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Quantitative Dextran Trafficking to the *Coxiella burnetii* Parasitophorous Vacuole

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Abstract

The gram-negative bacterium *Coxiella burnetii* causes human Q fever, a disease characterized by a debilitating flu-like illness in acute cases and endocarditis in chronic patients. An obligate intracellular pathogen, *Coxiella burnetii* survives within a large, lysosome-like vacuole inside the host cell. A unique feature of the *Coxiella* parasitophorous vacuole (PV) is high levels of fusion with the host endocytic pathway, with PV-endosome fusion critical for *Coxiella* survival within the host cell. This unit describes quantitating PV-endosome fusion by measuring delivery of the fluid phase endosome marker dextran to the PV using live cell imaging. To study the effect of host cell proteins involved in PV-endosome fusion, details are provided for using siRNA knockdown host cells. This method is a powerful tool to understanding mechanisms underlying *Coxiella's* ability to manipulate host cell trafficking pathways.

Keywords

Coxiella burnetii; endocytic trafficking; parasitophorous vacuole; quantitative microscopy

Introduction

The *Coxiella* parasitophorous vacuole (PV) is known to be highly fusogenic with host vesicular trafficking pathways, including endosomes, lysosomes, and autophagosomes (reviewed in (Larson et al., 2016)). In epithelial cells such as HeLa cells, the bacteria initially reside in a small, tight-fitting vacuole. Around 24–48 hours, the PV expands, followed by bacterial replication. While the source of membrane during PV expansion has not been definitively determined, it is believed to occur through fusion with endocytic vesicles. Further, *Coxiella* actively manipulates endocytic trafficking by secreting effector proteins into the host cytosol through the *Coxiella* type IVb secretion system (T4BSS). When PV-endosome fusion is inhibited through either depletion of host proteins involved in fusion or a *Coxiella* T4BSS mutant, *Coxiella* growth is significantly inhibited.

Given the importance of PV-endosome fusion during *Coxiella*'s intracellular growth, the ability to examine PV-endosome fusion is critical to further understanding the dynamics of this interaction. Fluorescent dextran is internalized by fluid-phase endocytosis and known to be delivered to the PV (Heinzen, Scidmore, Rockey, & Hackstadt, 1996). This unit describes a method for quantitating trafficking of fluorescent dextran to the PV using confocal live cell microscopy (Basic Protocol 1). Support Protocols 1 and 2 describe *Coxiella* infection of HeLa cells and replating onto optical grade chambered slides. Supporting Protocol 3 uses siRNA to knockdown host genes of interest. Support Protocol 4 describes setup and testing of the live cell chamber, with data analysis provided in Support Protocol 5.

BASIC PROTOCOL 1. Trafficking of Fluid Phase Marker to the CPV

The basic principle of this protocol is a classic pulse-chase, where a fluorescent fluid phase marker (fluorescent dextran) is internalized by *Coxiella*-infected cells through endocytosis. After a pre-set time (or pulse), the fluid phase marker is removed from the media and the dextran-positive endosomes traffic and fuse with the PV (chase). The increase in PV dextran fluorescence is measured during the chase. The effect of depletion or overexpression of proteins, drug treatment, or bacterial mutant on dextran delivery to the PV can be measured to demonstrate a role in PV-endosome fusion.

This protocol uses wild type *Coxiella burnetii* expressing mCherry red fluorescent protein (mCherry-*Coxiella*) and the HeLa epithelial cell line. If other cell types are used, pulse-chase conditions may need to be determined empirically. When using *Coxiella* strains not expressing mCherry, alternative methods will be needed to identify PVs (e.g., phase microscopy). This protocol uses an automated Nikon microscope and Yokogawa spinning disk confocal, but other microscope platforms can also be used. A spinning disk confocal microscope is essential in order to minimize cellular toxicity that occurs with excessive illumination while enabling fast and efficient imaging. Further, confocal imaging minimizes fluorescence from outside the focal plane and improves accuracy. An automated stage is required to image multiple PVs per experiment.

