs that allow users to quickly access all genomic features, genomes, protein families and pathways associated with the current taxon level. Note that the four workflows described below can be initiated through these tabs and the analyses will be purposefully scoped within that taxon. Along the left hand side of the page, PATRIC provides quick links to the same searches and tools that are available via the top main navigation bar. However, as with the tabs, accessing the searches and tools from the left hand side of a taxa page will purposefully scope analysis to within that specified taxa (although the scope can be expanded on-the-fly if needed).

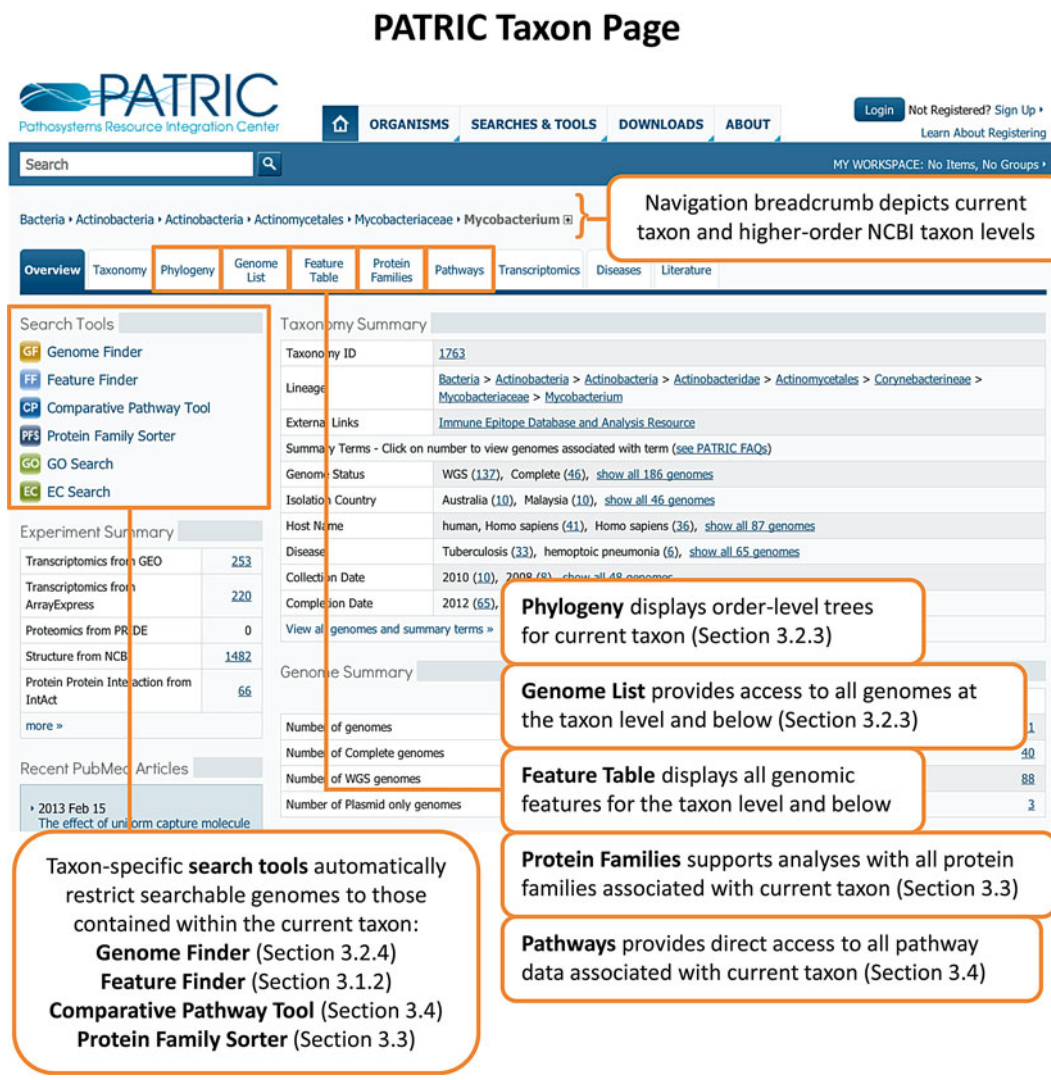

Fig. 2 PATRIC taxon pages summarize all genomes and features (e.g., proteins) contained within that taxon. At the top of each taxon page is a navigation breadcrumb that depicts both the current taxon and lineage. Data-specific tabs afford quick access to all genomic features, genomes, protein families, and pathways associated with the current taxon level. Along the *left* hand side of the page are quick links to the relevant searches and tools

3 Methods

3.1 Finding Genes of Interest

All the bacterial genomes available at PATRIC are re-annotated using RAST [2]. In addition, PATRIC also provides original annotations submitted to RefSeq/GenBank by individual researchers, who may have used a variety of annotation methods. Many of the comparative analysis tools at PATRIC, such as Protein Family Sorter and the Comparative Pathway Tool, primarily use the PATRIC-RAST annotations.

Often, researchers come to PATRIC to see information for a specific gene of interest. The information that they usually have is a RefSeq locus tag or a gene symbol. Researchers also come with functional names of a gene of interest. There are three ways to find genes of interest at PATRIC: using Global Search, using Feature Finder Tool, and using the ID mapping tool.

3.1.1 Global Search

Global Search allows users to quickly find specific genes or genomes of interest using simple keywords. It is located in the ribbon at the top of the page (below main navigation panel, Fig. 1) and can be accessed from anywhere in the PATRIC Web site.

1. Enter a gene symbol, locus tag, or the functional name of a gene of interest in the search box and click the search icon. Additional keywords related to genome name can also be provided to find matching genes in specific genomes.
2. The search returns a page with a summary of Features, Genomes, or Taxa matching the search terms and displays top hits (3a). Click on "Features" in the left hand panel to see all the features matching the search terms.
3. Click on a feature to navigate to the Feature Overview page, where one can see additional information about a gene and also access the genome browser and Compare Region viewer (*see* Subheading 3.3.10).

3.1.2 Feature Finder

The feature finder allows one to search for genes from specific annotation sources or from specific set of genomes using a locus tag or gene name. The feature Finder tool can be accessed in two ways: from the PATRIC main navigation bar, under "Searches & Tools," or from the left hand side of a PATRIC taxon page. Access via the main navigation allows users to find any bacterial genes contained in PATRIC. Access via a taxon page, automatically restricts the searchable genomes to those genomes contained within that specific taxon (Fig. 3b).

1. Enter a locus tag, gene symbol, or protein function of interest into the search box. The search can be restricted to a specific annotation source, but the default setting is for the RAST annotations provided by PATRIC. Similarly, the search can also be restricted to closed or incomplete genomes, with default being all genomes (Fig. 3b, top).
2. Click the search button. It returns a table that shows the features matching the search terms, along with information such as genome name, locus tag, RefSeq locus tag, gene symbol, and product description. Additional information (such as accession, genomic coordinates, and NA or AA length) can be displayed using the Show/Hide Columns button located in the table tool bar (Fig. 3b, bottom).

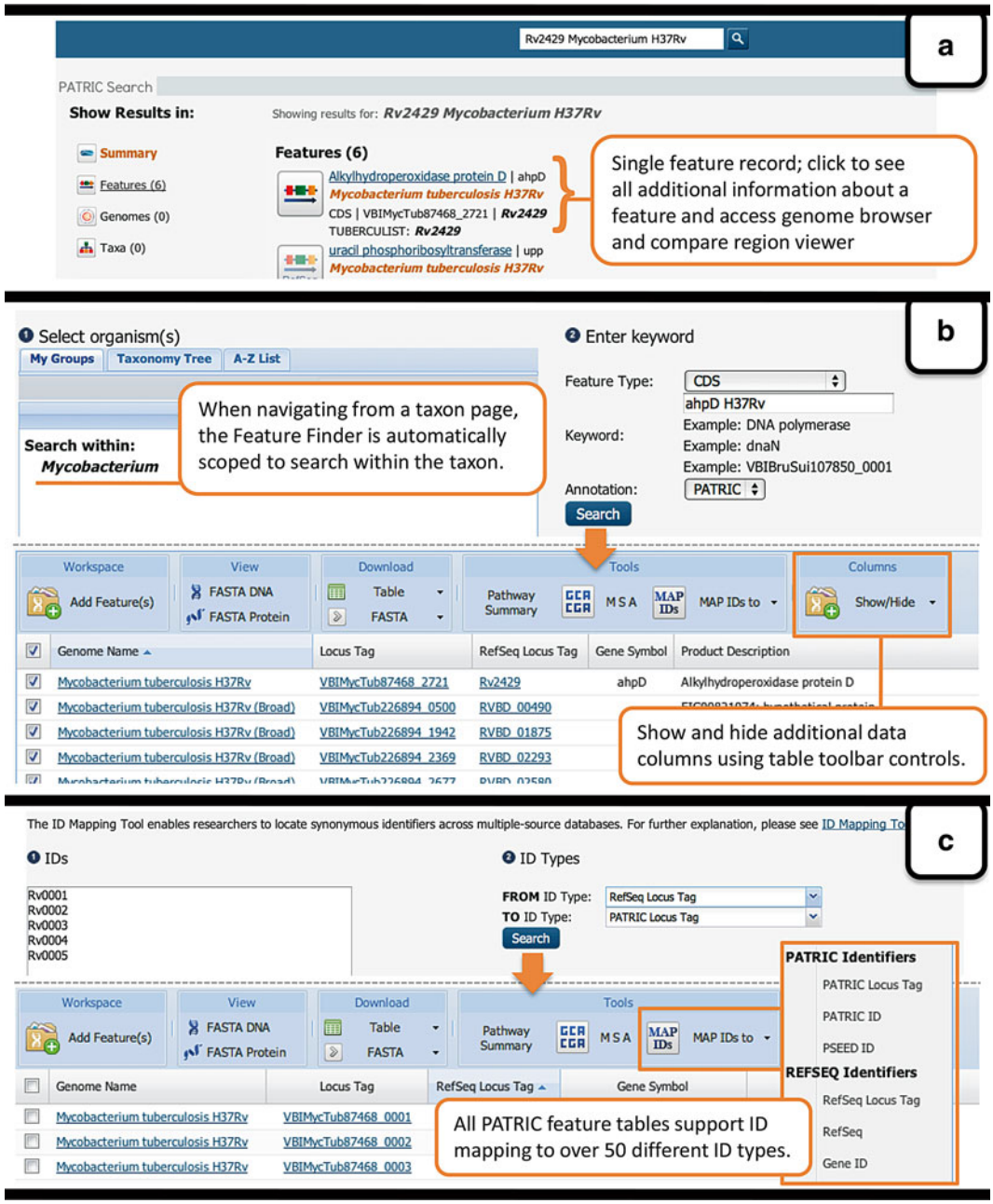

Fig. 3 PATRIC supports a number of methods for finding and collecting genomic features of interest (e.g., proteins). (**a**) The simple and quick, PATRIC search is available atop of ever PATRIC page. (**b**) Feature Finder allows more advanced searching, restricting searches to within user-specified taxa, and genomic feature types (e.g., CDS, rRNA, tRNA). (**c**) ID Mapping allows users to cut-and-paste a set of third party identifiers (e.g., RefSeq IDs) to find corresponding PATRIC features

3.1.3 ID Mapping

The ID Mapping Tool allows users to quickly map PATRIC identifiers to those from other prominent external databases, such as GenBank, RefSeq, UniProt, PDB, and others. Alternatively, users can start with a list of external database identifiers and map them to the corresponding PATRIC features. The ID Mapping

Tool can be accessed in two ways: from the PATRIC main navigation bar, under "Searches & Tools," or using "MAP IDs" button in most of the PATRIC tables showing features, including the feature table in the user workspace.

Using the ID Mapping Tool from the Main Navigation Bar

1. Select ID Mapping tool under "Searches and Tools" in the main navigation bar at the top.
2. Enter the identifier of interest (either a PATRIC locus tag, or any external database identifier of interest) into the search box. You may enter multiple identifiers as a list with one identifier per line or multiple identifiers in one line, separated by commas (Fig. 3c, top).
3. Choose appropriate options for "From ID Type" and "To ID Type" from the available drop down menu.
4. Click the search button. It will return a table that shows PATRIC locus tags and the specified identifiers, along with information such as genome name, locus tag, RefSeq locus tag, gene symbol, and product description (Fig. 3c, bottom).
5. Click on the locus tag to navigate to the protein overview page, where one can launch the genome browser, the Compare Region viewer, as well as other information associated with the protein.

Using the ID Mapping Tool from a Table Toolbar

1. Select features of interest in a PATRIC table by clicking on the check boxes to the left of the genome name. Or, select the check box next to "Genome Name" at the top to select all the features visible on the page.
2. Click the down arrow next to the "MAP IDs" icon in the blue tools section above the table (Fig. 3c, bottom).
3. Choose an ID type to display (like RefSeq locus tag). Clicking on this will redraw the table to include the specified identifier in its own column, along with other information, such as genome name, PATRIC locus tag, and the annotation source. Additional columns can be shown using the "Show/Hide" button the table toolbar.

3.2 Finding Genomes of Interest and Collecting Them into Groups for Subsequent Comparative Analysis

Since PATRIC contains thousands of comparable genomes, there are mechanisms in place that allow user-specific grouping of arbitrary sets of genomes including: single genomes that are of particular interest, whole genome sets within certain taxa, sets of genomes that share common metadata, and even closely related sets of genomes based on phylogeny. These user-specified collections of genomes are an important precursor to many of PATRIC's sophisticated analysis tools. Since the comparative analysis tools can operate on arbitrary genome sets, it is recommended that PATRIC users first create relevant sets of genomes based on criteria

that are important to their specific research goal. Below is a description of the many ways PATRIC supports finding and selecting genome sets.

3.2.1 Main Navigation Bar-Organisms

This feature allows users to directly access the taxon pages for the 22 genera containing NIAID Category A-C, emerging and reemerging bacterial pathogens, or easily go directly to the landing page that unites all bacteria. Clicking on one of these 22 genera in the main navigation bar (Fig. 1) will navigate users to the taxon landing page for that genera (Fig. 2), where users can select genomes in different ways (see Taxon Pages below, Subheading 3.2.3). If a user's genera (or genomes contained therein) of interest are not contained in this list, they can access their genera/genomes via global search (see Global Search below), or by using the "All Bacteria" link at the bottom of the "Organisms" main navigation bar, which takes users to the taxon page for all PATRIC bacteria (see Taxon Pages below for further instructions).

3.2.2 Global Search

This feature allows users to search on genome names and directly add the single genome to a group from the search results page. Users can also search by taxa (e.g., family, order, genus, etc. Fig. 4a), allowing users to navigate directly to taxon pages for subsequent genome selection (see Taxon Pages below for further instructions).

3.2.3 Taxon Pages

Using the Taxon pages allows researchers access to two different styles of genome selection methods (Fig. 2). The taxon pages can be accessed from the main navigation bar, from other taxon pages (navigating up and down the taxonomy tree), via PATRIC's Global Search, or by using the Genome Finder Tool.

Phylogeny

The phylogenetic trees at PATRIC, which are pre-computed for higher-level groups (typically at the order level), are based on concatenated alignments of multiple conserved protein families [5, 6]. The phylogenetic trees are currently available for 14 bacterial orders containing NIAID A-C pathogens. When accessing these trees from taxon pages (i.e., clicking on Phylogeny tab), researchers will see the entire phylogenetic tree for the order in which the current taxon belongs. If the current taxon page is presenting information for a bacterial family, genus, species or genome that is not in one of these 14 orders, the phylogenetic tree is not available at this time. If users are at the class, phylum or all bacteria taxon levels, the only order-level phylogeny trees available are those orders contained within these class or phylum levels. In these cases, order-level phylogenetic trees are viewable one order at a time.

When viewing an order-level tree, one can select arbitrary numbers of genomes and add them to a workspace group using checkboxes located to the right of the genome name. Once selected, click

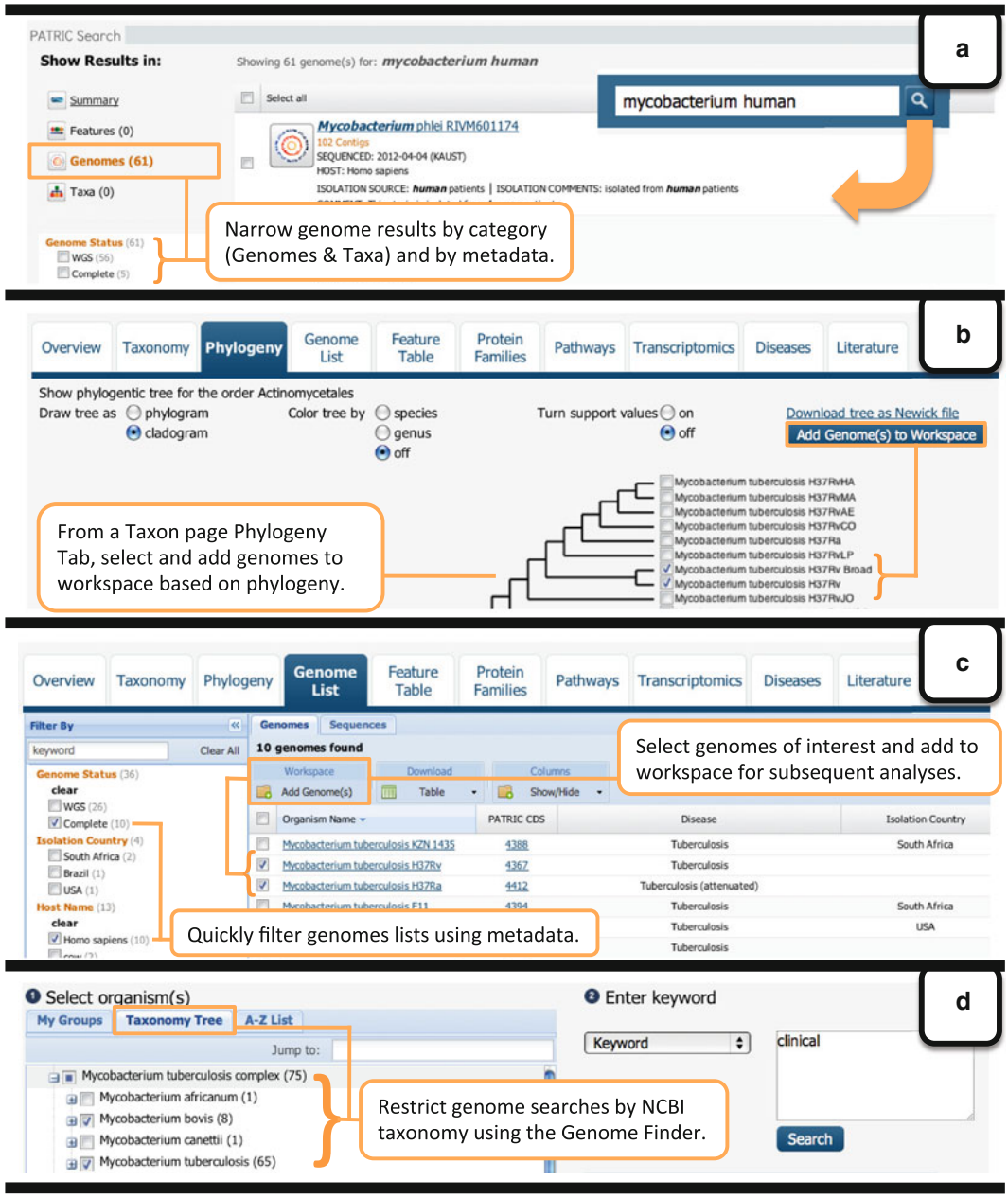

Fig. 4 There are many ways to find genomes of interest in PATRIC. (**a**) The PATRIC search affords searching on genome names and associated metadata. (**b**) The phylogeny tab organizes genomes within an order, to afford selections of genomes that are closely (or remotely) related. (**c**) The Genome List contains all genomes within a specific taxon, but supports quick filtering based on metadata, and collection of genome groups via workspace. (**d**) Genome Finder allows searching within or across user-specified NCBI taxonomy tree, and supports additional filtering based on keyword

"Add Genome(s) to Workspace" button (Fig. 4b). Selections can be made within one order, saved to a workspace group, and then another order's phylogenetic tree can be viewed to select subsequent genomes.