Materials

60× 1.4 NA oil immersion objective (Cat# MRD01605) on a motorized and fully automated inverted microscope-motorized TiE controlled by Elements (Nikon Instruments, Melville, NY)

Andor iXon 887 EM-CCD attached to a CSU10 spinning disk confocal (Yokogawa, Japan) with a MLC-400 Agilent laser launch with 488 and 561 nm lasers (Agilent, CA) and Lambda 10-3 controlled filter wheel (Sutter Instruments, Novato, CA) fitted with emission filters ET525/36m and ET605/70m (Chroma Technologies, Bellows Falls, VT) attached to the above microscope.

Live cell chamber, humidified and equilibrated to 37.1°C, 5% CO₂ (Support Protocol 4).

HeLa cells infected with mCherry-*Coxiella* and plated on Ibidi μ -Slides (Support Protocols 1 and 3). If desired, HeLa cells can be treated with siRNA (Support Protocol 2) prior to *Coxiella* infection.

Complete culture media (RPMI with 10% fetal bovine serum)

200 μ L pipette and sterile tips

2 mg/ml Alexa488-labeled dextran (10,000 molecular weight; D22910, ThermoFisher Scientific) in culture media (see reagents).

1. Warm culture media and dextran in culture media to 37°C.

This is critical to avoid flexing of the plastic that occur with temperature fluctuations.
2. In one channel of the μ -Slide, use mCherry-*Coxiella* to identify PVs and mark positions using microscope software.

To maximize the number of imaged PVs and avoid issues with oil in moving between channels, only one channel should be imaged at a time.
3. Determine the timepoints for sampling, total chase time, z-sample (number of z-stacks and size of slices), and camera settings (e.g., exposure time) for both Alexa488-dextran and mCherry-*Coxiella*. Setup the imaging experiment in the microscope software with the determined settings. In Elements, these setting are set within the “nd Acquisition” tool.

Set the timepoints to allow for the time it takes to collect the z-stacks at all the positions identified. Timepoints should be kept constant between experiments to facilitate comparisons.

Ideally, z-stacks will be sampled at or near the Nyquist criteria (~0.130 μ m for this 60 \times 1.4 NA oil immersion objective used here, <https://svi.nl/Nyquistcalculator>). We typically sample at ~0.3 μ m. Following the washes, the z-stack range and camera setting can be confirmed and adjusted as required as the dish may be move slightly during the washing steps.

In determining camera settings, it is imperative that there are no saturated pixels within the PV. If this happens, pixel intensities will be artificially cutoff and the mean intensities will be under-estimated.
4. With a 200 μ L pipette, carefully remove 120 μ L from the far channel. To the near channel, add 120 μ L of pre-warmed (37°C) Alexa488-dextran (2 mg/ml in culture media) and simultaneously start a timer. Incubate for 10 minutes.

HeLa cells are cultured on the channel slides in 150 μ L. Removing 120 μ L will leave 30 μ L in the culturing channel. Be careful to not disturb the Ibidi slide.
5. After ten minutes, use a 200 μ L pipette to remove 120 μ L of the labeling media from the far reservoir. Add 120 μ L of 37°C culture media to the near reservoir,

allow the media to reach the far reservoir and remove 120 μ L from the far reservoir.

6. Repeat at least 4 more times.

Five washes should be sufficient to remove uninternalized Alexa488-dextran. More washes may be performed if residual green fluorescence is observed in the extracellular media.
7. Check the PV positions, the z-stack range and the camera settings identified in step 1. Record this time as “dead-time.”

Typically, the ‘dead-time’ is 3–4 minutes. When comparing between experiments it is imperative that this is consistent to ensure comparable trafficking times.
8. Begin imaging experiment using the settings set in step 1 or as adjusted in step 7. The first set of captured images represents the “initial” intensity.