Genome List

Accessible at any taxon level throughout PATRIC via the "Genome List" tab, this list provides access to all genomes at that taxon level and below; from the "All Bacteria" taxon page (which includes ALL PATRIC genomes) down to a specific species taxon page (includes only those genomes contained in that species). From the genome list, users can filter by any number of metadata fields including genome status, isolation country, host name, disease, collection data, and so forth. Genomes in this list can then be arbitrarily selected and added to a users workspace group (Fig. 3c).

3.2.4 Genome Finder Tool

The Genome finder tool allows you to search for all PATRIC genomes based on genome names and available metadata. The Genome Finder can be accessed in two ways: from the PATRIC main navigation bar, under "Searches & Tools" (Fig. 1) or from the left hand side of a PATRIC taxon page (Fig. 2). Access via the main navigation allows users to find any bacterial genomes contained in PATRIC. Access via a taxon page, automatically restricts the searchable genomes to those genomes contained within that specific taxon. In both cases, searches can be further refined based on NCBI taxonomy (e.g., search within arbitrary sets of lower-level taxa), and by specifying any combination of keyword, or metadata (e.g., isolation country, host name, disease, etc.). Once the scope is set and keywords are entered, users can press "search" to execute the query (Fig. 4d). The resulting list of genomes is organized in exactly the same form as the genome list (see above), allowing users to filter by any number of metadata fields, to further winnow the list. Genomes can then be arbitrarily selected and added to a user's workspace group.

3.3 Comparative Genomics: Examining the Pan Proteome Using the Protein Family Sorter

PATRIC's compilation of all public bacterial genomes and their consistent annotation using RAST provides a powerful platform for comparative genomic analysis. The Protein Family Sorter tool at PATRIC allows users to select a set of genomes of interest, closely related or diverse, and examine distribution of protein families across the genomes, or a "pan proteome." It provides various filtering options to help quickly find protein families that are conserved across all the genomes ("core proteome"), conserved only in a subset of the selected genomes ("accessory proteome"), or match specified function. A tabular view shows protein families matching filtering criteria and an interactive heatmap viewer

provides a bird's-eye ("pan proteome") view of the distribution of the protein families across multiple genomes and relative conservation of synteny.

3.3.1 Accessing Protein Family Sorter and Selecting Genomes

The Protein Family Sorter Tool can be accessed in three ways: from the PATRIC main navigation bar under "Searches & Tools" (Fig. 1), from a PATRIC taxon page using "Protein Family Sorter" link on the left hand side (Fig. 2), or from "Protein Families" tab available on taxon pages (Fig. 2) for levels genus and below. Here is how you can access the Protein Family Sorter Tool in different ways at PATRIC.

1. From the main navigation bar under Searches and Tools, select Protein Family Sorter to navigate to the Tool page. Researchers can create customized genome groups of interest using methods described in Subheading 3.2, and compare protein families across group of genomes. Leaving the keyword box empty will shows all protein families across the selected genomes. At the current time, the heatmap view of the protein family sorter allows comparison of all protein families from up to 400 genomes. Entering a list of functions or related keywords in the "Enter Keyword" box allows one to compare a small number of protein families across the selected genomes. Clicking Search returns all the protein families within the selected group.
2. From the Taxon Overview Page: Click on the Protein Family Sorter button on the left panel. It navigates to the Protein Family Sorter search page, which is now restricted to include genomes from that taxonomy level only.
3. From the Protein Families Tab on Taxon Page: The tab is available for only taxon level genus or below. This tab directly shows comparison of all protein families across all the genomes under that taxon level.

The Protein Family Result page is divided into three sections: the filter panel on the left, Table Tab providing summary of the protein families matching filtering criteria, and Heatmap Tab showing presence/absence the protein families across all selected genomes as a two-dimensional interactive heatmap.

3.3.2 Filtering Protein Families Based on Presence or Absence in Genomes

Filter Panel (Fig. 5a) on the left allows for the following three selections to be made for each genome:

1. Selecting "Present in all Families" will show only protein families that include members from selected genomes.
2. Selecting "Absent from all Families" will show only protein families that do not include members from those selected genomes.

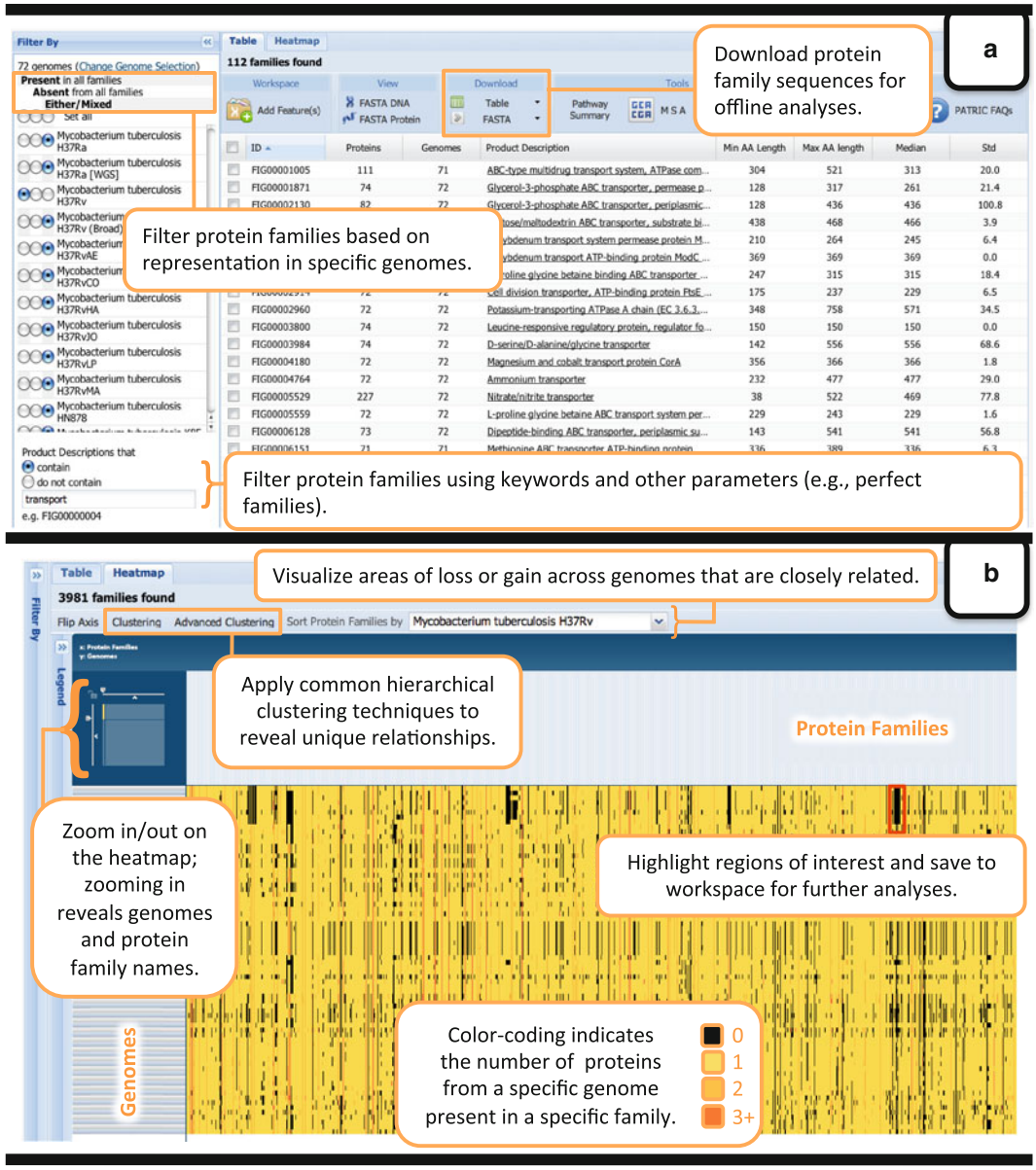

Fig. 5 PATRIC's Protein Family Sorter allows users to select a set of genomes of interest and examine the distribution of protein families across the genomes (i.e., a "pan proteome"). (**a**) It provides various filtering options to quickly find protein families that are conserved across all the genomes ("core proteome"), conserved only in a subset of the selected genomes ("accessory proteome"), or match specified function. (**b**) The heatmap viewer provides a visual overview of the distribution of proteins across a selected set of genomes; providing for example, a bird's-eye view of the conservation or divergence of protein families, or identification of multiple homologs or paralogs across a set of genomes

3. Selecting "Either/Mixed" specifies that proteins from selected genomes that may or may not be included in the resulting protein family list. This option is set by default, and allows users to focus on only those genomes they want to include and/or exclude without having to explicitly set one of these two options for every genome.

The Advanced Filter, located below the Genome Filter, enables narrowing of the results based on specific Product Descriptions, Perfect Families, and/or the number of proteins or genomes per protein family.

3.3.3 The Protein Family Summary Table

The Protein Family Table Summary is provided to the right of the Genome Filter (Fig. 5a). It assembles data across eight columns, all of which are sortable, that include a feature selection box, Protein Family Id, Proteins, Genomes, Product Description, Minimum AA Length (i.e., the smallest protein in that protein family), Maximum AA Length (the largest protein), Median, and Std (standard deviation). PATRIC uses FIGFams as the source of protein families [4]. FIGfams are protein families generated by the Fellowship for Interpretation of Genomes (FIG) [4], which are based on a collection of functional subsystems, as well as correspondences between genes in closely related strains. Further, all of the proteins within a single FIGfam are believed to implement the same function.

By default, tables show 20 rows of data. In a field at the bottom of the table, this number can be changed to any value up to 2,000 and the table can refreshed.

3.3.4 Downloading and Saving Data from the Protein Family Table View

Protein families of interest can be selected by clicking the radial button. Those families are saved to your workspace by clicking the "Add to Group" button above the radial buttons. In the download tab above the product description name, the down arrow next to "Table" offers the user the choice of getting the data in text or excel format (Fig. 5a). The entire summary information for all protein families is available for download by clicking on either "Text file" or "Excel file," which returns the appropriate format. All the supporting information, which includes all proteins within the individual family and the information about those proteins, can be obtained by clicking "Family Details. Text file" or "Family Details, Excel file."

3.3.5 Visual Analysis of the Protein Families Using the Heatmap Viewer

The Protein Family heatmap viewer is located on the second tab (Fig. 5b). The heatmap is an interactive visualization tool that provides an overview of the distribution of proteins across a selected set of genomes. Patterns visible in a heatmap can allow for many types of analyses that include a bird's-eye view of the conservation or divergence of protein families across genomes. It can also be used to discern how many proteins have the same function within a single genome and also to identify proteins with multiple homologs or paralogs across a set containing up to 400 genomes.

Each cell is colored according to how many proteins from a specific genome are members of a given protein family. Black cells indicate that there are no representative proteins from a specific genome for that protein family. Bright yellow cells indicate there is one representative protein, dark yellow cells indicate there are two representative proteins, and dark orange cells indicate there are

three or more representative proteins. Genomes are listed along the Y (vertical) axis and corresponding protein families are listed along the X (horizontal) axis. The scale of these rows and columns may be controlled by sliding the x and/or y slidebars located at the axis intersection in the upper left corner of the Heatmap, and individual rows or columns can be moved, allowing individualized grouping by the researcher.

3.3.6 Assembling Protein Families Along a Reference Genome

The entire visualization may be sorted by choosing a reference genome to act as an anchor. This will automatically sort all the protein families based on the gene order in that genome.

1. To select a reference genome, either chose one from the "Sort Protein Families by" field at the top of the Heatmap or click on any genome in the Heatmap and then click the "Sort Protein Families" button in the pop-up window. This functionality allows the user to quickly identify groups of contiguous genes present or absent in one or more of the selected genomes. In addition, the user can quickly visualize areas of loss or gain of genomic segments across genomes that are closely or more distantly related. Some of these areas may correspond to pathogenicity islands.
2. Regions of interest can be highlighted using the mouse or scroll button. On the computer screen, this will be visualized as a yellow box. This is followed by a prompt that directs the user to download the selected proteins (*see* Subheading 3.3.8).

3.3.7 Clustering Protein Families in the Heatmap Viewer

PATRIC allows the researcher to use hierarchical clustering in the heatmap view. This allows visual detection of similar protein families across multiple genomes, and can help find unique relationships, either by phylogeny or lateral transfer, without reorganizing the position of the genome in the heatmap. The default mode of cluster is a Pearson correlation for distance measure and pairwise average-linkage method for hierarchical clustering. Advanced clustering allows users to select from several distance measures and then perform the analysis. In the blue ribbon above the heatmap view, click "Clustering." The heatmap image will reload to show the clustered data.

3.3.8 Downloading Data from the Heatmap Viewer

Data for each protein, including the name of the genome, the locus tag, chromosomal location, length, and the name of the protein can be obtained in several ways.

1. Clicking the "Download Proteins" button will open the protein data in either a text or excel file.
2. Scrolling over an area of interest in the heatmap view will open a "Download Proteins" button that will open the selected data, containing the information about the individual proteins highlighted by the selection, in either a text or excel file.

3. Clicking the "Show Proteins" button will open the protein data within a Protein Families Table, where the Toolbar, located in the light blue row of the table may be utilized to perform several functions.
   (a) Users can view or download selected DNA and/or protein sequence data in FASTA format.
   (b) The table itself, or selected data within it, are also downloadable in both excel and txt file formats.

3.3.9 Multiple Sequence Alignments (MSAs) and Gene Trees

The multiple sequence alignment viewer is an interactive tool that displays a multiple sequence alignment and a gene family tree side-by-side. It allows the user to examine details of selected protein families, including the overall quality of the alignment as well as more specific details such as particular regions or motifs of interest.

1. From a workspace or row of proteins selected in the heatmap view or table, select all radial boxes on the left-hand side before the genome name.
2. In the tab above gene names, click the MSA icon. Up to 100 protein sequences can be aligned with this tool.
3. A gene tree and associated MSA are generated. Trees can be viewed as either a phylogram (default) or a cladogram, and the final nodes in the tree (leaves) can either be the name of the genome or the locus tag. Both the gene trees and the MSA can be downloaded in a printable format.

3.3.10 Follow Up Analysis: BLAST, Genome Browser, and Compare Region Viewers

Once proteins have been identified, PATRIC allows for further investigation. Researchers can look for other proteins of similar nucleotide or protein composition by BLAST, or can look at the nearest neighbors in a genomic context using the Genome Browser. PATRIC also provides the user with the ability to look at the gene neighborhood of the protein of interest, and look for a similar construction across all bacterial genomes using the Compare Region viewer.

1. BLAST. In the search tool tab, researchers will find the BLAST tool at PATRIC that allows blasting against nucleotides (BLASTn) or proteins (BLASTp) from a variety of different specially constructed databases. These include CDS, RNA or protein from all annotation sources available at PATRIC, and also against genomes or plasmids.
2. Genome Browser. The Genome Browser is a graphical representation of the alignment of genes and other genomic data depicted along a central horizontal axis of genome coordinates. Researchers can also access the Genome Browser from within any PATRIC table that contains the Genome Browser icon.

The Genome Browser will initially open with PATRIC annotated data tracks. These are depicted as blue (forward orientation) or yellow (reverse orientation) boxes with arrow lines within them. Additional annotation tracks, such as RefSeq, are located along the left side of the Genome Browser and can be added to the view by dragging the appropriate box (an example being "CDS RefSeq") into the browsing section on the right-hand side dragging tracks. Coding regions of genes (CDSs) and RNAs can also be viewed. Alternatively, tracks can be removed from the browser window by dragging the box to the left.

3. Compare Region Viewer. The Compare Region Viewer, implemented using JBrowse and data APIs provided by the SEED [7] allows researchers to compare genomic regions around a gene of interest across all other genomes, but it will display the most closely related genomes. This viewer can be accessed via the Compare Region Viewer tab available on feature-level pages that display PATRIC annotation. Using this viewer, one can quickly detect differences in translation start sites, potential frameshifts, or missing genes across multiple genomes. The viewer is interactive in the same manner as the genome browser, but with several additions. Coloring is based on protein functions and allows users to visually group proteins with similar functions. The red arrow indicates the protein whose feature page was used to launch the viewer (i.e., the protein the researcher initially selected). Users can also change the size of the region and number of genomes compared in fields located above the coordinate axis.

3.4 Comparative Metabolomics: Examining Pathways Across Multiple Genomes

PATRIC's Comparative Pathway Tool allows researchers to identify a set of pathways based on taxonomy, EC number, pathway ID, pathway name and/or specific annotation type (Fig. 6). All pathways at PATRIC come from the Kyoto Encyclopedia of Genes and Genomes, commonly known as KEGG [8, 9], and the Comparative Pathway Tool maps the RAST annotations to KEGG pathway maps. Pathways are classified according to major biological roles (e.g., carbohydrate metabolism, translation, biosynthesis of secondary metabolites, etc.) and are assigned identifications from a list of 137 unique cellular pathways. PATRIC displays a table of unique pathways (Fig. 6a) that match the search criteria (i.e., the genomes or proteins chosen by the researcher, or at any taxonomic level). From there, you can select specific pathways of interest and view the Comparative Pathway KEGG Map (Fig. 6b) and the Comparative Pathway Heatmap (Fig. 6c).

3.4.1 Choosing Genomes to Examine: Sorting Metadata in Genome List

1. In the pan proteome description, genomes were selected through the phylogenetic tree. PATRIC offers a variety of ways to choose genomes. From any taxon landing page, genomes

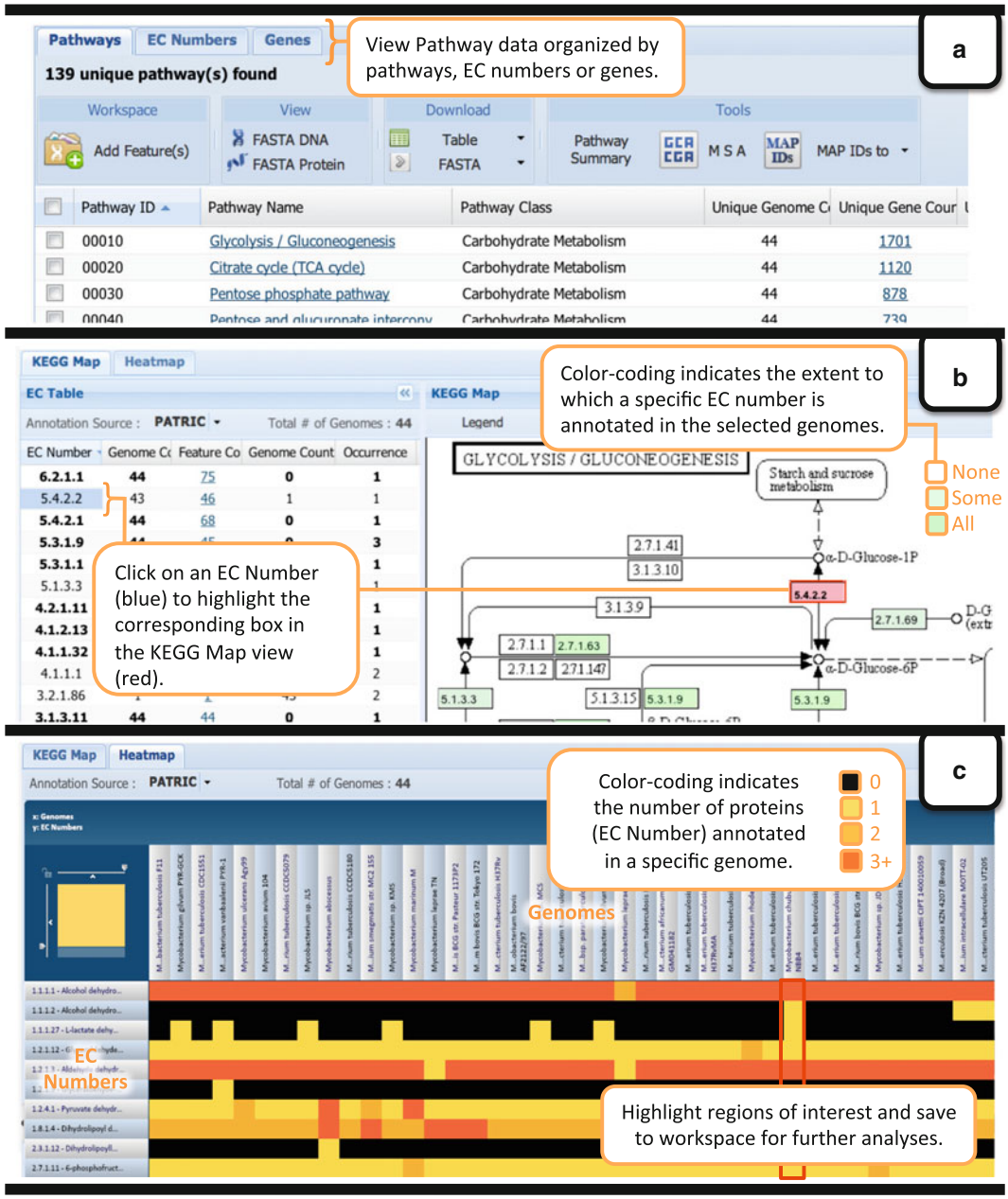

Fig. 6 (**a**) PATRIC's Comparative Pathway Tool allows you to search for a set of pathways within a set of genomes and subsequently view a list of individual proteins with a particular EC number or view a comprehensive list of all genes annotated across all pathways. (**b**) The KEGG map view affords quick visual identification of EC numbers annotated (or not) in the user-specified set of genomes. (**c**) The heatmap view provides a visual summary of the distribution of genes in a specific pathway across the selected genome set

can be selected by choosing the "Genome List" tab (Fig. 4c) from a taxon page (Fig. 2). The genome list provides the opportunity to filter on metadata associated with the genomes at the taxonomy level being examined, available on the left-hand column. For instance, clicking on the "Complete" box under the "Genome Status" metadata attribute.

2. Click the box in the top left of the table next to "Organism Name" to select all the complete genomes.
3. Selecting "Add Genomes" from the light blue Table toolbar allows users to save their selections. Researchers that are not logged in to the PATRIC site will be prompted to either login, or to register for a new account, or continue as a Guest.
4. In the modal, chose "Create New Group" and give the group a name. This group will always be available when logged in to PATRIC, and this grouping can be chosen when using PATRIC tools.

3.4.2 Examine Pathways Shared or Unique to Selected Genomes

1. Select "Comparative Pathway Tool" from the Searches and Tools tab in PATRIC's main navigation bar (Fig. 1). Alternatively, choosing the "Comparative Pathway Tool" button at the left-hand side of any taxon-landing page (Fig. 2) will sort and provide all pathway information at that selected level. There is also a pathway tab available on the taxon-landing page that does the same thing.
2. Under "My Groups," the researcher can select a group. Clicking the blue "Search" button deploys the tool. The Pathways Tool is now scoped to show results only for this specific list of genomes, and will return a Pathway Table.
3. A specific annotation source (PATRIC, RefSeq, or other) is chosen at the drill down box available to the right. In addition, pathways or genes of interest can be located by entering the KEGG ID, pathway name, EC number or specific keyword in the available box.
4. Clicking the "Search" button deploys the pathway finder.

3.4.3 Interpreting the Pathway Table

The Pathway Table has three tabs (Pathways, EC number, and Genes) at the top of the table (Fig. 6a). EC numbers are part of a numerical classification schema that has been developed for enzymes and the chemical reactions they catalyze. Clicking on any of these tabs goes to the sorted data within that grouping. All data can be downloaded in either text of excel formats.

1. The Pathways tab is the default page that first appears. It provides a summary of the data across the level selected (i.e., taxonomy level or special genome group) on a per pathway basis. This includes the KEGG id number, pathway name, class, the annotation source, the number of genomes the information is sorted across ("Unique Genome Count"), the number of genes involved in this pathway across all the genomes ("Unique Gene Count"), the EC numbers within that particular pathway across the selected level ("Unique EC Count), the percent of unique EC numbers present in all

selected genomes ("EC Conservation) and a way to estimate how many genes have the same EC number within a particular pathway ("Gene Conservation"). (Note: Gene conservation provides an estimate of pathways where there might be redundancies, or where EC numbers are missing. Numbers greater than one mean that in at least one genome, there is more than one gene that has been assigned a particular EC number. Numbers less than one mean indicate that in at least one genome, a particular EC number is missing.) There are several hyperlinks on these pages. Clicking on the blue, underlined text takes the researcher to that particular data. For example, clicking on Glycolysis/Glucogenesis will take the user directly to the pathway data.

2. The EC tab provides similar data to the Pathway tab, but at the EC level. This tab shows the individual proteins with a particular EC number, and provides information on how many genomes have a protein annotated with that particular EC destination ("Genome Count") and the number of genes across all the genomes that have that annotation ("Unique Gene Count"). From the EC Number tab you can navigate within rows to a specific PATRIC Pathway Map page (Pathway Name), a table with all the genes for a particular EC number (Unique Gene Count).
3. The Genes tab gives a comprehensive list of all genes annotated across all pathways. Depending upon the size of the genome group or taxonomy level initially chosen, this could be a very large group, and there may be a time delay in loading the page. From the Genes tab you can navigate within rows to specific PATRIC genome pages (Genome Name), NCBI nucleotide pages for that genome (Accession), specific PATRIC locus pages (Locus Tag), or a specific PATRIC Pathway Map page (Pathway Name).

3.4.4 Pathway View and Heatmap

Clicking on any pathway name allows the researcher to view results via the default KEGG map tab (Fig. 6b) or the heatmap Tab (Fig. 6c). The pathway view has both an EC table, and the information summarized on a KEGG map at the right.

3.4.5 Pathway View

1. The EC table (Fig. 6b, left) provides a summary of the data across the selected genomes in five columns. These columns include the EC Number, Genome Count, Feature Count, Genome Count Not Present (this shows how many genomes are missing a particular EC number, and Occurrence (this shows how many times this EC number occurs within a given pathway). As in all PATRIC columns, the data is sortable by clicking on it. Bold colors indicate that all genomes have that

particular EC number annotated, and clicking on a number will take its location in the KEGG map.

2. KEGG map view (Fig. 6b, right). The numbers in the boxes are Enzyme Commission, or EC numbers. These numbers are part of a numerical classification schema that has been developed for enzymes and the chemical reactions they catalyze. The box containing the EC number is one of three colors.
   (a) A white color indicates that this protein has not been annotated in any of the selected genomes.
   (b) A bright green color indicates that a protein with this EC number has been annotated in all the genomes chosen in the original Comparative Pathway Tool search.
   (c) A muted green color indicates that a protein with this EC number has been annotated in at least one, but not all, genomes chosen in your original Comparative Pathway Tool search by that specific annotation source.

3.4.6 Comparative Pathway Heatmap View

The comparative pathway heatmap is located on the second tab. Similar to the protein family heat map, it is an interactive visualization tool, providing a visual summary of the distribution of genes in a specific pathway across the selected genomes. The legend is located to the right of the heatmap view and can be closed by clicking a radial button.

Coloring of the cells is identical to the heatmap in the protein family viewer described above (*see* Subheading 3.3.5). A difference between these two viewers is that in the comparative pathway heatmap, the genomes are listed along the X (horizontal) axis and corresponding annotated EC numbers, as well as their name, is listed along the Y (vertical) axis. The scale of these rows and columns may be controlled by sliding the x and/or y slidebars located at the axis intersection in the upper left corner of the Heatmap, and individual rows or columns can be moved, allowing individualized grouping by the researcher. For example, some researchers might want to arrange the genomes in an order that follows the phylogeny (Note: Phylogenies are provided at the Order level for most of the genomes in PATRIC, and can be viewed by clicking on the "Phylogenetic Tree" tab from any taxon landing page) and this can be accomplished by clicking and dragging a column header (either the name of the genome, or the box containing the EC number and protein name) to the desired location.

Downloading the data from this viewer is identical to that described for the protein family heatmap view (*see* Subheading 3.3.8). Also identical are the selection methods for areas of interest, and deploying the MSA and gene tree tools (*see* Subheading 3.3.9).

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Part IV

Approaches for Difficult Bacteria

Chapter 18

A Markerless Deletion Method for Genetic Manipulation of *Burkholderia cenocepacia* and Other Multidrug-Resistant Gram-Negative Bacteria

Daniel F. Aubert, Mohamad A. Hamad, and Miguel A. Valvano

Abstract

Genetic manipulation of multidrug-resistant bacteria is often difficult and hinders progress in understanding their physiology and pathogenesis. This book chapter highlights advances in genetic manipulation of *Burkholderia cenocepacia*, which are also applicable to other members of the *Burkholderia cepacia* complex and multidrug-resistant gram-negative bacteria of other genera. The method detailed here is based on the I-SceI homing endonuclease system, which can be efficiently used for chromosomal integration, deletion, and genetic replacement. This system creates markerless mutations and insertions without leaving a genetic scar and thus can be reused successively to generate multiple modifications in the same strain.

Key words *Burkholderia cepacia* complex, Genetics, I-SceI homing endonuclease, Gene deletion, Chromosomal complementation

1 Introduction

The *Burkholderia cepacia* complex (BCC) is a group of gram-negative bacteria that are ubiquitous in the environment [1] and cause opportunistic infections in immunocompromised individuals [2, 3]. Treatment of these bacteria is very difficult due to their high level of intrinsic, multidrug antibiotic resistance [4, 5]. Genetic manipulation of bacteria remains a powerful tool to understand gene function and elucidate bacterial physiology and pathogenesis at the molecular level. Several factors have hindered the development of reliable genetic tools to manipulate *Burkholderia* species. They include lack of suitable selectable and counterselectable markers, as well as few reliable methods to introduce DNA into *Burkholderia*. Over the past decade several genetic systems for *Burkholderia* species became available [6–11], which help to deal with these shortcomings. The pGPI-SceI/pDAI-SceI-SacB system,

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developed in our laboratory, is based on the I-SceI homing endonuclease system and has become the most widely used method to genetically manipulate Bcc members [7, 12]. The methods can also be applied to other multidrug-resistant gram-negative bacteria.

Mutagenesis based on the homing endonuclease I-SceI involves cloning the sequences flanking the chromosomal region targeted for deletion into a suicide plasmid that cannot replicate in *B. cenocepacia* and carries the I-SceI recognition site. This plasmid is transferred to *B. cenocepacia*, resulting in its targeted insertion into the chromosome via homologous recombination and the introduction of a mutant allele. Next, a second plasmid that constitutively expresses the I-SceI nuclease is introduced. I-SceI causes a double strand break into the inserted plasmid sequence, which stimulates intramolecular recombination between the mutant and parental alleles. The resolution of this co-integrate can either restore the parental allele or introduce a gene deletion, depending on the site of the cross-over (Fig. 1).

This system allows for markerless genetic replacement, gene deletion (knock out) or gene insertion (knock in), through two independent crossover events by homologous recombination [7, 13, 14]. To construct a deletion plasmid, ~500 bp DNA fragments flanking upstream and downstream of the gene are cloned into the suicide vector pGPI-SceI, which contains the unique I-SceI recognition sequence. This deletion vector is conjugated into *B. cenocepacia* K56-2 where it integrates into the genome. A second crossover event is achieved by conjugating the pDAI-SceI-SacB plasmid into co-integrant clones. The I-SceI homing endonucleases, expressed from pDAI-SceI-SacB plasmid, catalyze double-strand DNA breaks in the chromosome at the specific I-SceI restriction site. As DNA double-strand breaks are lethal, only mutants undergoing second homologous recombination events are recovered. Colonies with a regenerated wild type gene will be obtained if the same flanking region is used for both the first and second crossover events, while deletion mutants are obtained if each of the flanks has been involved in a crossover reaction. Colonies are first screened for trimethoprim sensitivity to confirm excision of pGPI-SceI by a second crossover event and PCR is then used to identify deletion clones (Fig. 1).

Genomic complementation with a single copy of a gene is possible by reintroducing the gene of interest into a neutral site of the *B. cenocepacia* genome while leaving the original deletion mutation intact. This approach allows physiological gene expression from single copy, unlike multicopy expression systems, which can result in artifacts and non-physiological effects. The strategy we developed permits inserting a complementing gene by the unmarked swapping of the gene of interest with the gene locus (*BCAL1674-BCAL1675*), which encodes a nonessential aminoglycoside efflux pump. To construct a complementation plasmid, the gene of

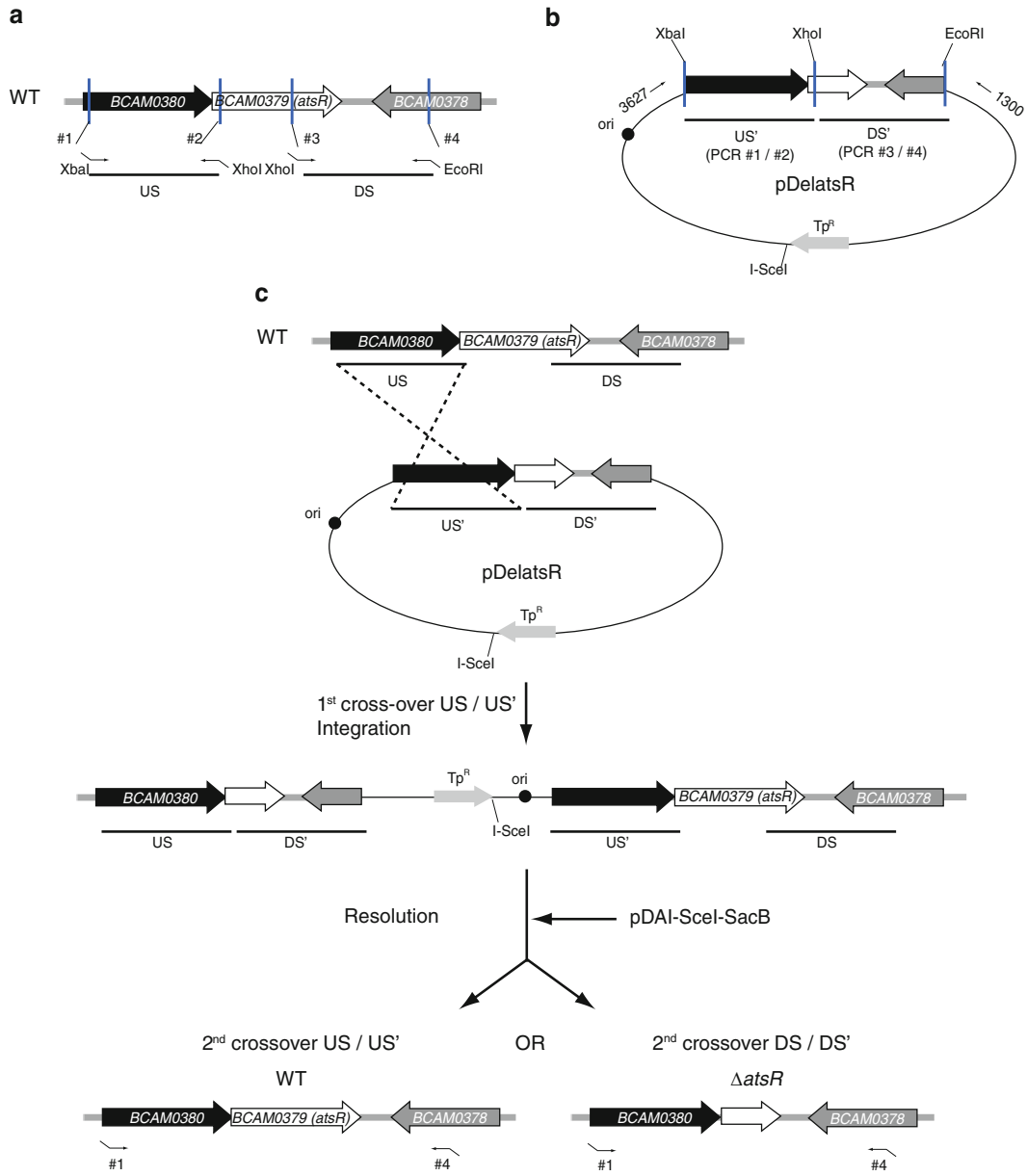

Fig. 1 Strategy for genetic replacement (deletion) using the pGPI-SceI/pDAI-Sce-I system. (**a**) Genetic organization of *atsR* in *B. cenocepacia* K56-2 wild-type (WT). Genes are indicated with an *arrow*, the location of the primers used to amplify the upstream (US) and downstream (DS) regions flanking *atsR* are shown. (**b**) The deletion plasmid (pDelatsR) is constructed by cloning of the upstream (US′) and downstream (DS′) flanking regions of the gene targeted for deletion (*atsR*) into the suicide plasmid pGPI-SceI, which has an I-SceI recognition site, a R6Kγ origin of replication (ori) and encodes trimethoprim resistance (Tp^R). The locations of the vector sequencing primers 1300 and 3627 are shown. (**c**) The suicide plasmid is introduced by conjugation into *B. cenocepacia* and integrates into the genome by homologous recombination (1st crossover) using either the US′ or DS′ fragments (only the result of pDelatsR integration using the US′ fragment is shown here). Plasmid pDAI-SceI-SacB, which expresses the I-SceI endonuclease, is then introduced. I-SceI expression results in a double strand cut in the chromosomal DNA at the I-SceI site, which favors the 2nd crossover event by stimulating the host DNA repair machinery. A 2nd crossover occurring between DS and DS′ will delete the intervening sequence in between these sites including the targeted gene and pGPI-Sce-I backbone with the trimethoprim resistance cassette. Resolution of the deletion plasmid and reversion to wild type occurs if recombination involves the same regions that have been involved in the 1st crossover step (here US and US′). PCR using primer #1 and primer #4 differentiates between wild type and deletion clones

interest is cloned into the vector pMH447, which also carries a unique I-SceI site. The complementation vector is conjugated into the *B. cenocepacia* recipient strain where it integrates into the genome at aminoglycoside efflux genes (*BCAL1674-BCAL1675*) giving rise to first crossover co-integrates. The second recombination event is mediated by I-SceI expressed from pDAI-SceI-SacB. As described before, a mixture of parental and complemented exconjugants will be recovered depending on the flanks combination used during homologous recombination. However, the colonies carrying the complementing gene of interest in single copy are also gentamicin sensitive, which provides an effective way to identify the appropriate clones by screening for gentamicin sensitivity (Fig. 2). Curing the replicative vector pDAI-SceI-SacB is easily achieved by sucrose counterselection (*see* **Note 1**). The genes integrated using this method are efficiently expressed due to the constitutive promoter upstream the efflux pump genes. This method is unique to *B. cenocepacia* since gene integration using the pMH447 system is based on homologous recombination and the recognition of the two flanking regions of *BCAL1674-BCAL1675*. However, a similar approach can be used in other species if a similar site is identified.

Therefore, the I-SceI system is very versatile and can be adapted to introduce or swap any DNA sequences into the chromosome allowing promoter swapping [14], introduction of point mutations [14], and creation of in-frame deletions that eliminate specific protein motifs allowing the expression of the rest of the protein, as well as protein fusions to reporter tags [15].

In this chapter we describe in detail the method used to create a markerless gene deletion and single copy gene complementation in *Burkholderia cenocepacia* K56-2 using the gene *atsR* (BCAM0379) as an example.