SUPPORT PROTOCOL 1. Infection of HeLa cells with *Coxiella*

Coxiella readily infects a wide range of cell types, including epithelial cells and macrophages. Once internalized by the host cell, *Coxiella*-containing phagosomes can undergo homotypic fusion. As it has not been established if vacuole maturation proceeds differently in cells infected with multiple bacteria, this protocol uses infection conditions that have been optimized for ~1 bacterium per cell, with 30–40% of the cells being infected. The infection conditions are determined empirically for each bacteria stock, cell type, and culture vessel being used. For basic culturing of HeLa cells, see Current Protocols in Microbiology Unit 11A.2, “Sphingolipid trafficking and purification in *Chlamydia trachomatis*-infected cells” (Elizabeth R. Moore, 2013). For preparation of *Coxiella* Nine Mile II (NMII) stocks from Vero cells, see Cockrell *et al* (Cockrell, Beare, Fischer, Howe, & Heinzen, 2008). These steps should be done in a biosafety cabinet with sterile reagents.

Materials

Trypsinized HeLa cells

Hemocytometer

Tissue culture scope with 10X objective

Complete growth medium (RPMI containing 10% FBS)

24 well tissue culture plate

Coxiella burnetii stock [Copy Editor: Please ask the author to clarify... Is this labeled with mCherry? If so, where can you obtain this? Or, if you have to label it yourself, how is that done? Furthermore, it seems that mCherry should be mentioned in the protocol introduction.]

Sterile 1X PBS (phosphate buffer saline)

1. Count HeLa cells with hemacytometer (Appendix 4A) and dilute to 1×10^5 cells/ml in complete growth medium. Add 500 μ L cells per well of 24 well plate, for a final number of 5×10^4 cells per well. Let adhere 4–6 hours.

Unless over-trypsinized, HeLa cells should attach and spread out within 4 hours. The cells can also be left overnight; however, cell growth must be considered when calculating the number of bacteria to use during the infection. This can be determined by trypsinizing and determining cell number in several extra wells right before the infection.

2. In complete growth medium in a sterile 15 ml conical, dilute *Coxiella* stock to 4×10^7 bacteria/mL. The final number of bacteria being added is 200 per cell.

Vortex the bacteria stock well before removing an aliquot, making sure to look for clumps. After adding to growth medium, vortex for another 30–60 seconds.

3. Remove media from well and replace with 250 μ L of diluted bacteria.

Incubating in a smaller volume increases the efficiency of uptake.

4. Incubate at 37°C, 5% CO₂ for 1 hour.

5. Wash wells extensively with sterile PBS.

Coxiella NMII bacteria are hydrophobic, and tend to stick to the plastic. To remove uninternalized bacteria, it may be necessary to wash with 15 – 20 exchanges of PBS. To ensure that the cells do not dry out, wash wells one at a time, always leaving enough PBS to just cover the cells.

6. Replace with 500 μ L complete growth media.

7. Incubate at 37°C, 5% CO₂.

SUPPORT PROTOCOL 2: Transfection of HeLa cells with siRNA

Transfection is a method to deliver nucleic acids into cells, typically using a lipid carrier. During a “forward” transfection, cells are seeded onto plates and transfected with nucleic acid-lipid complexes the following day. A “reverse” transfection involves adding the nucleic acid-lipid complexes while the cells are being seeded. Reverse transfection not only saves time but also often increases knockdown efficiency. This protocol uses reverse transfection to deplete cells of the protein of interest using small-interfering RNA (siRNA) with Dharmacon Smartpool siRNA and Dharmafect transfection reagent (GE Healthcare). These steps should be done in a biosafety cabinet with sterile reagents.

Materials

Sterile 1.5 ml microcentrifuge tubes

Serum-free RPMI

Dharmafect 1 (GE Healthcare)

Control (*e.g.*, non-targeting) and experimental siRNA at 20 μ M stock concentration

24 well tissue culture plate

Trypsin

T25 or T75 of HeLa cells, depending on the number of wells to be transfected

Hemocytometer

Tissue culture scope with 10X objective

Antibiotic-free complete growth medium (RPMI with 10% fetal bovine serum)

1. Make a master mix of diluted Dharmafect in a 1.5 ml microcentrifuge tube. For each well of a 24 well plate, add 0.62 μ L Dharmafect to 130 μ L serum-free RPMI.