Fig. 2 (continued) which contains the homology regions US′ and DS′ for recombination within cluster encoding the aminoglycoside efflux pump, an I-SceI recognition site, a R6Kγ origin of replication (ori), and encodes trimethoprim resistance (Tp^R). The locations of the sequencing primers #5885 and #5886 are shown. (**c**) The suicide plasmid is introduced by conjugation into *B. cenocepacia* Δ*atsR* and integrates into the genome by homologous recombination (1st crossover) using either the US′ or DS′ fragments (only integration using the US′ fragment is shown here). Plasmid pDAI-SceI-SacB, which expresses the I-SceI endonuclease, is then introduced. I-SceI expression results in a double strand cut in the chromosomal DNA at the I-SceI site, which favors the 2nd crossover event by stimulating the host DNA repair machinery. A 2nd crossover occurring between DS and DS′ deletes the intervening sequence in between these sites including *BCAL1674* and part of *BCAL1675* as well as the suicide vector containing the trimethoprim resistance cassette but leaves the complementing gene. The complemented strain (Δ*atsR atsR*$^+$) is trimethoprim sensitive (Tp^S) and gentamicin sensitive (Gen^S). Reversion to the parental strain (Δ*atsR*, trimethoprim sensitive (Tp^S) but gentamicin resistant (Gen^R) occurs if recombination involves the same regions that have been involved in the 1st crossover step (here US and US′). Specific integration of the complementation gene can be confirmed by PCR using internal primers within the gene of interest that are absent in the deletion mutant (here primer #7 and primer #8)

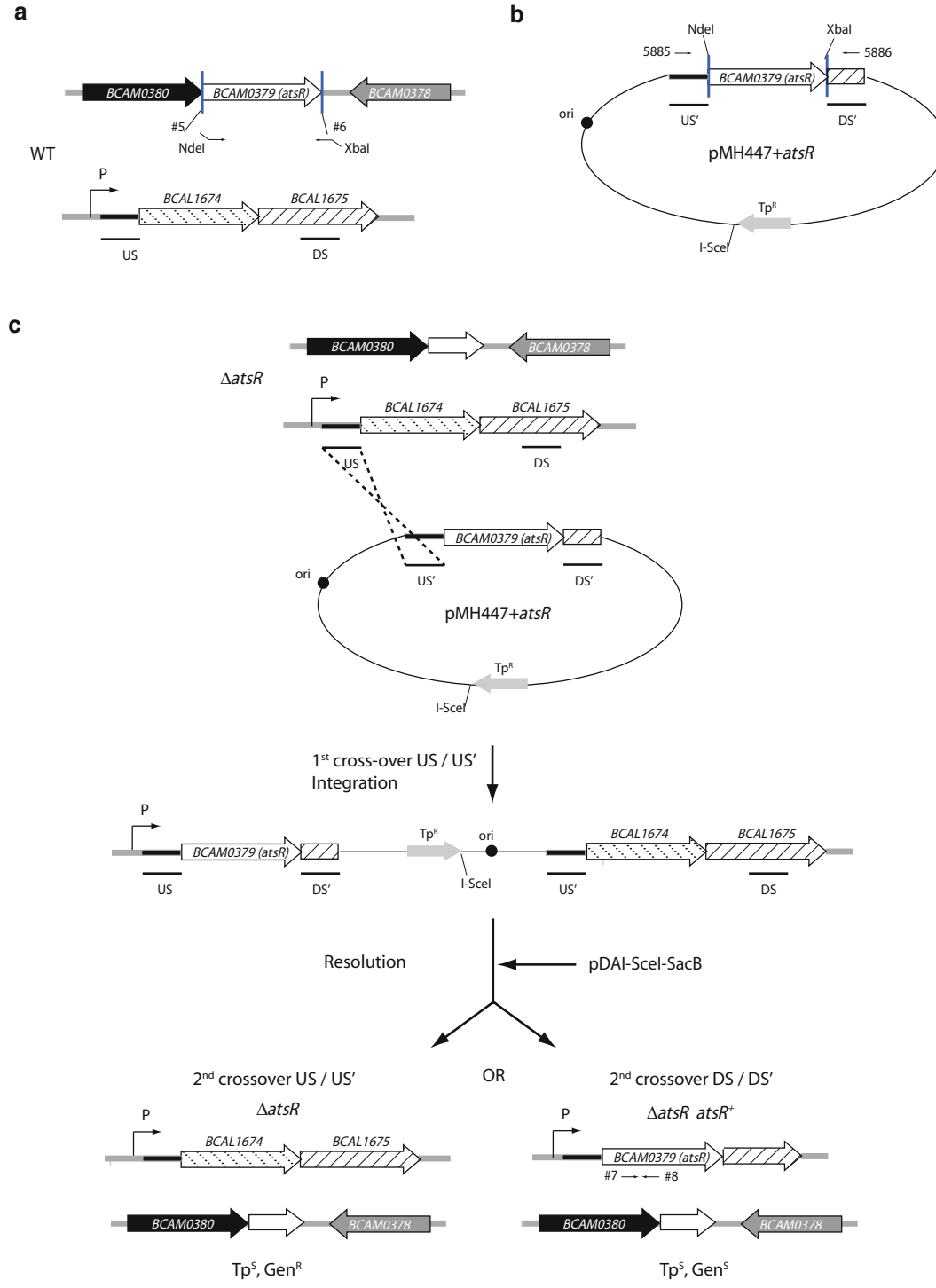

Fig. 2 Strategy for genetic replacement (complementation) using the pMH447/pDAI-Sce-I system. (**a**) Genetic organization of *atsR* and of *BCAL1674/1675* encoding components of the aminoglycoside efflux pump, which confers gentamicin resistance in *B. cenocepacia* K56-2 wild-type (WT). Genes are indicated with an *arrow*; the location of primer #5 and primer #6 used to amplify *atsR* are shown. The homology regions US and DS, which are located upstream of *BCAL1674* and within *BCAL1675*, respectively and used during recombination are shown. The broken *arrow* represents the promoter (P) of the aminoglycoside efflux pump. (**b**) The complementing plasmid (pMH447 + atsR) is constructed by cloning the gene of interest (*atsR*) into the suicide plasmid pMH447,

2 Materials

2.1 Growth Media, Antibiotics, and Chemicals

1. LB broth.
2. Difco SOB medium (Difco).
3. LB agar plates: LB with 1.5 % Bacto Agar (Difco).
4. SOB-agar plates: SOB with 1.5 % Bacto Agar.
5. Phosphate buffered saline (PBS) sterilized by autoclaving.
6. Kanamycin sulfate (stock solution 20 mg/mL, gentamicin sulfate (100 mg/mL), ampicillin sodium salt (100 mg/mL). Prepare in distilled water (dH_2O) and filter sterilize by passage through a 0.22-μm filter. Store at 4 °C.
7. Polymyxin B sulfate (25 mg/mL). Prepare in dH_2O and store at −20 °C.
8. Trimethoprim (50 mg/mL), prepare in *N*,*N*-Dimethyl-acetamide and store at room temperature (*see* **Note 2**).
9. Tetracycline hydrochloride (10 mg/mL). Prepare in 50 % ethanol and store at 4 °C away from direct light.
10. 5 % sucrose LB agar plates lacking salt: 0.5 g sucrose, 0.5 g yeast extract, 1 g tryptone, and 1.6 g agar into 100 mL dH_2O. Sterilize by autoclaving.
11. 37 °C incubator.
12. Rotary shaker (37 °C).

2.2 Bacterial Strains

1. *Burkholderia cenocepacia* K56-2 (CF clinical isolate, *B. cepacia* Research and Referral Repository for Canadian CF Clinics [16]) (*see* **Note 3**). For manipulation of extremely antibiotic resistant *Burkholderia* strains, like *B. cenocepacia* J2315 or BC7 (*see* **Note 4**).
2. *Escherichia coli* DH5α, used to maintain plasmids pDAI-SceI-SacB (*see* Subheading 2.4, **step 3**) or pRK2013 (*see* Subheading 2.4, **step 4**).
3. Chemically competent *E. coli* GT115 (Invivogen), which expresses the λ Pir protein required for cloning and propagation of plasmids with the R6K origin of replication such as the suicide vector pGPI-SceI and derivatives (*see* **Note 5**).

2.3 Nucleic Acid Sequence

The sequenced genome from *B. cenocepacia* J2315 (ET-12 clone related to K56-2 [17]) is used as a reference for primer design for *B. cenocepacia* K56-2. DNA sequences are available from GenBank (accession numbers: NC_011000 for chromosome 1, NC_011001 for chromosome 2, NC_011002 for chromosome 3, and NC_011003 for plasmid PBCJ2315 NC_011003).

2.4 DNA Vectors

1. pGPI-SceI (accession number EU372690): mobilizable suicide plasmid containing a multiple cloning site (MCS) to clone DNA flanks of gene of interest, a R6Kγ origin of replication (*oriR6K*) and includes an I-SceI restriction site. Maintain in *E. coli* GT115 in LB media supplemented with trimethoprim at a final concentration of 50 μg/mL [7].
2. pMH447: pGPI-SceI derived plasmid for unmarked single copy genomic complementation by recombination into an aminoglycoside efflux pump (BCAL1674-BCAL1675). Maintain in *E. coli* GT115 in LB media supplemented with trimethoprim at a final concentration of 50 μg/mL [14].
3. pDAI-SceI-SacB: broad host range replicative plasmid expressing the I-SceI homing endonuclease and the counterselectable marker SacB [7, 18]. Maintain in *E. coli* DH5α in LB media supplemented with tetracycline at a final concentration of 20 μg/mL.
4. pRK2013: helper plasmid that provides *tra* and *mob* genes required for the mobilization of plasmids containing an *oriT* such as pDAI-SceI-SacB, pGPI-SceI, and derivatives by triparental mating. Maintain in *E. coli* DH5α in LB media supplemented with kanamycin at a final concentration of 40 μg/mL [19].

2.5 Primers

1. Primers used to create pDelatsR (listed 5′-3′, restriction site is underlined).
   - Upstream forward primer, Primer #1 TACGTCTAGA AAAAGCCTGCTGACAACCTG (XbaI).
   - Upstream reverse primer, Primer #2 TTTTCTCGAG CGAGGACCAGGATGATTTTT (XhoI).
   - Downstream forward primer, Primer #3 TTTTCTCGAG GTGCTCGATCTCGAACTGC (XhoI).
   - Upstream reverse primer, Primer #4 TTTTGAATTC CGCGATATCGAACGCTATTT (EcoRI).
2. pGPI-SceI sequencing primers.
   - Forward primer # 1300. TAACGGTTGTGGACAACAAGCCAGGG.
   - Reverse primer # 3627. GCCCTACACAAATTGGGAGATATATC.
3. Primers used to amplify *atsR* and create pMH447 + *atsR*.
   - Forward primer # 5. TTTTCATATGCCGCTCGGCGAAGCCAAGT (NdeI).
   - Reverse primer # 6. GTTTTCTAGATCAGGCGAGCAGTGTCTCGA (XbaI).

4. pMH447 sequencing primers.
   - Forward primer # 5885. TTGATGGCGAGCGATTCTTC.
   - Reverse primer # 5886. CCAGTTCTTCAGCGTGACGA.
5. Primers used to screen for *atsR* complementation.
   - Forward primer # 7. GTCAACGAGGGCGTGCTC.
   - Reverse primer # 8. GCGCTGGGCGAATTCATGAC.

2.6 Enzymes, Kits and Materials for Molecular Cloning

1. HotStar HiFidelity Polymerase Kit (Qiagen).
2. Purified genomic DNA from *B. cenocepacia* (*see* **Note 6**).
3. Taq DNA Polymerase.
4. dNTP mixture: containing the appropriate nucleotides and provided with the kit.
5. QIAquick PCR purification kit (Qiagen).
6. QIAprep Spin miniprep Kit (Qiagen).
7. Restriction enzymes (from any appropriate supplier) and Antarctic™ phosphatase (New England Biolabs).
8. Boiling water bath.
9. Microfuge.
10. Spectrophotometer.
11. T4 DNA ligase.
12. Thermocycler.
13. PCR tubes.
14. Agarose gel electrophoresis.
15. Gel electrophoresis equipment.
16. Sterile toothpicks.

3 Methods

3.1 Construction of Deletion Plasmid (pDelatsR)

1. Retrieve the DNA sequence of the gene of interest (*atsR*) plus at least 1,000 bp on each side.
2. Design two sets of primers (primers #1/#2 and primers #3/#4) to amplify 300–700-bp regions upstream and downstream of the gene of interest. Primer design can be achieved manually or using computer software such as Primer3 (http://primer3.wi.mit.edu/, [20]) that helps choose compatible primers. Primers should contain restriction sites not found within the target DNA fragments. Primer #1 and primer #4 must contain

a restriction site present in the MCS of pGPI-SceI (e.g., XbaI and EcoRI). Restriction site for primer #2 and primer #3 should be identical or compatible (e.g., XhoI). Primers should have a 4–6 nucleotides extension upstream the restriction sites to allow for effective restriction digestion of PCR fragments (*see* **Note 7**).

3. Perform PCR amplification of upstream and downstream fragments (*see* **Note 8**). The following conditions are routinely used in our laboratory to perform PCR used for cloning purposes.

   Reaction mix (Total volume 50 µL):

   - 26 µL dH_2O.
   - 10 µL 5× Q-Solution.
   - 10 µL 5× HotStar HiFidelity reaction buffer (dNTPs included).
   - 1 µL of 100 µM forward primer.
   - 1 µL of 100 µM reverse primer.
   - 1 µL of genomic *B. cenocepacia* DNA (50–200 ng/mL).
   - 0.5–1 µL HotStar HiFidelity Polymerase.

   Mix well by gentle pipetting up and down few times and place in thermocycler.
   Thermal cycling conditions: 95 °C for 10 min, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min/kb with a final extension step at 72 °C for 5 min.
   The upstream fragment of *atsR* is amplified with primer #1 and primer #2 while the downstream fragment is amplified with primer #3 and primer #4 (Fig. 1a). Check for successful and specific amplification of each fragment using agarose gel electrophoresis (2 µL of the PCR mix used to load the agarose gel).

4. Clean upstream and downstream fragments with a PCR purification kit and purify pGPI-SceI with a miniprep kit.
5. Digest pGPI-SceI DNA and PCR products with appropriate restriction enzymes (e.g., Digest pGPI-SceI with EcoRI and XbaI, the upstream PCR fragment with XbaI and XhoI, and the downstream PCR fragment with XhoI and EcoRI) for 2–24 h at 37 °C (*see* **Note 9**).
6. Clean digested fragments and pGPI-SceI with a PCR purification kit.
7. Optional but recommended: Treat digested pGPI-SceI with Antarctic™ phosphatase for 30 min at 37 °C (*see* **Note 10**).
8. Ligate digested plasmid DNA with digested upstream and downstream fragments. The ligation ratio of vector–insert 1–insert 2 should be 1:2:2. Ligation is performed at 4 °C overnight, typically in a volume of 20 µL.

9. Transform chemically competent *E. coli* GT115 with the entire ligation reaction following manufacturer's recommendations. Plate transformation reaction on LB agar supplemented with trimethoprim at a final concentration of 50 μg/mL, and incubate at 37 °C overnight.
10. Screen for positive cloning by colony PCR using screening method described below:

    Reaction mix (Total volume 50 μL):

    - 30 μL dH_2O.
    - 10 μL 5× Q-Solution.
    - 5 μL 10× Taq polymerase reaction buffer.
    - 1.5 μL of 100 mM dNTPs.
    - 1 μL of 100 μM forward primer (e.g., primer #1).
    - 1 μL of 100 μM reverse primer (e.g., primer #4).
    - 0.5–1 μL Taq DNA polymerase.

    Mix well by gentle pipetting and aliquot 10 μL into PCR tubes.

    DNA templates are prepared as follows:

    - Suspend one colony in 50 μL dH_2O.
    - Boil for 10 min.
    - Pellet debris by centrifugation for 1 min at 14,000 × *g*.
    - Add 1 μL of supernatant to PCR reaction mix and use the same thermal cycling conditions as in Subheading 3.1, **step 3**.
11. Extract plasmid from 1 to 3 positive clones and confirm that the DNA fragments cloned are devoid of any point mutation using DNA sequencing (*see* **Note 11**) with sequencing primers (primer # 1300 and primer # 3627) located on each side of the MCS of pGPI-SceI (*see* **Note 12**).
12. Prepare a bacterial stock of the correct clone (Fig. 1b) and store at –80 °C for future use (*see* **Note 13**).

3.2 Conjugation of Deletion Plasmids into Burkholderia

1. Grow donor strain (*E. coli* GT115 plus deletion vector) in LB broth supplemented with 50 μg/mL trimethoprim. Grow helper strain (*E. coli* DH5α carrying pRK2013) in LB broth supplemented with 40 μg/mL of kanamycin. Grow recipient bacteria (*B. cenocepacia*) in LB Broth. Overnight cultures should be grown at 37 °C with shaking (*see* **Note 14**).
2. After overnight growth, bacterial cultures should have an optical density between 2.5 and 5 OD_{600}. The ratio of donor: helper: recipient should be 3:3:1. Pellet 1 mL of donor strain, 1 mL of helper strain, and 330 μL of recipient strain by centrifugation (14,000 × *g*, 1 min). Combine and resuspend cell pellets in 1 mL of LB broth. Spin down and remove LB broth. Resuspend the pellet containing the mixture of the three strains

in 100 μL SOB broth and spot onto an SOB agar plate. Incubate SOB plate right face up at 37 °C overnight.

3. The next day scrape the bacterial spot and resuspend in 1 mL of sterile PBS. To ensure the development of isolated colonies, perform serial dilution in 1 mL PBS for up to 10^{-3}. Plate 100 μL of each dilution onto LB agar containing trimethoprim 100 μg/mL (to select for *Burkholderia* co-integrants) and gentamicin 50 μg/mL (to kill helper and donor *E. coli* containing helper and deletion plamids (*see* **Note 15**). Incubate plates at 37 °C for 2 days.
4. By day 2, trimethoprim resistant colonies should appear on plates (*see* **Note 16**). Pick 3–5 isolated colonies with sterile toothpicks and patch onto a fresh LB agar plate containing the same antibiotics. Incubate at 37 °C for 1 day. The next step is to resolve the co-integrates using the I-SceI endonuclease. To prepare for this step grow *E. coli* carrying pDAI-SceI-SacB in LB broth plus 20 μg/mL tetracycline and helper *E. coli* carrying pRK2013 in LB broth supplemented with kanamycin 40 μg/mL.
5. By day 3 scrape recipient bacteria (*Burkholderia* plus integrated suicide vector) from 3 to 5 patches and resuspend in 1 mL PBS. Centrifuge 1 mL of donor *E. coli* DH5α pDAI-SceI-SacB and 1 mL of helper *E. coli* pRK2013, 300 μL of recipient suspension at an OD_{600} of 2 and remove spent media. Resuspend three strains in 100 μL SOB broth and spot onto an SOB agar plate. Incubate SOB plate containing the mating reaction right face up at 37 °C overnight.
6. By day 4 scrape the bacterial spot and resuspend in 1 mL of PBS. Perform serial dilution in 1 mL PBS for up to 10^{-3}. Plate 100 μL of each dilution onto an LB agar containing tetracycline 150 μg/mL to select for *Burkholderia* carrying pDAI-SceI-SacB and gentamicin 50 μg/mL to kill helper or donor *E. coli*. Incubate plate at 37 °C for 2 days.
7. Patch 20 colonies onto two LB agar plates, one containing tetracycline at 150 μg/mL and gentamicin 50 μg/mL and the other containing trimethoprim 100 μg/mL. This step is performed to confirm excision of the deletion plasmid. Screen tetracycline resistant but trimethoprim sensitive colonies using upstream forward primer and downstream reverse primer (e.g., primer # 1 and primer # 4) (Figs. 1c and 3a).
8. To cure the replicative vector pDAI-SceI-SacB from *Burkholderia*, grow deletion mutants in LB broth without antibiotics. The next day perform serial dilution for up to 10^{-5}. Plate 50 μL of 10^{-3}, 10^{-4}, and 10^{-5} onto 5 % sucrose LB agar plates lacking salt (*see* **Note 17**). Incubate sucrose plate at 37 °C overnight.
9. Patch resulting isolated colonies onto LB agar and LB agar plus 150 μg/mL tetracycline. Tetracycline sensitivity is indicative of loss of pDAI-SceI-SacB. Streak cured mutants for isolation, stock and store at −80 °C for future use.

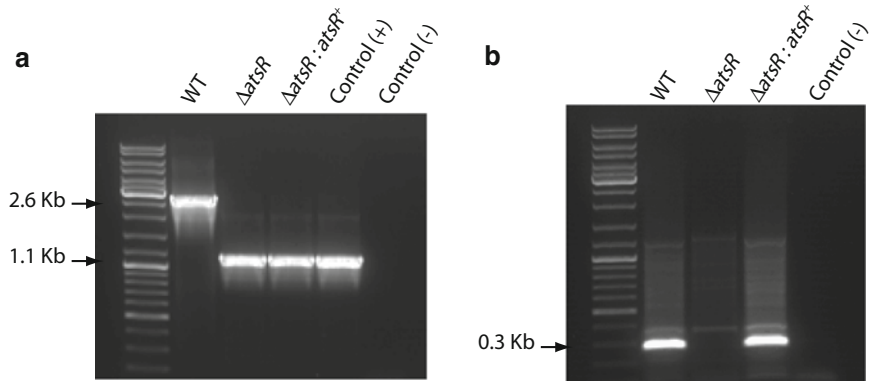

Fig. 3 PCR confirmation of successful gene deletion/gene complementation. (**a**) Confirmation of successful gene deletion. PCR analysis of K56-2 wild type (WT), *atsR* deletion mutant (Δ*atsR*), and complemented *atsR* deletion mutant (Δ*atsR* : *atsR*$^+$) using primer #1 and primer #4. Plasmid pDelatsR and no template were used as PCR Control (+) and Control (–), respectively. (**b**) Confirmation of successful gene complementation. PCR analysis of K56-2 wild type (WT), *atsR* deletion mutant (Δ*atsR*), and *atsR* deletion mutant complemented (Δ*atsR* : *atsR*$^+$) using primer #7 and primer #8. No template was used as PCR Control (–). Band of expected PCR product size is indicated by *arrows*

3.3 Construction of Complementation Plasmid (pMH447 + atsR)

1. Design primers to amplify the gene of interest (e.g., *atsR*). Forward primer should be designed with an NdeI restriction site while reverse primer should contain an XbaI site. The ATG start codon of the gene should be included as part of the NdeI site.
2. Perform PCR as indicated in Subheading 3.1, **step 3** using a High fidelity DNA polymerase and the forward and reverse primers (e.g., primer #5 and primer #6).
3. Check for successful and specific PCR amplification by agarose gel electrophoresis.
4. Digest PCR product and pMH447 with NdeI and XbaI.
5. Clean digested PCR product and pMH447 using QIAquick PCR purification kit.
6. Treat digested pMH447 with Antarctic™ phosphatase as indicated in Subheading 3.1, **step 7**.
7. Ligate digested inserts and pMH447 at a ratio of 1 vector to 2 insert in 20 μL.
8. Transform chemically competent *E. coli* GT115 with ligation reaction following manufacturer's recommendations. Plate transformation reaction on LB agar supplemented with trimethoprim at 50 μg/mL.