 Serum-free media is required to form siRNA-lipid complexes. To keep the lipid from sticking to the tube walls, pipet the Dharmafect directly into the media.

2. Add stock siRNA to diluted Dharmafect master mix to a final concentration of 50 nM. For example, for each well add 1.6 μ L stock siRNA (20 μ M) to 130 μ L diluted Dharmafect master mix. Vortex briefly to mix.

 Make a mastermix of siRNA if doing multiple wells.

3. To each well of a 24 well plate, transfer 125 μ L of siRNA-lipid mix.
4. Incubate siRNA-Dharmafect mix 30 minutes at room temperature.
5. While incubating, trypsinize HeLa cells, count with a hemacytometer, and dilute to 5×10^4 cells/ml in antibiotic-free complete growth media.

 For best transfection efficiency, use cells in log-phase and less than 20 passages. The use of antibiotics will decrease efficiency and may cause toxicity. For basic culturing of HeLa cells, see Current Protocols in Microbiology Unit 11A.2, "Sphingolipid trafficking and purification in Chlamydia trachomatis-infected cells" (Elizabeth R. Moore, 2013).

6. Following the 30 minute incubation of siRNA-lipid mix, add 500 μ L of diluted cells to each well, pipetting up and down several times to mix.
7. Transfer cultures to a 37°C, 5% CO₂ incubator for desired amount of time.

 For each targeted protein, the amount of time required for efficient knockdown should be determined, along with how long the knockdown lasts and if Coxiella infection affects knockdown stability. It may be necessary to transfect a second time to achieve knockdown. In this case, we typically re-transfect with siRNA after infection with Coxiella.

SUPPORT PROTOCOL 3. Replating infected cells from 24 well plate onto ibidi μ -slides

Cells treated with siRNA and infected with *Coxiella* are replated onto imaging dishes 24 hrs before imaging. Slides or dishes from Ibidi (Fitchburg, WI) are used because of their: 1) low volume and surface area, meaning fewer cells and less dextran are required; 2) optical grade plastic that is amenable to cell attachment while being compatible with high resolution fluorescence microscopy; 3) standard slide size. Here we describe plating onto the Ibidi μ -Slide VI^{0.1}, which has six channels to facilitate multiple samples in one experiment. When using for the first time, consult the manufacturer's product information sheet for tips on handling and culturing cells in Ibidi μ -Slides.

Materials

Infected HeLa cells in a 24 well plate from Supporting Protocol 1

Sterile 15 ml conical tubes

Complete growth medium (RPMI containing 10% FBS)

Trypsin

Swinging bucket centrifuge

Hemocytometer

Tissue culture scope with 10X objective

200 μ L pipetter with sterile tips

μ -Slide VI^{0.1} (catalog number 80296, Ibidi, Fitchburg, WI)

1. Transfer 10 mL complete growth medium to a 15 mL conical tube.
2. Trypsinize cells in a single well with 150 μ L trypsin, and transfer to the 15 mL conical tube with complete growth medium.

It is often necessary to pool multiple wells of a 24 well plate, depending on how many cells are needed to plate the desired number of channels. To concentrate cells for plating onto the Ibidi μ -slide, the cells most often need to be spun down. This is because the trypsin must be diluted with 5 volumes of 10% RPMI to inactivate enzymatic activity and avoid killing the cells.

3. Centrifuge 250 \times g for 5 min.
4. Carefully remove the media, and resuspend in a small volume of complete growth medium (<500 μ L).
5. Count cells with hemocytometer, and adjust volume to 2–3 \times 10⁵ cells/ml with complete growth medium.
6. Add 30 μ L of resuspended cells to one channel of ibidi μ -slide.

Consult the manufacturer's product information sheet for tips on plating and removing bubbles.

7. Place in incubator at 37°C, 5% CO₂ for ~2 hours to allow the cells to adhere.

Because Ibidi μ -Slides exchange gas through the bottom, make sure to incubate according to the instructions.