9. Screen resulting colonies for positive cloning using Taq DNA polymerase and the same forward and reverse primers (*see* Subheading 3.3, **step 1**).
10. Pick 3 positive clones, extract plasmids, and confirm the accuracy of the sequence using DNA sequencing and primers (primer #5885 and primer #5886) which are located on each side of the MCS of pMH447 within the flanking regions from *BCAL1674-BCAL1675*.

3.4 Conjugation of Complementation Plasmids into Burkholderia

Transfer of complementation plasmids is identical to transfer of deletion plasmid indicated in Subheading 3.2 except for the antibiotic selection (*see* **Note 18**).

1. During the first crossover (*see* Subheading 3.2, **step 3**) use a combination of trimethoprim 100 μg/mL and ampicillin 100 μg/mL, polymyxin B 25 μg/mL to select for *Burkholderia* co-integrates and kill *E. coli* helper and donor strains, respectively.
2. During the second crossover (*see* Subheading 3.2, **step 6**) use a combination of tetracycline at 150 μg/mL, ampicillin 100 μg/mL, and polymyxin B 25 μg/mL to select for *Burkholderia* containing pDAI-SceI and to kill *E. coli* helper or donor strains.
3. To identify complemented clones, patch 20 colonies each onto 3 LB agar plates; one containing tetracycline 150 μg/mL, ampicillin 100 μg/mL, and polymyxin B 25 μg/mL, the second containing trimethoprim 100 μg/mL and the third containing gentamicin 50 μg/mL. Colonies that are tetracycline resistant but gentamicin and trimethoprim sensitive are positive clones and represent the replacement of the gene of interest into the efflux pump. Confirm complementation by PCR using internal primers that are absent in the truncated/deleted target gene (e.g., primer #7 and primer #8) (Fig. 3b).
4. Cure the replicative vector pDAI-SceI-SacB as indicated in Subheading 3.2, **steps 8**, **9**. Streak cured mutants for isolation and stock at −80 °C for future use.

4 Notes

1. The *sacB* gene encodes a levansucrase from *Bacillus subtilis* that when expressed in gram-negative bacteria grown in the presence of 5 % (w/v) sucrose causes a lethal phenotype [21].
2. In our experience *N*,*N*-Dimethylacetamide is the best solvent for trimethoprim. However, due to its toxicity it is important to handle it with gloves and under a chemical hood to prevent both skin irritation and inhalation.

3. LMG 18863 in LMG collection (Ghent).
4. For manipulation of extremely antibiotic resistant *B. cenocepacia* strains such as *B. cenocepacia* J2315 or BC7 we recommend using the suicide plasmid pGPI-SceI-XCm (Accession number: KC409641) to construct deletion vectors. pGPI-SceI-XCm is derived from pGPI-SceI and carries a chloramphenicol resistance determinant and a *xylE* reporter gene [18]. pGPI-SceI-XCm is not stably maintained in *E. coli* GT115 and therefore should be maintained in *E. coli* SY327 instead of *E. coli* GT115 [22]. Selection for co-integrates in *B. cenocepacia* J2315 is achieved using trimethoprim and chloramphenicol at 200 and 400 μg/mL, respectively. To confirm co-integrates, bacterial colonies arising from the first crossover event should be sprayed with catechol, which turns colonies carrying pGPI-SceI-XCm vector into bright yellow color due to activity of *xylE* gene [18].
5. The R6K origin of replication (*oriR6K*) requires for its function a protein called π, which is encoded by the *pir* gene. This gene is in *Escherichia coli* by a prophage (λ *pir*) [22, 23].
6. *B. cenocepacia* genomic DNA is purified with a DNeasy Blood & Tissue Kit (Qiagen), according to manufacturer's instructions, but any other method to obtain high quality genomic DNA may be used.
7. Ideally choose restriction enzymes that have the same buffer compatibility to perform double digest of the PCR products or plasmid in a single reaction. Check after adding the restriction site and nucleotides extension that no hairpin can be formed with the rest of the primer sequence.
8. PCR of DNA sequences from *B. cenocepacia* genome are sometimes difficult due to its high GC content. We highly recommend using PCR kits specifically designed to amplify GC rich DNA. To minimize unwanted mutation during the amplification reaction, PCR products used for cloning are performed using a high fidelity DNA polymerase, like HotStar HiFidelity Polymerase Kit (Qiagen). We highly recommend using Q-solution supplemented with Qiagen PCR kits because it often enhances PCR amplification yields and specificity. Alternatively, if PCR fails to yield products or gives nonspecific amplification we recommend optimizing PCR by doing gradient PCR to identify optimal annealing temperatures. If the problem persists we recommend constructing a new second set of primers.
9. The time of digestion depends on the specific amplicon and is usually determined empirically by inability to ligate. Longer digestion times ensure complete digestion and better efficiency in the ligation step.
10. To prevent recircularization of partially digested vector during the ligation reaction and to improve cloning efficiency. Antarctic™ phosphatase can be heat-inactivated at 65 °C for

15 min and dephosphorylated plasmid can be used directly in the ligation mix, alternatively Antarctic™ phosphatase can be removed using a PCR purification kit.

11. Deletion plasmids should be sequenced to ensure that no unwanted mutations in the upstream and downstream flanks are introduced into the genome.
12. We have constructed a second version of pGPI-SceI vector named pGPI-SceI-2 (accession number: KC409642). pGPI-SceI-2 carries more unique restriction site that can be used for cloning allowing for more flexibility during the construction of deletion vectors [14].
13. For long-term storage, bacteria are suspended from heavily inoculated plates into LB and mix 1:1 with LB + 40 % glycerol to give a final glycerol concentration of 20 %.
14. Plasmids are transferred into *Burkholderia* by triparental mating. Triparental mating is performed by combining the donor strain (*E. coli* carrying a mobilizable vector, pGPI-SceI derivatives or pDAI-SceI-SacB), the helper strain (*E. coli* carrying pRK2013), and the recipient strain (*B. cenocepacia* K56-2). Following vector transfer into *Burkholderia*, exconjugants or co-integrates are selected for using antibiotic markers expressed from the mobilized vector while donor and helper *E. coli* is selected against using antibiotics to which *Burkholderia* is naturally resistant. Generally gentamicin is used to select against donor and helper *E. coli*. However, if deletion is performed in the gentamicin sensitive variant of *B. cenocepacia* K56-2 (MH1K) [18], or if complementation is performed using the suicide vector pMH447, ampicillin and polymyxin B should be used instead of gentamicin.
15. If the gene to be deleted encodes a protein that if absent is suspected to affect membrane permeability we recommend using ampicillin 100 μg/mL, polymyxin B 25 μg/mL instead of gentamicin 50 μg/mL to counterselect against donor and helper *E. coli* during conjugation step.
16. Failure to obtain deletion is often indicative that the gene to be deleted is essential for viability. To test if a gene is essential for viability we recommend constructing a conditional mutant using the rhamnose-inducible vector pSC200 [24, 25].
17. pDAI-SceI-SacB should be cured using sucrose counterselection since it can induce a growth lag and affect phenotypic characterization of mutant strains. We have found that sucrose counterselection is more efficient if bacterial suspensions are plated on LB without salt.
18. Since complementation integrates into the aminoglycoside efflux pump responsible for gentamicin resistance in *B. cenocepacia*, ampicillin and polymyxin B should be used instead of gentamicin to select against donor and helper *E. coli*.

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Chapter 19

Gene Inactivation in *Coxiella burnetii*

Paul A. Beare and Robert A. Heinzen

Abstract

Coxiella burnetii, the agent of human Q fever, is a zoonotic bacterial pathogen with a worldwide distribution. Owing to an historic lack of methods for genetic manipulation, virulence factors deployed by this bacterium for disease pathogenesis are poorly understood. However, the recent advance of host cell-free (axenic) growth of *C. burnetii* has coincided with development of several new genetic technologies including site-specific and random transposon systems, shuttle vectors, and an inducible gene expression system. We have recently added two methods for targeted gene inactivation to the expanding *C. burnetii* genetics toolbox. Here, we describe a "loop in/loop out" gene inactivation system for *C. burnetii*. This procedure allows for generation of site-directed mutants in approximately 10 weeks and has been used by our laboratory to generate more than 50 individual *C. burnetii* mutants. The collection of *C. burnetii* genetic tools now allows for conventional mutation and complementation strategies to define virulence factors.

Key words *Coxiella burnetii*, Electroporation, Genetic transformation, Targeted gene inactivation, Axenic media, Transposon, Counterselection, Homologous recombination

1 Introduction

Coxiella burnetii is a gram-negative bacterium that causes human Q fever. Q fever normally presents as an acute febrile illness but in rare cases *C. burnetii* infection can result in chronic disease that most frequently manifests as endocarditis [1]. The bacterium is an intracellular pathogen that replicates in a parasitophorous vacuole (PV) with phagolysosome-like characteristics [2, 3]. *C. burnetii* resists the degradative activities of this vacuole and modifies host cell functions, such as apoptosis signaling, that promote pathogen growth [4, 5]. Type IVA and IVB secretion systems are essential virulence factors of gram-negative bacteria that translocate proteins directly into the host cytosol where they alter eucaryotic functions [6]. To date, over 50 *C. burnetii* Type IVB secretion system (T4BSS) effector proteins have been identified [7–10], with two having defined anti-apoptotic activity [11, 12]. Moreover, *Himar1* transposon mutagenesis confirms type IVB secretion is essential for intracellular replication of *C. burnetii*. Specifically, mutation of the

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T4BSS structural genes *icmL* or *icmD* results in bacteria that are deficient in translocation of effectors and growth inside mammalian host cells [9, 13].

The original *Himar1* mutagenesis system for *C. burnetii* was developed using host cell-based propagation of the organism [14]. Although successful in generating mutants, the procedure was time consuming with technical hurdles associated with antibiotic selection, expansion, and cloning of genetic transformants. In addition, any mutant with significantly lowered fitness for growth in a eucaryotic cell was unlikely to be recovered. *C. burnetii*'s obligate reliance on a host cell for propagation was abolished upon development of an axenic (host cell-free) method of growth in acidified citrate cysteine medium (ACCM) [15, 16]. Under microaerobic conditions, a second generation ACCM (ACCM-2) now supports approximately 4 logs ($\log_{10}$) of *C. burnetii* growth in liquid media and the formation of small (~0.5 mm in diameter) colonies on solid media [16]. ACCM-based culture enabled rapid development of genetic tools for *C. burnetii* including RSF1010 *ori*-based shuttle vectors [8, 10], an improved *Himar1* transposon system [16], a Tn7-based system for single-copy, site-specific *in cis* complementation [13, 17], and a system for inducible gene expression using the anhydrotetracycline-inducible promoter, *tetA* [13].

We have recently added two procedures for generating targeted gene deletions to the *C. burnetii* genetic toolbox [18]. One method for gene deletion exploits Cre recombinase. Sequential rounds of transformation with suicide plasmids containing 34 bp *loxP* sites are first performed to incorporate *loxP* sites next to a gene targeted for deletion. A third transformation is then conducted using a suicide plasmid expressing Cre recombinase that promotes recombination between the *loxP* sites and subsequent deletion of the gene of interest (GOI). The other method involves a "loop in/loop out" strategy (Fig. 1). In this method, a suicide plasmid carrying an antibiotic resistance marker (e.g., Kan^r) cassette flanked by upstream and downstream regions of the GOI, and a counterselectable marker (the *sacB* gene), is inserted by homologous recombination into the genome (Fig. 1a). The plasmid is then removed by a second recombination event between the plasmid-coded flanking region and the reciprocal region in the genome, resulting in deletion of the wild type (WT) gene (Fig. 1b). This loop-in/loop-out strategy uses the counterselectable marker *sacB* that confers sensitivity to sucrose and allows for positive selection of bacteria that have undergone the second recombination event. Our laboratory has found this method to be highly effective in deleting genes in both virulent and avirulent *C. burnetii*. Here, we describe the loop in/loop out protocol for creating marked gene deletions in *C. burnetii* (Fig. 2).

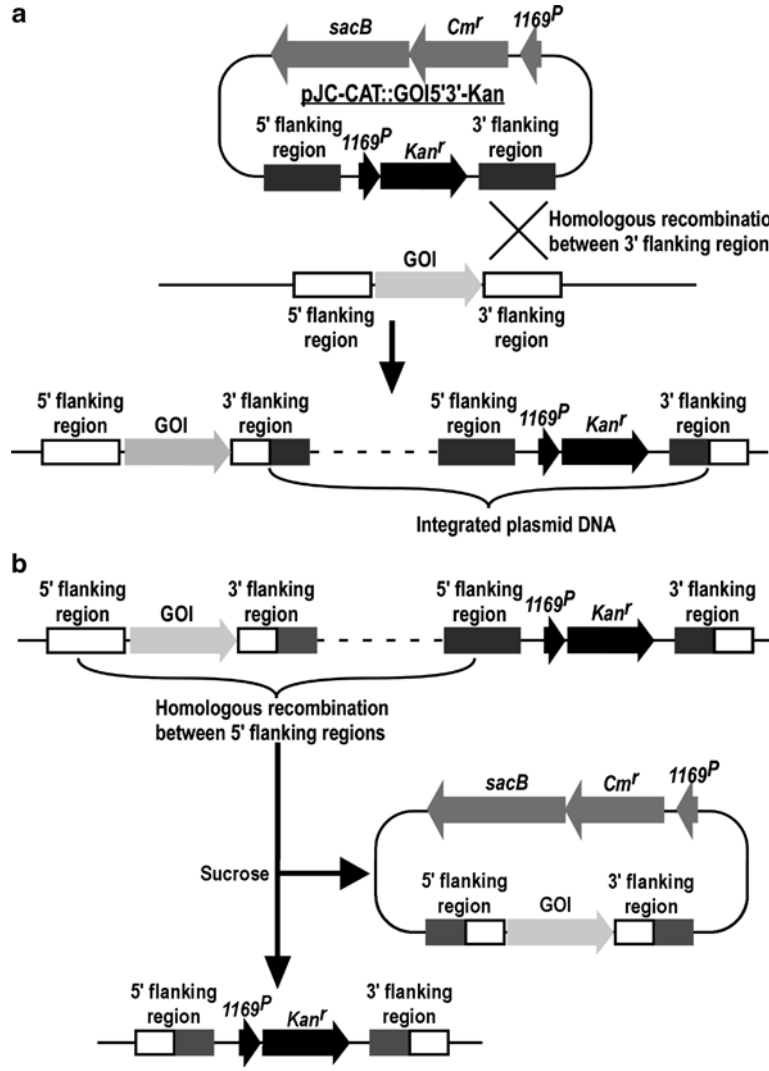

Fig. 1 Schematic of the loop in/loop out gene deletion method. (**a**) Integration of pJC-CAT::GOI5′3′-Kan by homologous recombination between the 3′ flanking region on the plasmid and the corresponding region in the genome. Integration can also be mediated by the 5′ flanking region (not shown). (**b**) Resolution of the co-integrant by a second homologous recombination event between the 5′ flanking region of the integrated plasmid and the reciprocal region in the genome. Selection in sucrose-containing media aids in recovery of transformants having undergone the second recombination event by eliminating bacteria that carry *sacB*. The kanamycin cassette now replaces the gene of interest (GOI)

2 Materials

2.1 Construction of Suicide Plasmids

1. DNA oligonucleotide primers (*see* **Notes 1** and **2**). Primers designed to amplify the 5′ (5′F and 5′R) and 3′ (3′F and 3′R) flanking regions (~2,000 bp) (Fig. 3a).
2. In-Fusion kit (BD Clontech).
3. Stellar chemically competent *E. coli* cells (BD Clontech) (*see* **Note 3**).

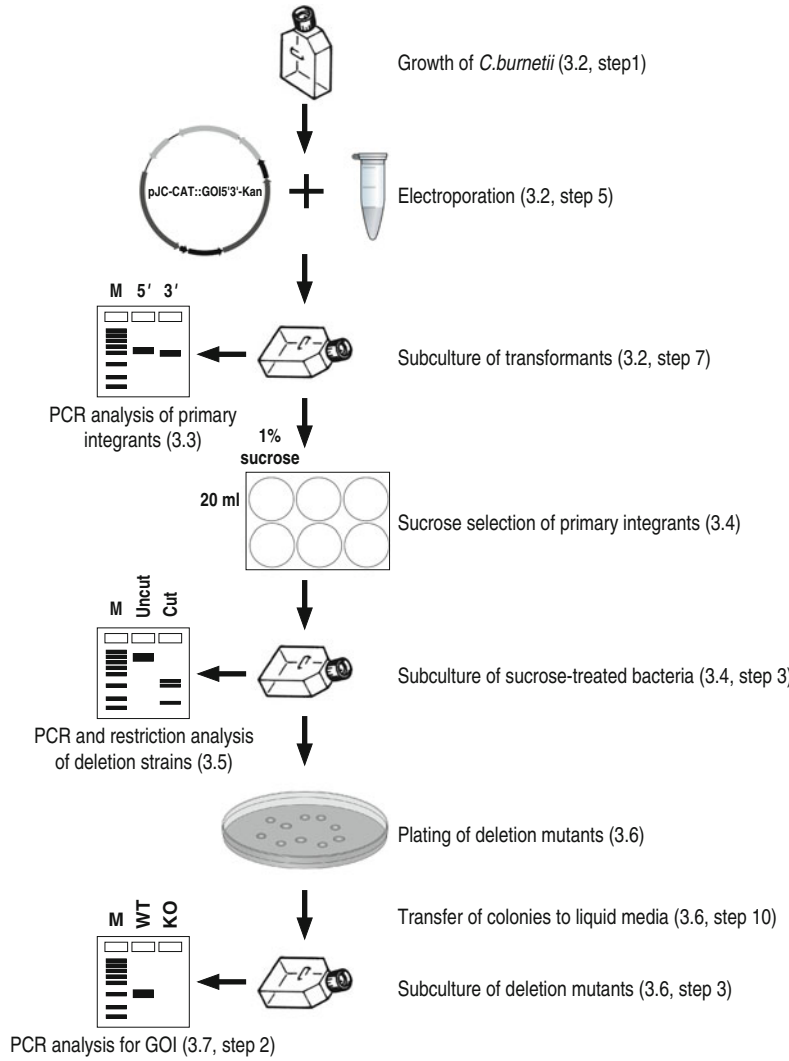

Fig. 2 Strategy for the creation of targeted gene deletions in *C. burnetii.* Numbers in parentheses denote the specific subheading or step in Subheading 3 where each procedure in described

4. Chloramphenicol and kanamycin for selection of *E. coli* transformants: 10 μg/mL and 50 μg/mL final concentrations, respectively.
5. Transformation vectors pJC-CAT and pJB-Kan (available from the Heinzen lab).
6. *C. burnetii* Nine Mile (phase II) genomic DNA (gDNA) (*see* **Note 4**).
7. Accuprime *pfx* DNA polymerase for PCR.
8. Nucleospin PCR and Gel Cleanup kit or other agarose gel DNA extraction and PCR Cleanup kits.