8. After the cells have adhered, add 120 μ L of 10% RPMI to each channel. Remove bubbles if necessary, and place back in the incubator.

SUPPORT PROTOCOL 4. Setup of live cell imaging chamber

The objective in live-cell microscopy is to maintain a tissue culture model, such as HeLa cells, in their normal growth conditions while conducting experiments that are best followed using microscopy. It is essential that the temperature be stable at 37.1°C, as membrane and cytoskeletal dynamics can change dramatically with temperature. Temperature (37.1°C), CO₂ (5%) and humidity are controlled using commercially available chambers that can be adapted to a wide variety of microscope stages and culture dishes. This protocol describes imaging of Ibidi μ -Slides using a slide adapter installed in the stage top incubator Bold Line from OkoLabs (Burlingame, CA). The temperature in the OkoLab chamber is regulated either by its own temperature or by an auxiliary thermistor that can be placed inside the chamber in a dish filled with water or media. We choose to use the auxiliary thermistor approach as we find the temperature at the dish cannot be maintained at 37.1°C by setting just the chamber temperature. In addition, an objective heater set to 37.1°C ensures the sample is not cooled by a room temperature objective.

Materials

Okolab Bold Line universal stage top incubator with CO₂ module, objective heater and OkoTouch for control (H301 Bold Line, OkoLabs, Burlingame, CA)

Distilled water

2 well Ph+ imaging dish (80296, Ibidi, Fitchburg, WI)

μ Slide VI^{0.1} (80666, Ibidi, Fitchburg, WI)

Type 37 Oil immersion fluid (Type 37, Cargille Laboratories)

1. Fill the gas humidification chamber with distilled water.

This step may be common to live cell stage-top incubators. Distilled water, as opposed to pure water (i.e., Milli-Q) is used to avoid corrosion of the thermistor.

2. To image Ibidi μ -Slides, install the two-position slide adapter (*2xGS-M*) for the OkoLab chamber and install on microscope.

When using the OkoLab chamber, select the slide insert (*2xGS-M*) in the stage setup menu on the OkoTouch controller

3. Place a 2 well Ph+ imaging dish filled with distilled water in the live cell chamber and thread the thermistor into one of the wells and under the inset plastic.

The cap for the 2 well Ph+ can be used to secure the thermistor in place. The distilled water may be preheated to accelerate the equilibration of the imaging chamber.
4. Install objective heater on 60× 1.4 NA oil immersion objective (MRD01605, Nikon Instruments, Melville, NY) and turn objective heater on and set to 37.1°C.
5. Place a μ -Slide VI^{0.1} with water in the chambers in the live cell chamber, and contact the objective with 37.1°C immersion oil (Type 37, Cargille Laboratories).
6. Run calibration routines for the temperature control. OkoLab touch provides access to calibration when selecting different control approaches or stage inserts under the “Settings” menu. Calibration may take 1–2 hours with the OkoLab Bold Line.

This step may be skipped if a recent calibration is valid for a given room temperature. We find it beneficial to work with building engineers to minimize room temperature fluctuations and ensure that appropriate cooling capacity is available in the room.

7. Set the humidity to 75% and temperature to 37.1°C and allow the chamber to equilibrate.

The temperature and humidity progress may be followed graphically on the OkoLab Touch and can take up to an hour.

SUPPORT PROTOCOL 5. Analysis of trafficking time course

The experimental data is analyzed using the open source imaging analysis software FIJI (4), and plotted with the graphing software Prism (GraphPad, San Diego, CA). The microscope software (*i.e.*, Nikon Elements) records an annotated dataset with metadata that should be archived and backed-up for future reference. FIJI has the basic tools for quantitative analysis and can open most proprietary software formats via Bio-Formats (Linkert et al., 2010). As such, this protocol is relevant if data is collected on a platform other than Nikon Elements.