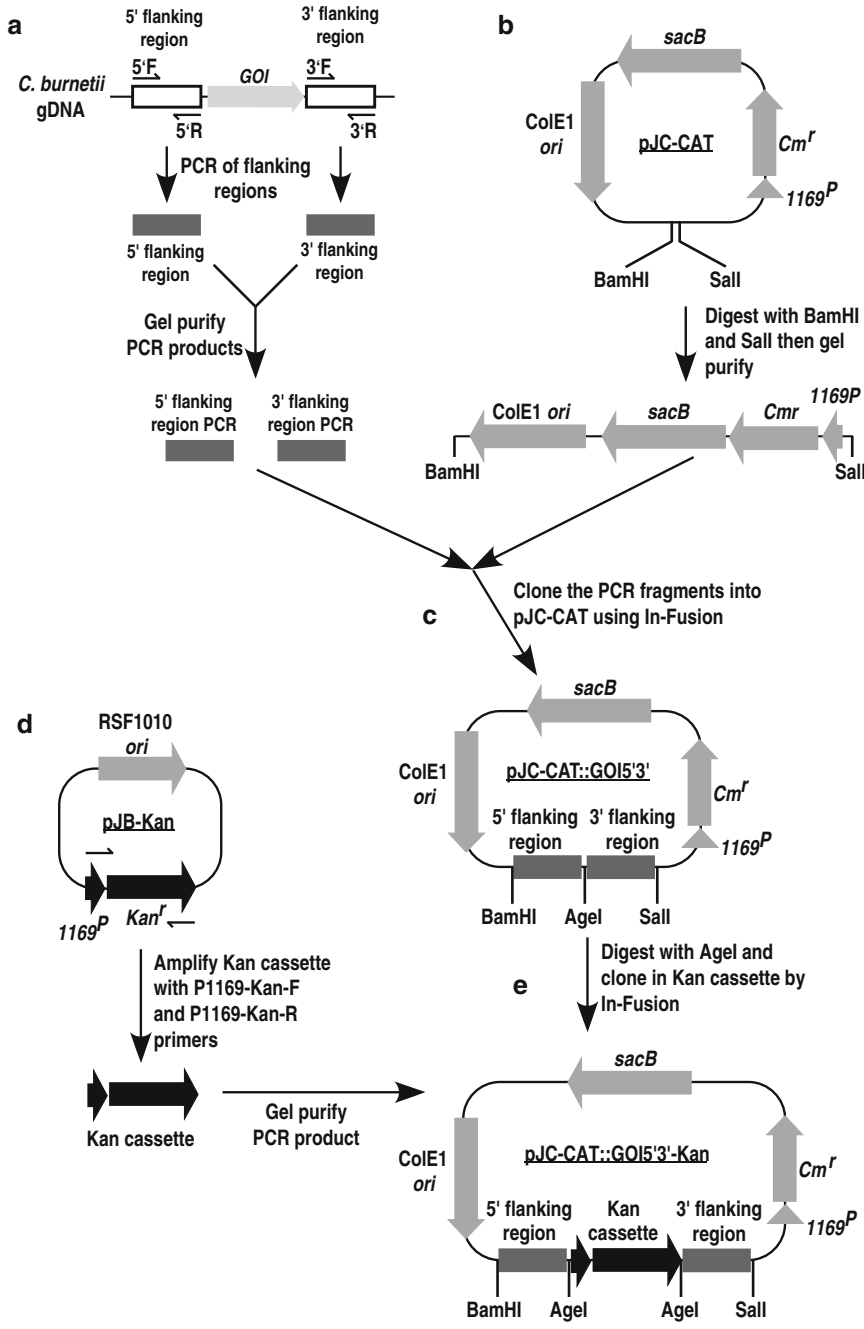

Fig. 3 Construction of the pJC-CAT-based gene deletion suicide plasmid. (**a**) PCR is conducted with primer pairs 5′F/5′R and 3′F/3′R to amplify ~2,000 bp of 5′ and 3′ DNA flanking the *C. burnetii* gene of interest (GOI). Each PCR product is purified using a Nucleospin PCR and Gel cleanup kit. (**b**) The plasmid pJC-CAT is digested with BamHI and SalI and the linearized 3,754 bp plasmid is purified using a Nucleospin PCR and Gel cleanup kit. The locations of BamHI and SalI restriction sites are shown. (**c**) The 5′ and 3′ fragments are cloned into pJC-CAT using an In-Fusion cloning kit. A unique AgeI restriction site is created in construction of pJC-CAT::GOI5′3′. (**d**) The kanamycin cassette is amplified from pJB-Kan by PCR using primers P1169-Kan-F and P1169-Kan-R. The ~1,000 bp kanamycin cassette is purified using a Nucleospin PCR and Gel cleanup kit. (**e**) The plasmid pJC-CAT::GOI5′3′ is digested with AgeI and the linearized plasmid purified using a Nucleospin PCR and Gel cleanup kit. The kanamycin cassette is cloned into AgeI-digested pJC-CAT::GOI5′3′ using an In-Fusion kit. The locations of BamHI, SalI, and AgeI restriction sites are shown

9. BamHI, SalI, and AgeI restriction enzymes.
10. Luria Broth for growth of *E. coli*: 10 g tryptone, 5 g yeast extract, 10 g NaCl. Add to 1 L of deionized water and autoclave. For LB agar, add 15 g/L of agar prior to autoclaving.
11. 300 mL Erlenmeyer flasks for growing 100 mL cultures of *E. coli* transformants.
12. Microfuge and floor model high-speed centrifuge.
13. Plasmid DNA can be purified using various kits. We routinely use the QIAprep Miniprep kit (Qiagen) for small scale plasmid purification and the GenElute HP endotoxin-free plasmid maxiprep kit (Sigma) for large scale plasmid purification.
14. Amicon Ultra-0.5, Ultracel-30 columns (Millipore) for plasmid DNA concentration.

2.2 Transformation of C. burnetii and Selection of Deletion Mutants

1. ACCM-2 (1 L) for broth culture of *C. burnetii*: 2,568 mg citric acid, 4,740 mg sodium citrate, 500 mg potassium phosphate, 200 mg magnesium chloride, 13.2 mg calcium chloride, 2.78 mg iron sulfate, 7,280 mg sodium chloride, 263.4 mg l-cysteine, 100 mg Bacto Neopeptone, 2,500 mg casamino acids, 1,000 mg methyl-β-cyclodextrin, 125 mL RPMI w/GlutaMAX, 875 mL deionized H_2O. Sterilize by filtration and store at 4 °C (*see* **Note 5**).
2. O_2/CO_2 MCO-19 M tri-gas incubator (Sanyo). Set to 37 °C, 5.0 % CO_2 and 2.5 % O_2.
3. *C. burnetii* freezing medium: RPMI containing 10 % DMSO and 10 % FBS.
4. Biosafety cabinet (*see* **Note 6**).
5. 6-well tissue culture plates, 75-cm^2 (T75) and 25-cm^2 (T25) cell-culture flasks.
6. Sterile 50 and 250 mL O-ringed screw cap centrifuge tubes/bottles.
7. Electroporation cuvettes (0.1-cm gap width).
8. BTX ECM Exponential Decay Wave Electroporation System (*see* **Note 7**).
9. 10 % glycerol electroporation solution. Sterilize by filtration and store at room temperature.
10. Chloramphenicol and kanamycin for selection of *C. burnetii* transformants: 3 μg/mL and 350 μg/mL final concentration, respectively (*see* **Note 8**).
11. 50 % sucrose: 50 g sucrose in 100 mL of sterile deionized water. Sterilize by filtration and store at room temperature.
12. Oligonucleotide primers 2 (5′-CGGAGAACCTGCGTGC AATCCATC) and 3 (5′-TGCTGAAGAGCTTGGCGGCG)

specific to the kanamycin resistance gene (*nptII*). Also design and purchase primers specific to regions upstream (primer 1) and downstream of the 5′ and 3′ GOI flanking regions, respectively, for PCR analysis of potential primary integrants (Fig. 4a).

13. Accuprime *taq* DNA polymerase for PCR.
14. Phosphate-buffered saline at pH 7.2: 1.54 mM potassium phosphate monobasic, 155.17 mM sodium chloride, 2.71 mM sodium phosphate dibasic.

2.3 Analysis of Coxiella Gene Knockouts

1. 2× ACCM-2 (500 mL) and agarose for *C. burnetii* plating: 2,568 mg citric acid, 4,740 mg sodium citrate, 500 mg potassium phosphate, 200 mg magnesium chloride, 13.2 mg calcium chloride, 2.78 mg iron sulfate, 7,280 mg sodium chloride, 263.4 mg l-cysteine, 100 mg Bacto Neopeptone, 2,500 mg casamino acids, 1,000 mg methyl-β-cyclodextrin, 125 mL RPMI w/GlutaMAX, 375 mL deionized H_2O. Sterilize by filtration and store at 4 °C. Agarose (0.5 %, 500 mL): 2,500 mg of Ultrapure agarose, 500 mL deionized H_2O. Sterilize by autoclaving and store at 55 °C prior to use.
2. 100 mm × 20 mm petri dishes (Corning).
3. 5 mL Falcon tubes.
4. 6-well and 24-well tissue culture plates, T75 and T25 cell culture flasks (Corning).
5. MO BIO Microbial DNA kit or another genomic DNA isolation kit.
6. Accuprime *taq* DNA polymerase for PCR.
7. Agarose gel electrophoresis.

3 Methods

3.1 Construction of Gene-Specific Suicide Plasmids

1. Design forward (F) and reverse (R) primers to amplify the 5′ and 3′ regions (~2,000 bp) flanking your GOI (*see* **Notes 1** and **2**). Add the following linkers to the 5′ end of each primer: 5′F—CGGTACCCGG**GGATCC**, 5′R—CACC<u>ACCGGT</u>CG ACGCGAGCGTCGAG, 3′F—CGTCG<u>ACCGGT</u>GGTGCG CATGTACGTC, 3′R—GAACCTGTTT**GTCGAC**. The bolded nucleotides of 5′ and 3′ primers indicate the BamHI and SalI restriction sites (*see* **Note 9**), respectively, while the underlined nucleotides indicate the AgeI restriction site used later for cloning of the kanamycin gene cassette (*see* **Notes 10** and **11**).
2. Amplify by PCR the 5′ and 3′ regions flanking regions using *C. burnetii* gDNA as template (Fig. 3a) (*see* **Note 12**). Use the following cycling conditions: 94 °C (2 min) followed by 35 cycles of 94 °C (30 s), 55 °C (45 s), and 68 °C (3 min), then 68 °C (4 min).

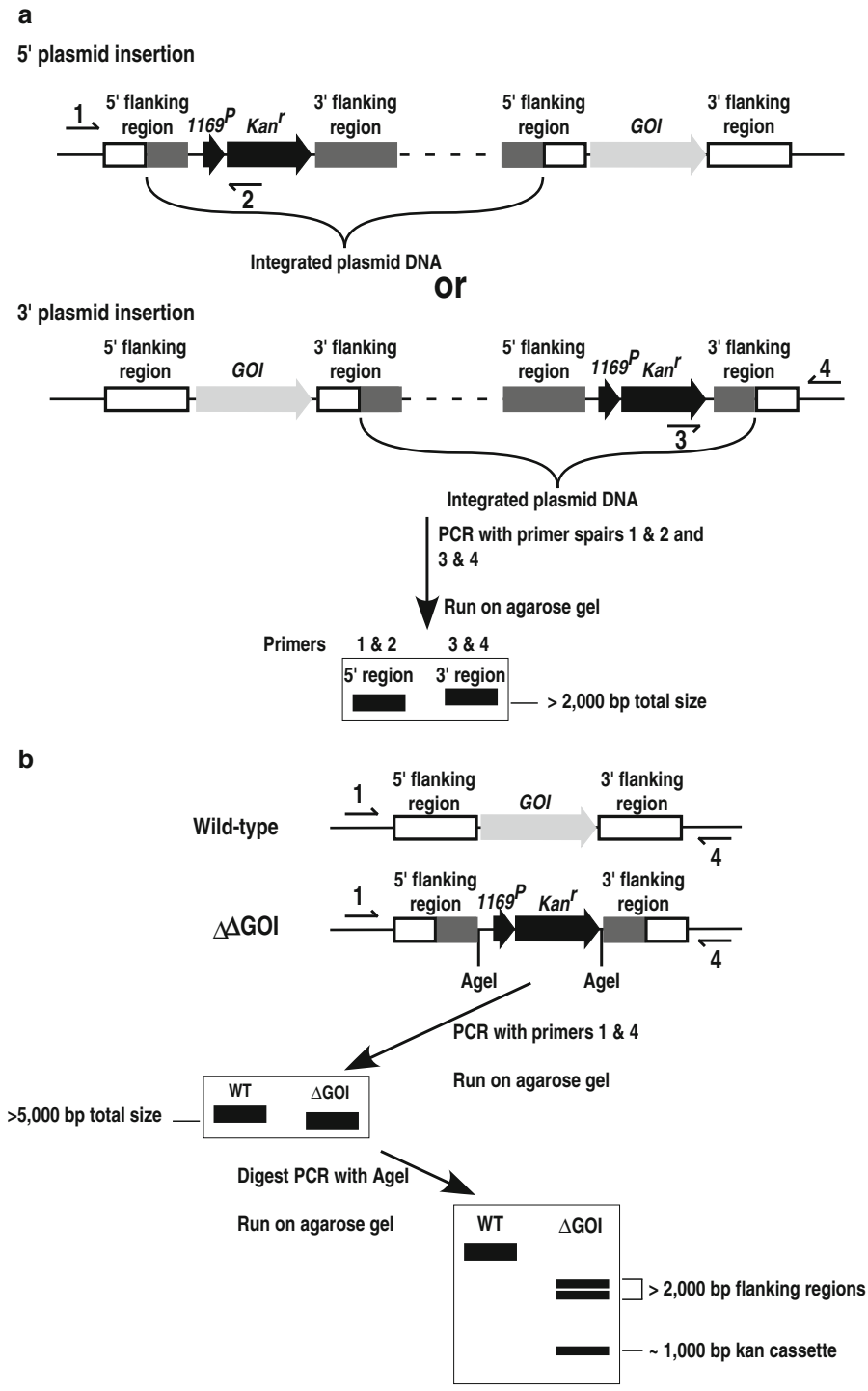

Fig. 4 PCR analysis of *C. burnetii* containing a plasmid co-integrant or gene knockout. (**a**) Predicted PCR results following a 5′ or 3′ flanking region-mediated insertion of pJC-CAT::GOI5′3′-Kan into the *C. burnetii* genome (Subheading 3.2). PCR is conducted with primer pairs 1/2 and 3/4 to determine whether a 5′ or 3′ plasmid insertion, respectively, has occurred. PCR products are visualized on an agarose gel. A band >2,000 bp indicates the presence of an integrated plasmid. (**b**) Predicted PCR results following sucrose counterselection

3. Purify PCR fragments by gel purification using a Nucleospin PCR and Gel Cleanup kit.
4. Linearize 10 μg of pJC-CAT in a 100 μl reaction with BamHI/SalI using a buffer recommended by the supplier for 4 h at 37 °C. Purify using a Nucleospin PCR and Gel Cleanup kit (Fig. 3b).
5. Clone PCR products containing the 5′ and 3′ flanking regions into linearized pJC-CAT by In-Fusion. Cloning results in the formation of an internal AgeI site between the 5′ and 3′ flanking regions and creation of pJC-CAT::GOI5′3′ (Fig. 3c).
6. Transform competent *E. coli* cells with 2 μl of the In-Fusion reaction and plate bacteria on LB-agar containing chloramphenicol. Incubate overnight at 37 °C (*see* **Note 3**).
7. Pick an *E. coli* transformant, grow in Luria broth overnight at 37 °C, and isolate plasmid DNA (*see* **Note 13**).
8. Amplify the *1169^{P}-Kan* cassette (~1,000 bp) from pJB-Kan by PCR using the primers P1169-Kan-F (5′-GCTCGCGTCG ACCGGTATGGCTTCGTTTCGCAGCGAACTTGG-3′) and P1169-Kan-R (5′-CATGCGCACCACCGGTTTATCAGAAG AACTCGTCAAGAAGGC-3′) (Fig. 3d). The AgeI restriction site is underlined. Use the following cycling conditions: 94 °C (2 min) followed by 35 cycles of 94 °C (30 s), 55 °C (45 s), and 68 °C (2 min), then 68 °C (3 min).
9. Purify the kanamycin cassette by gel extraction using a Nucleospin PCR and Gel Cleanup kit.
10. Linearize 2 μg of pJC-CAT::GOI5′3′ in a 50 μl reaction with AgeI using a buffer recommended by the supplier for 4 h at 37 °C and gel purify with a Nucleospin PCR and Gel Cleanup kit.
11. Clone the kanamycin cassette into AgeI-digested pJC-CAT::GOI5′3′ by In-Fusion to create pJC-CAT::GOI5′3′-Kan (Fig. 3e).
12. Transform competent *E. coli* cells with 2 μl of In-Fusion reaction and plate bacteria on LB plates containing kanamycin.
13. Pick an *E. coli* transformant, grow in Luria broth, and isolate plasmid DNA.

Fig. 4 (continued) using primers 1 and 4 (Subheading 3.5) and DNA from transformed bacteria and wild type (WT) DNA. Different sized bands will be amplified if the GOI and kanamycin cassette (~1,000 bp) are different sizes. The resulting PCR products are digested with AgeI and run on an agarose gel. A PCR product generated from WT DNA should remain uncut (depending on the presence of AgeI cut sites). The presence of a ~1,000 bp band indicates the presence of the kanamycin cassette and a deleted GOI. In deletion strains, at least two additional bands will also be present. Representative genome structures of WT and deletion strains are shown

14. Inoculate 100 mL of Luria broth containing kanamycin with the cloned *E. coli* transformant and incubate overnight at 37 °C with shaking at 220 rpm.
15. Isolate plasmid DNA using the GenElute maxiprep kit. Elute DNA in 2.5 mL deionized H_2O.
16. Concentrate plasmid DNA using an Amicon Ultra-0.5, Ultracel-30 column. Add 500 µl of the plasmid solution to the column, centrifuge at 14,000 × *g* for 6 min, then discard the flow-through. Repeat until all plasmid DNA solution has been run through the column. Wash twice with 500 µl of deionized H_2O. Place column upside down in a new microfuge tube and spin at 1,000 × *g* for 1 min to collect the concentrated plasmid DNA.

3.2 Transformation of C. burnetii

1. Add *C. burnetii* to 200 mL of ACCM-2 at a concentration of ~2×10^6 *C. burnetii* genome equivalents per mL. Aliquot 20 mL of the bacterial suspension to each of ten T75 flasks. Incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days (*see* **Note 14**). *C. burnetii* should grow to a final density of 1×10^9–1×10^{10} genome equivalents per mL.
2. Combine cultures from the T75 flasks and pellet bacteria by centrifugation (16,000 × *g*, 16 min) in a sterile 250 mL O-ringed screw cap centrifuge bottle. Wash bacteria by resuspending the pellet in 30 mL of 10 % glycerol.
3. Pellet bacteria again by centrifugation (16,000 × *g*, 16 min) in a sterile 50 mL O-ringed screw cap centrifuge tube. Resuspend pellet in 1 mL of 10 % glycerol and transfer to a sterile 1.5 mL microfuge tube, and store on ice (*see* **Note 15**).
4. Aliquot 50 µL of the *C. burnetii* suspension into a 1.5 mL microfuge tube and store on ice. Add 10–20 µg of plasmid DNA.
5. Transfer the *C. burnetii*/DNA mix to a prechilled 0.1-cm gapped electroporation cuvette. Electroporate at 18 kV, 25 µF, and 500 Ω (*see* **Note 7**).
6. Add 950 µL of RPMI tissue culture medium to the cuvette and mix.
7. Add 150 µL of electroporated bacteria to 6 mL of ACCM-2 containing 1 % fetal bovine serum (FBS) in a T25 flask and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 24 h (*see* **Note 16**).
8. Add chloramphenicol and kanamycin to the culture and incubate for an additional 6 days.
9. Transfer 1 mL of transformation culture to 6 mL of fresh ACCM-2 (containing chloramphenicol and kanamycin) in a T25 flask and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days.

10. Transfer 50 μL of culture to 6 mL of fresh ACCM-2 (containing chloramphenicol and kanamycin) in a T25 flask and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days (*see* **Note 17**).
11. Pellet bacteria by centrifugation (4,000 × *g*, 15 min), resuspend in 250 μL *C. burnetii* freezing medium, and freeze at −80 °C.

3.3 PCR Confirmation of Primary Integrants

1. Pellet 1 mL of *C. burnetii* culture from Subheading 3.2, **step 10** (16,000 × *g*, 10 min) and resuspend bacteria in 30 μL of PBS.
2. Add 3 μL of the *C. burnetii* suspension directly to Accuprime *taq* PCR reaction mix containing either primer 2 and a primer that anneals upstream of the 5′ flanking region (primer 1), or primer 3 and a primer that anneals downstream of the 3′ flanking region (primer 4) (Fig 4a). Use the following cycling conditions: 94 °C (10 min) followed by 35 cycles of 94 °C (30 s), 55 °C (45 s), and 68 °C (3 min), then 68 °C (4 min).
3. Electrophorese PCR products on a 0.8 % agarose gel. A plasmid integration event 5′ of the GOI will result in a band slightly larger than 2,000 bp when using primers 1 and 2. A band of similar size is expected for 3′ plasmid integrants when using primers 3 and 4 (*see* **Note 18**).

3.4 Sucrose Selection of Deletion Strains

1. Add 50 μL of culture from Subheading 3.2, **step 10** (or 1 μL of frozen stock culture from Subheading 3.2, **step 11**) to 6 mL of fresh ACCM-2 containing only kanamycin in a T25 flask and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 6 days.
2. Add 3 mL of ACCM-2 containing kanamycin and 1 % sucrose to one well of a 6-well plate, inoculate with 20 μl of culture and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 4 days (*see* **Note 19**).
3. Inoculate 6 mL of fresh ACCM-2 (containing kanamycin) in a T25 flask with 200 μL of the sucrose-treated culture. Incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days.
4. Transfer 50 μL of culture into 6 mL of fresh ACCM-2 (containing kanamycin) in a T25 flask and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days.
5. Pellet bacteria by centrifugation (4,000 × *g*, 15 min), resuspend in 250 μL of *C. burnetii* freezing medium, and store at −80 °C.

3.5 PCR Confirmation of Gene Deletion

1. Pellet 1 mL of bacteria from Subheading 3.4, **step 4** (centrifuge at 16,000 × *g* for 10 min) and resuspend in 30 μL of PBS.
2. Add 3 μL of resuspended bacteria directly to Accuprime *taq* DNA polymerase mix containing primers specific to sequences upstream and downstream of the 5′ (primer 1) and 3′ flanking regions (primer 4). As a control, carry out PCR using primers 1 and 4 on 100 ng of WT DNA. Conduct PCRs using the

following cycling conditions: 94 °C (10 min) followed by 35 cycles of 94 °C (30 s), 55 °C (45 s), and 68 °C (6 min), then 68 °C (7 min).

3. Electrophorese PCR products on a 0.8 % agarose gel. Expect a single band of greater than 5,000 bp for each PCR (*see* **Note 20**).
4. Digest the PCR products with AgeI using buffer 1 for 2 h at 37 °C to release the kanamycin gene cassette.
5. Electrophorese PCR products on a 0.8 % agarose gel. Expect a band of approximately 1,000 bp containing the kanamycin gene cassette. Other band(s) will be present depending on the presence of AgeI sites in the PCR fragment (*see* Fig. 4b).

3.6 Cloning of Deletion Mutants

1. Mix 10 mL of 2× ACCM-2 (preheated to 37 °C and containing appropriate antibiotics) with 10 mL of 0.5 % agarose. Pour into a 100 mm by 20 mm petri dish and allow to cool for ~30 min (*see* **Note 21**).
2. Air-dry the petri dish for ~20 min uncovered in a biosafety cabinet.
3. Mix 1.25 mL of 2× ACCM-2 and 0.75 mL sterile water in a 5 mL Falcon tube. Add appropriate antibiotics, place in 37 °C water bath.
4. Make tenfold serial dilutions of the sucrose-treated culture (*see* Subheading 3.4, **step 4**) in ACCM-2 and add 10 μL of each dilution to the 5 mL Falcon tubes prepared in **step 3** (*see* **Note 22**).
5. Add 0.5 mL of 0.5 % agarose (stored at 55 °C), mix and immediately pour on top of 0.5 % base agar prepared in **step 2**.
6. Allow petri dishes to cool until top agarose solidifies (~20 min). Place at 4 °C for 20 min.
7. Air-dry for ~20 min with the lid off in a tissue culture hood.
8. Incubate petri dishes at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 6 days (*see* **Note 23**).
9. Under a stereomicroscope, demark location of ~0.5 mm individual *C. burnetii* colonies on the bottom of the petri dish with a marker pen.
10. Enlarge the end of a P200 Rainin pipette tip by cutting off the tip. Within a biosafety cabinet, use a P200 Rainin pipettor to remove individual colonies and place each in 1 mL of ACCM-2 in individual wells of a 24-well plate.
11. Disrupt each colony by mixing with a Rainin P1000 pipette and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days (*see* **Note 24**).

12. Transfer the entire sample into 3 mL of ACCM-2 containing kanamycin in one well of a 6-well plate and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days.
13. Transfer 50 μl of culture to a T25 flask with 6 mL of ACCM-2 containing kanamycin and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days. Repeat passage until growth is visible (this may require 2–3 more passages in ACCM-2).
14. Once growth is visible, inoculate 20 mL of ACCM-2 containing kanamycin in a T75 flask with 100 μl of culture and incubate at 37 °C in 5.0 % CO_2 and 2.5 % O_2 for 7 days.
15. Pellet (centrifuge at 4,000 × *g* for 15 min) 10 mL of the bacteria and resuspend in 250 μL of *C. burnetii* freezing medium and store at −80 °C (*see* **Notes 25–29**).

3.7 Verification of GOI Deletion

1. Pellet (centrifuge at 4,000 × *g* for 15 min) 10 mL of the remaining culture from Subheading 3.6, **step 14** and use the MO BIO Microbial DNA isolation kit to extract genomic DNA. We add an additional heating step (85 °C for 30 min) after **step 3** of the manufacturer's protocol. Elute the DNA in 50 μL of sterile deionized H_2O.
2. Use 5 μL of the gDNA sample in a PCR reaction with primers specific to the deleted gene and a PCR reaction with primers for a control gene (this can be any other *C. burnetii* gene). Use the following cycling conditions: 94 °C (2 min) followed by 35 cycles of 94 °C (30 s), 55 °C (45 s), and 68 °C (2 min), then 68 °C (3 min). Electrophorese 20 μL of the PCR reaction on a 0.8 % agarose gel. Only the control PCR should produce a product. Also run control PCRs for each GOI primer set using *C. burnetii* gDNA.

4 Notes

1. Primers must be compatible with the In-Fusion HD cloning system.
2. The In-Fusion cloning system is a homologous recombination-based method that fuses a PCR product with a linearized plasmid vector by recombination with a common 15 bp sequence in the 5′ and 3′ ends of the PCR product and linearized vector. The following Web site has a primer design tool for In-Fusion cloning: http://bioinfo.clontech.com/infusion/convertPcrPrimersInit.do.
3. Other chemically competent *E. coli* cells can be substituted throughout this protocol. However, if using competent *E. coli* strains other than Stellar for transformation with In-Fusion

reactions, the reaction mix should be diluted 1:10 before transformation because the In-Fusion enzyme is toxic for other *E. coli* strains.

4. The *C. burnetii* Nine Mile, phase II, (clone 4) strain (RSA439) is exempt from US Centers for Disease Control (CDC) and Prevention, Division of Select Agents and Toxins (DSAT) regulations and can be manipulated under biosafety level 2 laboratory conditions [19]. All other *C. burnetii* isolates are considered biosafety level 3 organisms and a CDC, DSAT select agent (*see* the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories, 5th edition).
5. Avoid using ACCM-2 more than 1 week old. Media should be made fresh.
6. All manipulations of *C. burnetii* should be conducted in a biosafety cabinet.
7. A Bio-Rad Gene Pulser XL Electroporation system can be substituted.
8. Genes conferring resistance to chloramphenicol, kanamycin, or ampicillin are approved for *C. burnetii* genetic transformation studies in our laboratory by the Rocky Mountain Laboratories Institutional Biosafety Committee and the CDC, DSAT program. Researchers should follow their local and national regulations.
9. BamHI and SalI are used to linearize the pJC-CAT plasmid prior to cloning in the 5′ and 3′ flanking regions, but BglII, SacI, AvaI, XmaI, SmaI, and XbaI could also be used. Use of the In-Fusion cloning system eliminates problems with internal BamHI or SalI restriction sites as the 5′ and 3′ flanking region PCR products are not cut with restriction enzymes prior to cloning.
10. The AgeI restriction site used to insert the kanamycin cassette into pJC-CAT::GOI5′3′ can be changed to any other restriction site that is absent in the plasmid. However, 5′ and 3′ flanking region and kanamycin cassette PCR primers (5′R, 3′F, P1169-Kan-F, and P1169-Kan-R) must be redesigned accordingly by changing the sequence of the underlined region of these primers. This site is introduced during cloning of the 5′ and 3′ flanking regions.
11. The *1169^{P}-Kan* cassette consists of the 185 bp CBU1169 promoter fused to the 795 bp kanamycin resistance gene (*nptII*).
12. The 5′ and 3′ flanking region PCR products contain complementary sequences at their 3′ and 5′ ends, respectively. Thus, the flanking regions can be fused by splice overlap PCR prior to cloning into pJC-CAT. Complementary sequences allow for annealing of flanking region PCR products, that then serve as

primers for subsequent round of PCR that splices together the two fragments. the following cycling conditions: 94 °C (2 min) followed by 35 cycles of 94 °C (30 s), 55 °C (45 s), and 68 °C (4 min), then 68 °C (5 min).

13. Sequencing should be conducted to verify all constructs.
14. 200 mL of bacterial suspension is split into ten T75 flasks to provide a greater surface to air interface, which improves *C. burnetii* growth. This volume is enough for ~20 individual transformations.
15. Approximately 1×10^{10} genome equivalents per milliliter of *C. burnetii* are used per electroporation.
16. The addition of 1 % FBS helps prevent sticking of hydrophobic avirulent phase II *C. burnetii* to the bottom of the T25 flasks and improves transformation frequency. FBS is not necessary for transformation of virulent phase I *C. burnetii*, which are hydrophilic.
17. Multiple passages are required to reduce the level of untransformed bacteria.
18. The *C. burnetii* transformant mix will contain both 5′ and 3′ integrants; thus, expect positive PCR reactions for both pairs of primers. For some GOIs, the suicide plasmid will incorporate predominantly into only one flanking region. This will be reflected in the resulting PCR products.
19. Due to the unknown number of genome equivalents in the bacterial suspension and the frequency of homologous recombination required for "looping out" the plasmid containing the GOI, we routinely use two different volumes of bacterial suspension (20 and 50 μL) in 3 mL of ACCM-2 containing kanamycin and treat these with either 0.5 or 1 % sucrose to make certain we get the desired gene deletion.
20. If there is a significant size difference between the GOI and the kanamycin cassette, a deleted GOI may be detected prior to cloning by comparing the size of PCR products generated with WT and transformant DNA. An AgeI digest of the transformant PCR product will result in release of the ~1,000 bp kanamycin cassette and two other bands of ~2,000 bp. AgeI digest of the WT PCR product will result in a single >5,000 bp band, unless an internal AgeI site is present in the GOI, in which case multiple kb bands may be visible.
21. ACCM-2-agarose plates should be made fresh on the day you plate *C. burnetii*.
22. Cloning can also be conducted by limiting dilution. A 6 day culture is diluted 1:10,000 in ACCM-2, and then twelve 1:3 dilutions are made in a 96-well plate containing 120 μL of ACCM-2 per well. Wells with detectable growth at the highest

dilution are then expanded and tested for a deleted GOI (as per Subheading 3.7).

23. Although *C. burnetii* colony size varies, there appears to be no correlation between colony size and the ability for expansion of the bacterium.
24. Disruption of the agarose plug containing the *C. burnetii* colony is very important. We often repeat this process 2 days after the initial extraction.
25. The steps in Subheading 3.2 can be used for transforming *C. burnetii* with other DNA's (e.g., shuttle vectors, Tn7 or *Himar1*), with the only change being the antibiotic used at each step.
26. Ampicillin can also be used for selection in ACCM-2 (at a concentration of 350 μg/mL).
27. When transforming with the *C. burnetii* shuttle vector, 6-well plates can be used in Subheading 3.2, **step 7**. Add 100 μL of the RPMI/electroporation mix to 3 mL of ACCM-2 containing 1 % FBS in one well of a 6-well plate. This is due to the increased frequency of transformation with replicating plasmid DNA.
28. For unmarked deletions, kanamycin is not added in **steps 1–5**, Subheading 3.4 or in all steps of Subheading 3.6. You may need to screen more clones for a deletion mutant but one should expect a ~50:50 ratio of WT to deletion mutant.
29. To store samples for subsequent infection of tissue culture cells, a 6 mL, 6 day ACCM-2 culture is washed three times in PBS and resuspended in 250 μl cell freezing media (RPMI, 10 % FBS, 10 % DMSO).

Acknowledgement

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Chapter 20

A Chemical Mutagenesis Approach to Identify Virulence Determinants in the Obligate Intracellular Pathogen *Chlamydia trachomatis*

Bidong Nguyen and Raphael Valdivia

Abstract

Our understanding of how most microbes "work" is hindered by the lack of molecular genetic and recombinant DNA tools to manipulate their genomes. We devised an approach to perform genetic analysis in one such microbe, the obligate intracellular bacterial pathogen *Chlamydia trachomatis*. Comprehensive libraries of clone-purified mutants with distinct plaque morphologies were generated through chemical mutagenesis. Whole-genome sequencing (WGS) was then employed to identify the underlying genetic lesions and to draw correlations between mutated gene(s) and a common phenotype. Taking advantage of the ability of *Chlamydia* to exchange DNA in co-infection settings, we then generated recombinant strains after co-infection of mammalian cells with mutant and wild type bacteria. In this manner, causal relationships between genotypes and phenotypes were established. The pairing of chemically induced gene variation and WGS to establish correlative genotype–phenotype associations should be broadly applicable to a large list of medically and environmentally important microorganisms currently not amenable to genetic analysis.

Key words *Chlamydia*, Genetic analysis, Whole genome sequencing, Chemical mutagenesis, Genetic mapping

1 Introduction

Recombinant DNA and molecular genetics have accelerated our understanding of many biological processes by providing the means to specifically inactivate genes and to analyze the resulting phenotypes. Unfortunately, many important microbes remain intractable to routine molecular genetic manipulation. For example *Chlamydia trachomatis*, a pathogen responsible for most sexually transmitted infections and infectious blindness (trachoma), remains poorly characterized and it is unclear the extent to which individual *Chlamydia* genes contribute to evasion of innate immunity, replication within infected tissues, transmission, or other processes important for the pathogen's survival within a mammalian host.

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To address this problem, we developed a methodology that combines classical chemical mutagenesis approaches with Next Generation DNA Sequencing to draw phenotype–genotype correlations in *Chlamydia*.

The relatively small size (~1 Mb) and lack of repetitive sequences make genomes of *Chlamydia* spp. ideal for whole genome sequencing (WGS) by a variety of Next Generation DNA sequencing platforms. Indeed, over 60 Chlamydiae species and biovars have been sequenced, providing unique insights into the evolution of chlamydial species and their adaptation to their hosts [1–3]. In some instances, mechanistic information as to the potential function of virulence factors has also been inferred [4, 5]. Unfortunately, the genetic diversity displayed by clinical isolates still does not provide the resolution required to systematically map the function of all *Chlamydia* genes.

Mutagen-induced gene variation, coupled with defined assays that measure defects in virulence, can expand the spectrum of mutations than can be surveyed without confounding effects from natural selection. Chemical mutagens, in particular, are useful as they can generate null, conditional, hypomorphic, and hypermorphic alleles. These mutants can then be clonally isolated by adapting plaquing techniques used for animal viruses [6], and the resulting DNA lesions mapped by WGS. Overall, mutagenesis, clone purification, and DNA lesion mapping enable the application of routine forward genetic approaches to study *Chlamydia* virulence.

The protocol described below addresses two separate steps in *Chlamydia* genetic analysis [7]. Firstly, we describe how to generate and clone mutants, and how to derive *correlations* between mutations in a gene or genetic pathway from among many mutants that share a phenotype. Secondly, we describe how to generate *Chlamydia* recombinants based on a system for natural DNA exchange within infected cells. In this manner true *genetic linkage* can be established between a mutation and a phenotype (Fig. 1).

2 Materials

2.1 Bacteria and Cell Culture

1. Vero cells (ATCC CCL-81).
2. DMEM (Dulbecco's Modified Eagle Media) supplemented with 10 % FBS (Fetal Bovine Serum).
3. *Chlamydia trachomatis* (strain *L2*/434/Bu/*ATCC* VR902B) (*see* **Note 1**).
4. *Chlamydia trachomatis* strains that are resistant to one of the following: rifampin, spectinomycin, trimethoprim.
5. Antibiotics: rifampin, spectinomycin, and trimethoprim.

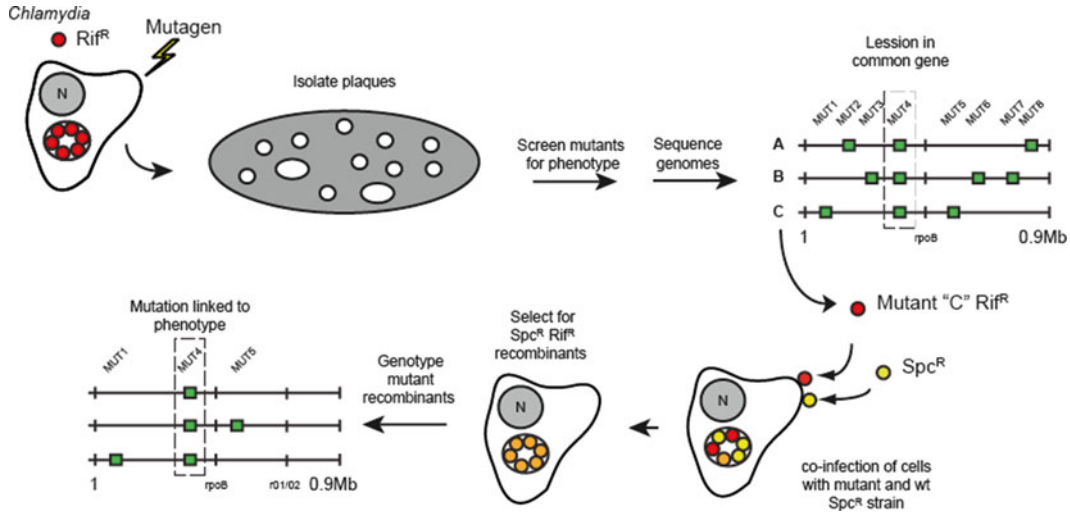

Fig. 1 Overall strategy for forward genetic analysis and recombination-based mapping in *Chlamydia*. Rifampin resistant (Rif^R) *C. trachomatis* was mutagenized during its replicative stage and mutagenized EBs harvested. These EBs were used to infect Vero cell monolayers until visible plaques formed. Individual mutant clones were collected and assayed for specific phenotypes, such as altered plaque morphologies. The genomes of mutants sharing a common phenotype were sequenced to identify common genetic lesions. To establish linkage between these gene lesions and the phenotype of interest, recombinant Rif^R Spc^R strains were selected in the presence of rifampin and spectinomycin after co-infection of Vero cells with mutants generated in a Rif^R and a wild-type Spc^R strain ("crosses"). The segregation of individual mutations present in the parental mutant strain—as defined by WGS—among the recombinant bacteria displaying the desired phenotype was determined by targeted DNA sequencing. (Reproduced with permission from PNAS [7])

2.2 Chemical Mutagenesis

1. Ethyl methyl sulfanate (EMS). In a chemical safety hood, prepare 20 mg/mL ethyl methyl sulfanate (EMS) in sterile phosphate buffered saline containing (PBS) supplemented with 0.493 mM $MgCl_2$ and 0.901 mM $CaCl_2$. (*see* **Note 2** for safety considerations and proper disposal of mutagens).
2. 1 and 5 M NaOH.
3. Cycloheximide (200 μg/mL in dH_2O).
4. Gentamicin (25 mg/mL in dH_2O).
5. PBS + $MgCl_2$/$CaCl_2$: Phosphate buffered saline (PBS) with 0.493 mM $MgCl_2$ and 0.901 mM $CaCl_2$, pH 7.4 (sterile, readymade).
6. 5× SPG buffer: 1.25 M sucrose, 50 mM sodium phosphate, 25 mM glutamic acid, pH 7.4.
7. 25 cm^2 tissue culture flasks (T25 flasks).
8. Microfuge tubes (sterilized).