Materials

FIJI, Version 2.0.0-rc-59/1.51j with Java 8 installed for your computer platform
(<http://imagej.net/Downloads>)

Digital datasets from microscope

Excel (Microsoft, Redmond, WA) or other spreadsheet software

Prism (GraphPad, San Diego, CA) or other graphing software

NOTE: This protocol is demonstrated in Video1.mp4.

1. Open dataset in FIJI: “File” -> “Import” -> ”Bio-Formats” and in the “Bio-Formats” interface select “HyperStack” from the “View stack with” option.
If the digital dataset contains many image datasets, such as multiple cells or stage positions, select each one you wish to open in the “Bio-Formats” interface.
2. Separate the imaging channels: “Image” -> “Color” -> “Split Channels”.
3. Select the channel that contains the fluorescent dextran by clicking on the relevant window.
4. Perform a background subtraction: “Process” -> “Subtract Background...” with a radius of 50 pixels and process all images in stack when asked by the “Process Stack?” dialog window.

This pixel radius is a compromise between the smallest and largest PVs that may be encountered under these infection conditions at 3 days post infection. If the experiment is done at different times post-infection, when the PVs are significantly smaller or larger, this may need to be adjusted.

5. Sum all the z-stack slices: “Image” -> “Stacks” -> “Z-Project...” select “Sum Slices” and check “All time frames.”

Stacks are summed to avoid introducing unintended sampling bias which could be introduced by selecting a single z-slice.

6. Open “Analyze” -> “Set Measurements...” and select “Mean gray value”, “Area” and “Display Label”.

The Label field recorded in the “Results” table will include the timepoint following the final colon.

7. Move to the first timepoint by dragging the slider at the bottom of the image window all the way to the left. Open “Analyze” -> “Tools” and select “ROI Manager...”
8. Make a circle selection with a diameter of at least 20 pixels inside the *Coxiella* PV but preferably not overlapping *Coxiella* and add the circle selection to the “ROI Manager” by selecting “Add” in the “ROI Manager” window.

By adding the selection to the “ROI Manager” it can be recalled later by clicking on the selection in the “ROI Manager”, if the selection is removed accidentally. Further, a selection may be recalled from the “ROI Manager” on additional datasets to maintain consistency in the area measured.

9. Record the fluorescent intensity in the circle selection by pressing the ‘m’ key. This is the “Initial” average intensity.

NOTE: The PV may be labeled overnight with a spectrally non-overlapping labeled dextran (e.g. Alexa647) to aid in selection of an

area inside the PV or the entire PV. The circle ROI should only need to be moved as the cell moves during the experiment.

10. Move to the next time point. Move circle selection to inside the PV (if required) and record the value by pressing the “m” key. Repeat for every time-point.
11. Copy the results from the FIJI “Results” table and paste into an Excel spreadsheet by using the right-click menu on the “Results” table or the “Edit” menu when selecting the “Results” table.
12. The fold change in intensity is calculated by dividing the average intensity (“mean gray value” at each timepoint by the “Initial” average intensity.

This assumes the volume of the PV does not change dramatically over the time course. If this a concern, the PV may be labeled overnight with a spectrally non-overlapping labeled dextran as mentioned above to enable measurement of the entire PV volume at every time-point.

13. Repeat for additional PVs and plot the fold change in Prism as column data for a given treatment condition. Statistical test such as *t*-test or ANOVA may be performed as appropriate.

REAGENTS AND SOLUTIONS

Dextran stock solution

Resuspend lyophilized Alexa488-dextran (D22910, ThermoFisher Scientific) at 10 mg/ml in sterile 1X PBS. Make 100 μ l aliquots and store at -20°C , protected from light. To make the working stock immediately prior to live cell imaging, dilute to 2 mg/ml in 10% RPMI and vortex well. Centrifuge at 12,000 \times g for 2 minutes to pellet insoluble material. Transfer supernatant to a new sterile microcentrifuge tube.