2.3 Plaque Purification

1. 2 % Molecular Biology Grade Agarose in dH_2O. Sterilize by autoclaving.
2. 2× DMEM buffered with a final concentration of 0.74 % sodium bicarbonate, pH 7.4. Filter sterilized.
3. Cycloheximide (200 μg/mL in dH_2O).
4. 0.54 % agarose/DMEM: 18.9 mL cold buffered 2× DMEM pH 7.4, 4.2 mL Fetal Bovine Serum (FBS), 0.42 mL 100 Nonessential amino acids mix (NEAA), 0.042 mL 200 μg/mL cycloheximide, 0.021 mL 50 mg/mL gentamicin. Mix well.
   Mix in 18.9 mL of hot sterile 1.2 % agarose/H_2O, stirring to prevent clumps. Mixture should be warm to touch. Keep in a warm water bath at 55 °C before adding to infected cells.
5. Phosphate buffered saline (PBS), pH 7.4.
6. Gentamicin (50 mg/mL in dH_2O).
7. SPG buffer, pH 7.4: 0.25 M sucrose, 10 mM sodium phosphate, 5 mM glutamic acid.
8. 96-well tissue culture plates.
9. 6-well tissue culture plates.
10. 1.0 mL sterile barrier pipette tips.
11. Ice.

2.4 Whole Genome Sequencing

1. Genomic DNA purification kits (e.g., Qiagen).
2. Fluorometer.
3. Adaptive Focused Acoustics S220 instrument (Covaris) (*see* **Note 3**).
4. Construction kit for preparation of genomic sequencing libraries.
5. Optional: barcoded primers.
6. Graphic user interface software (*see* **Note 4**).

2.5 Equipment

1. Chemical safety hood.
2. Class 2 Microbiological Safety Cabinet.
3. Centrifuge and adaptors for centrifuging tissue culture plates.
4. Dissection microscope.
5. Humidified 37 °C incubator with 5 % CO_2.
6. Hemocytometer.

3 Methods

3.1 Chemical Mutagenesis

1. For a schematic of mutagenesis procedures refer to Fig. 2. Seed approximately 1×10^6 Vero cells (as tittered in a hemocytometer) per T25 flask in 3 mL of DMEM/10 % FBS.

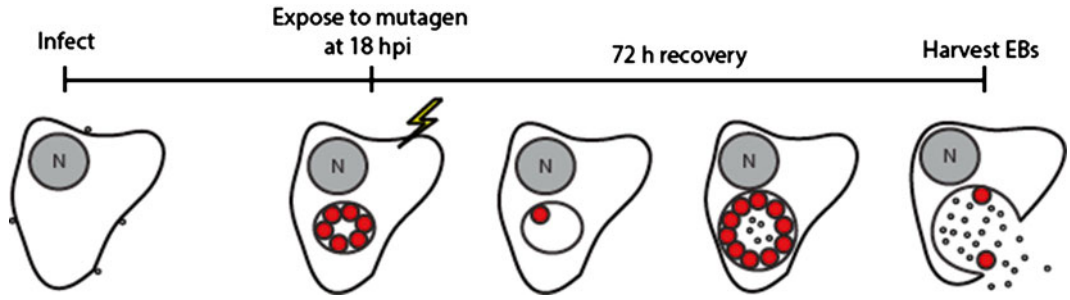

Fig. 2 Schematic representation of EMS mutagenesis protocol. *Chlamydia trachomatis*-infected cells were exposed to EMS during the RB stage of the infectious cycle, and the infection was allowed to proceed for 72 h to allow for the generation of infectious elementary bodies (EB). Mutagenized EB pools were harvested and tittered for inclusion forming units (IFU) and plaque-forming units on Vero monolayers. *N* nucleus. (Reproduced with permission from PNAS [7])

Incubate in a humidified cell culture incubator at 37 °C 5 % CO_2. Cells should form a confluent monolayer within 24 h (*see* **Note 5**).

2. Aspirate media. Infect confluent T25 flasks of Vero cells with *Chlamydia* at a multiplicity of infection (moi) of five ($\sim 14 \times 10^6$) in a total volume of 3 mL of media (DMEM, 200 ng/mL cycloheximide, 10%FBS) (*see* **Note 6**).
3. Begin mutagenesis at 18 h post infection (hpi). Aspirate media and wash cells once with 3 mL of PBS + $MgCl_2$/$CaCl_2$. Incubate cells with 3 mL of 20 mg/mL EMS in PBS + $MgCl_2$/$CaCl_2$ for 1 h in the chemical hood at room temperature (*see* **Notes 2** and 7).
4. Remove media with EMS and place it in a closed container containing NaOH, to detoxify the mutagen (*see* **Note 2**).
5. Wash cells three times in 3 mL PBS + $MgCl_2$/$CaCl_2$ to remove residual mutagen. Treat washes as EMS waste and place them in a closed container containing NaOH.
6. Add 6 mL of DMEM/10 % FBS containing 200 ng/mL cycloheximide and 25 μg/mL gentamicin, and incubate at 5 % CO_2 37 °C for 48–72 h (*see* **Note 8**).
7. Completely aspirate media. *Hypotonic Lysis*: Add 1.0 mL H_2O and rock to dislodged cells for 10 min. Lyse Vero cells by pipetting up and down at least ten times.
8. Collect lysates in 1.5 mL microfuge tubes and add 0.250 mL of the concentrated storage buffer 5× SPG to bring it to a 1× final concentration. Store at −80 ° C.

3.2 Clonal Isolation of Chlamydia Mutant Strains by Plaque Purification

1. Seed ~0.4×10^6 cells in 3 mL DMEM/10 % FBS per well in a 6-well plate and in a humidified cell culture incubator at 37 °C 5 % CO_2. Allow cells to form a confluent monolayer over the next 24 h.
2. Thaw stock solution of mutagenized *Chlamydia* on ice. Perform 6×10-fold serial dilutions in a total volume of 200 µl per dilution in DMEM/10 % FBS media and place them on ice (*see* **Note 9**).
3. Wash Vero cells twice with 3 mL PBS per well.
4. Add 3 mL DMEM to each well for 6-well plates and 100 µl of the bacterial dilutions in duplicate. Swirl the plate to ensure even mixture.
5. Centrifuge infected plates at $2{,}700 \times g$ for 30 min at 15 °C then incubate at 37 °C in a humidified, 5 % CO_2 incubator for 1–2 h.
6. Prepare 0.54 % agarose/DMEM (for 6 wells).
7. Aspirate bacterial suspension from dishes and apply 6 mL 0.54 % agarose/DMEM per well. Allow agarose to solidify completely at room temperature outside the hood for 15 min. Dry the plates with the lids removed in the Microbiological Safety Cabinet for 15 min (*see* **Note 10**).
8. Incubate at 37 °C in CO_2 incubator for 7–10 days. Plaques should be visible with the aid of a dissecting microscope. Refer to Fig. 3 for typical images of plaques.
9. The day prior to clone purifying mutant plaques, seed 1×10^4 Vero cells in 100 µl per well in a 96-well plate. Cells will be confluent within 24 h.
10. Using a dissection microscope, mark plaques to be picked. Plug plaques using a sterile 1.0 mL barrier pipette tip.
11. Resuspend plaques in 100 µl of DMEM supplemented with 10 % FBS, 400 ng/mL cycloheximide, 50 µg/mL gentamicin.
12. To expand mutant strains, overlay the suspension onto confluent monolayers of Vero cells. Centrifuge plates at $2{,}700 \times g$, 15 °C for 30 min.
13. Incubate plates at 37 °C 5 % CO_2 in a humidified incubator. Harvest EBs when >50 % cells show visible inclusions, which can occur as early as 48 hpi and up to 14 days (*see* **Note 11**).
14. Extract EBs by Hypotonic Lysis of infected cells: Completely aspirate media. Add 160 µl of sterile water. Incubate at room temperature for 10 min. Disrupt cells by pipetting up and down several times to ensure complete lysis, use barrier tips to avoid cross contamination. Transfer 160 µl lysates to microfuge tubes.

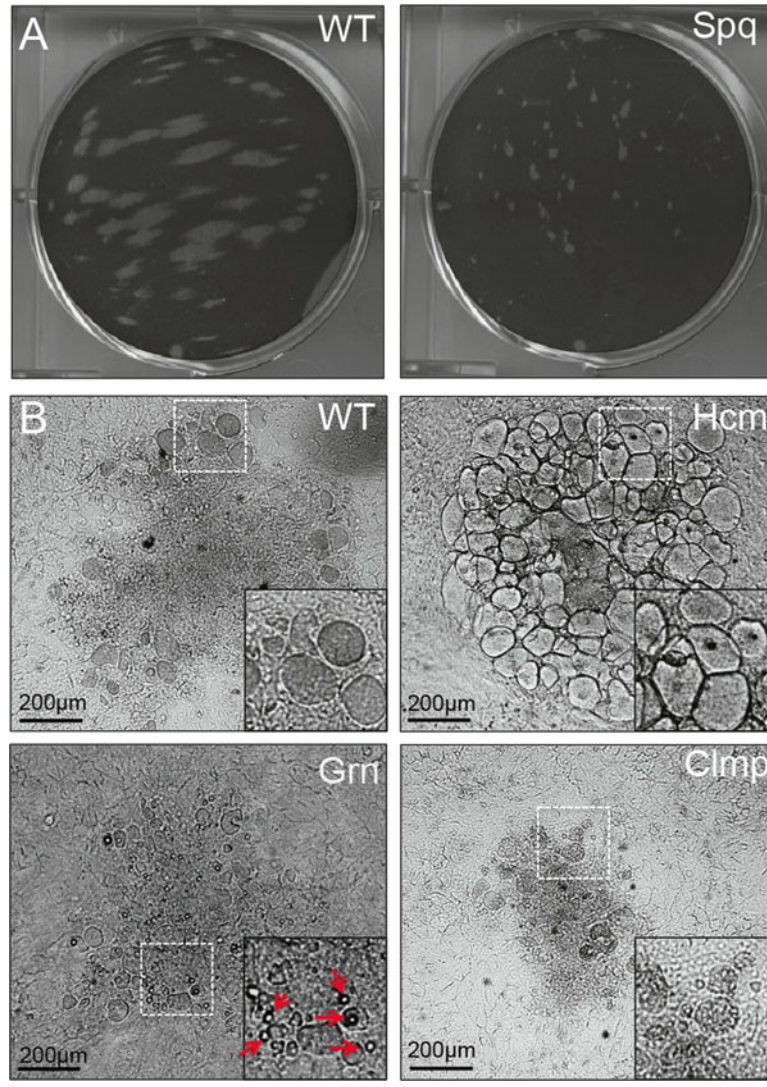

Fig. 3 Examples of common plaque morphologies among EMS-mutagenized *C. trachomatis*. Vero cell monolayers were infected with limited dilutions of mutagenized *C. trachomatis* and overlaid with agar. Infected monolayers were incubated for 10–14 days until visible plaques began to form. Plaques variants with altered size (**a**) and morphologies (**b**) were picked, amplified in Vero cells, and re-plaqued on Vero monolayers to confirm the stability of the plaque morphotypes. Examples of common phenotypes are shown including honeycomb (*Hcm*), clumped (*Clmp*), small plaque (*Spq*), and granular (*Grn*). *Arrows* indicate large granular deposits within a Grn plaque. A majority of Grn plaques were determined to bear mutations in the glycogen branching enzyme GlgB (Reproduced with permission from PNAS [7])

15. Mix in 40 µl of 5× SPG. Store at −80 °C.
16. Assay and screen mutants for phenotype(s) of interest.

3.3 Whole Genome Sequencing of Selected Chlamydia Mutants

1. Extract genomic DNA from gradient-purified EBs. Protocols for large scale culture of *Chlamydia* and purification of EBs can be found in ref. [8]. Contaminating host DNA is first removed from crude EB extracts by DNase treatment:
   (a) Lyse a T25 flask of Vero cells infected with mutant strain of interest for 2 days by hypotonic lysis: Completely aspirate media. Quickly rinse with 1 mL of sterile water. Aspirate liquid completely. Add 1.0 mL of sterile water and incubate at room temperature for 10 min, occasionally rocking the flask. Disrupt cells by pipetting up and down several times to ensure complete lysis.
   (b) Transfer lysates to 1.5 mL microfuge tube and pellet by centrifugation at top speed (25,000 × *g*) for 5 min. Decant supernatant and resuspend pellet in a total volume of 180 µl of dH_2O.
   (c) Add 20 µl of 10× DNase buffer and 4 U/mL RQ1 DNase and incubate at 37 °C for 60 min.
   (d) Inhibit the DNase by adding EDTA to a final concentration of 2 mM and incubate at 65 °C for 10 min. Samples should be ready for genomic DNA purification.
2. Use commercial genomic DNA purification kits to extract DNA from 2×10^9 bacteria following manufacturer's instructions.
3. Use a fluorometer to accurately measure the amount of DNA. 1–5 µg of purified DNA is required for the Illumina sequencing platform (*see* **Note 12** for whole genome sequencing platforms).
4. Fragment DNA to appropriate size (300–400 bp for Illumina sequencing) using an Adaptive Focused Acoustics S220 instrument (Covaris) according to the manufacturer's suggested settings.
5. Prepare the genomic sequencing libraries using commercial construction kits, following the instructions of the manufacturer. Libraries can be indexed using barcoded primers such that multiple samples can be pooled and sequenced simultaneously.
6. Run sequencing samples. This step is usually performed by commercial services or core facilities operated by dedicated technical staff. Follow their procedures and recommendations.
7. Illumina reads (FASTQ format) can be assembled to a reference genome using user-friendly graphic user interface software (*see* **Note 4**) which can be also used for SNP/mutation identification. To map mutations, set parameters to 90 % for minimum variant frequency and 50× for minimum coverage.

Open source genome assemblers, such as MAQ [9] and BWA [10] can also be used.

8. Confirm mutations by Sanger sequencing: use genomic DNA as template for PCR amplification of 300–500 bp regions flanking identified mutations and sequence purified or diluted (1:20) PCR products by Sanger sequencing (*see* **Note 13**).

3.4 Generation of Chlamydia Recombinants (See Note 14)

1. Co-infect wild type and mutant strains, each with a different antibiotic resistance marker, by centrifuging 6×10^5 bacteria from each strain onto confluent monolayers of Vero cells grown on a 24-well plate. In parallel, infect monolayers with each strain alone (*see* **Notes 1** and **15** for rationale of using marked strains).
2. At 44 hpi, harvest EBs by hypotonic lysis of infected cells: Completely aspirate media. Add 0.4 mL of sterile water and let stand for 10 min. Lyse cells by vigorous pipetting up and down for at least ten times. Transfer lysates to a microfuge tube.
3. Mix in 0.1 mL of 5× SPG for a final concentration of 1× SPG.
4. Crude EB lysates can be used immediately or stored at 80 ° C.
5. Plaque 50 µl of the crude EB preps and 5 × 1:10 serial dilutions in agarose/DMEM supplemented with appropriate antibiotics to select for recombinants. (*see* Table 1 for concentrations of typical antibiotics for selection of recombinants).
6. Plug out plaques, isolate and enrich recombinant strains as in Subheading 3.2. Score recombinants for presence or absence of phenotype. Genotype recombinants for the presence or absence of mutations present in the parental strains.
7. Crosses can be repeated to further segregate mutations, and generate isogenic strains. Co-infect selected recombinants to another wild type strain bearing a different antibiotic marker.

Table 1
Antibiotic concentrations for selection of recombinants

Final concentration	Preparation instructions
200 ng/mL rifampin	Make 25 mg/mL storage stock in dimethyl sulfoxide (DMSO). Make up 200 µg/mL working solution by diluting the storage stock with H_2O. Store at –20 °C in the dark
200 µg/mL trimethoprim	Make up 100 mg/mL in DMSO
200 µg/mL spectinomycin	Make up 100 mg/mL stock in water and aliquot into 100 µL stocks and store at 20 °C. Avoid repeated freeze–thaw

4 Notes

1. *Chlamydia trachomatis* is a BSL2 pathogen. Refer to your institutions' standard operating procedures (SOP) for handling such pathogens. Segregation of mutations by recombination requires selection for antibiotic resistant recombinant progeny. We recommend using antibiotic resistant strains (e.g., rifampin, spectinomycin, or trimethoprim) for generating mutants for the ability to perform recombinant analysis and to isolate isogenic strains. Antibiotic resistant strains can be generated by a stepwise selection process [11, 12].
2. EMS is a potent mutagen and carcinogen. Work with mutagen, including solution preparation, incubation, and washes should be performed in a chemical safety cabinet as EMS is a volatile liquid. Neutralize EMS waste and spills in a final concentration of 1 M sodium hydroxide, which can be done by adding 1.0 mL of 5 M sodium hydroxide to 4 mL of EMS waste in a closed container. Decontaminate any dry waste, such as plastic, paper, and glassware, with 1 M sodium hydroxide in an open beaker. Allow 24 h for complete neutralization. Consult institutional guidelines for proper disposal of chemical wastes.
3. This machine is generally available by commercial services or core facilities. If not available, nebulization or transposon-based methods for DNA fragmentation would be acceptable.
4. We recommend Geneious (Biomatters) for genome assemblies but other opens source genome assemblers, such as MAQ [9] and BWA [10], can also be used.
5. Vero cells are able to survive this concentration of EMS and conditions outside the incubator for short periods. Remember to include a control sample that has not been mutagenized.
6. For *Chlamdyia* infections of tissue culture cells we recommended supplementing the growth medium with the eukaryotic translation inhibitor cycloheximide in order to maximize bacterial replication (as host cell resources will be diverted away due to inhibition of protein synthesis) and improve the recovery of bacteria after mutagen treatment.
7. We found that the replicative form, the reticulate bodies (RB) is more amenable to chemical mutagenesis than the infectious form, the elementary body (EB). At mid-cycle (between 18 to 20 hpi), RBs are at the greatest numbers prior to RB-EB transition. Because *Chlamydia* is an obligate intracellular pathogen the effects of the mutagen on the host health can limit bacterial recovery. Vero cells were found to be more resistant to the adverse effects of high levels of EMS than other cell lines tested.
8. Inclusions appear devoid of bacteria about 3–6 h after mutagenesis. At 72 h, about 10 % of inclusions are filled with bacteria while the rest remain empty.

9. Bacterial titer in inclusion forming units (IFU) is a good estimate of plaque forming units (pfu). Aim to plaque 10, 100, and 1,000 pfu.
10. Vibration from the microbiological safety cabinet can distort the agarose.
11. This amount of infected cells allows enough viable bacteria to be stored. Mutant strains differ in growth rates and infectivity. Allow slow growing strains to naturally reinfect neighboring cells until enough cells are infected.
12. Several platforms can be used to sequence bacterial genomes, including Illumina/Solexa- and SoliD-based systems. We chose to use HiSeq (Illumina) as it produces high density of short, but high quality sequencing reads. Smaller scale genome sequencers, such as IonTorrent (Life Technologies) and MySeq (Illumina) are suitable too for multiplexed sequencing of up to four *Chlamydia* genomes at a time.
13. *Chlamydia* exchanges DNA during infection [13–15]. Recombinant progeny can be recovered by co-infection of cells with two genetically independent strains bearing natural variants that lead to antibiotic resistance, followed by selection for dual antibiotic resistance [13, 14]. In this manner, we can segregate mutations to generate co-isogenic strains. Hence, EMS mutagenesis was performed in a rifampin resistance (rif^R) H471Y in CTL0567 (RpoB) background such that they can be "crossed" to wild type strain bearing a different antibiotic resistant allele (e.g., spectinomycin resistance (spc^R), G1197 in r01/r02 (16SRNA)).
14. At this step, it will be apparent how many mutations per genome where introduced by the EMS-treatment protocol. We suggest tittering plaque-forming units of mutagenized *Chlamydia* on Vero cells supplemented with 200 ng/mL rifampicin so as to assess the efficiency of mutagenesis by monitoring the frequency of Rif^R plaques that arise. The greater the mutagenesis rate, the greater the number of genes that can be included in the association studies to find correlations between common gene lesions and unique phenotypes (e.g., plaque morphologies).
15. To establish linkage between any one mutation and a defined phenotype, we take advantage of natural DNA exchange that occurs in *C. trachomatis* in a co-infection setting [11–13] to generate recombinant strains whose genomes are mosaics of the parental strains. These strains can then be classified based on their phenotype and individually tested for the segregation of individual mutations, as identified by WGS, to define causal linkage between any one mutation and its corresponding phenotype.

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