COMMENTARY

Background Information

An obligate intracellular pathogen, *Coxiella* first targets alveolar macrophages during natural infection, but can infect a wide range of cells (Khavkin & Tabibzadeh, 1988; Stein et al., 2005; Voth & Heinzen, 2007). Following phagocytosis by a host cell, the *Coxiella*-containing phagosome matures through the endocytic pathway into an acidic and hydrolytic phagolysosome. Approximately 24–48 hours after host cell internalization, the *Coxiella* parasitophorous vacuole (PV) expands, forming a mature PV that is large, dynamic, and highly fusogenic with endosomes, lysosomes, and autophagosomes (Voth & Heinzen, 2007). *Coxiella* extensively modifies the PV through insertion of bacterial proteins into the phagosome membrane, as well as recruitment of host cell proteins. Host vesicular trafficking pathways are known to be manipulated by the *Coxiella* T4BSS, which secretes bacterial proteins across the PV membrane and into the host cytosol.

While interactions between the PV and host endocytic trafficking pathways are essential for PV formation, only recently have some of the host cell factors been identified and described.

The small GTPases Rab1b, Rab5, and Rab7 are found on the PV, with siRNA knockdowns or overexpression of dominant negatives preventing PV formation (Beron, Gutierrez, Rabinovitch, & Colombo, 2002; Campoy, Zoppino, & Colombo, 2011; McDonough et al., 2013; Romano, Gutierrez, Beron, Rabinovitch, & Colombo, 2007). Depletion of clathrin or the clathrin-adaptor protein AP2 also blocks PV expansion (Larson, Beare, Howe, & Heinzen, 2013), and the lysosomal membrane proteins LAMP-1 and LAMP-2 increase the rate of endosome fusion (Schulze-Luehrmann et al., 2016). The endocytic SNAREs syntaxin-17 and VAMP7 have also emerged as a critical component of PV formation (Campoy, Mansilla, & Colombo, 2013; McDonough et al., 2013).

Several approaches have been used to demonstrate heterotypic fusion between host vesicular trafficking pathways and the PV. The most common approach is measuring PV size, as the amount of fusion is thought to directly relate to formation and maintenance of the large PV (Larson et al., 2013; Romano et al., 2007). A second approach is determining the percentage of PVs with host vesicular markers, for example lysosomal cathepsin D (Romano et al., 2007), assuming that these proteins are delivered directly through fusion. Both of these approaches are simple and do not require specialized techniques; however, changes in PV size or marker acquisition could be due to factors other than PV-endosome fusion.

A third method examines delivery of vesicular cargo into the PV lumen, which is a direct measure of fusion between the PV and cargo-carrying vesicles. Both fluorescent dextran and BSA are used as fluid-phase endosome markers (Campoy et al., 2013; Campoy et al., 2011; Justis et al., 2017; Mulye, Samanta, Winfree, Heinzen, & Gilk, 2017), while fluorescent heat-inactivated *Staphylococcus aureus* and latex beads mark phagosomes (Colonne et al., 2016; Schulze-Luehrmann et al., 2016). Traditionally, cargo delivery to the PV has been quantitated by determining the percentage of cargo-positive PVs, and has been used almost exclusively in fixed cells. While straightforward, quantitation is based strictly on the presence or absence of the marker, and cannot account for variations in fusion. As a result, more subtle phenotypes can be easily missed. This protocol quantitates the change in PV dextran fluorescence over time using live cell imaging, providing a continuous measure of fusion. By sampling at regular intervals during the timecourse, this approach can also identify conditions where the rate of fusion may vary with time, even if the amount of fusion is of the same final magnitude. The major disadvantage of this approach is that it requires a sophisticated spinning disk confocal microscope platform with live cell capabilities. Further, technical precision is critical, as the assay is very sensitive to timing precision and consistency between experiments.

Critical Parameters

Working with *Coxiella*—Avirulent *Coxiella* Nine Mile II (NMII) must be handled at biosafety level 2 (BSL2). Aseptic technique is required, as antibiotics traditionally used in cell culture (e.g., penicillin, streptomycin) inhibit *Coxiella* growth. Historically, *Coxiella* stocks were prepared from aged cultures in Vero cells, which allows for isolation of the stable small cell variant (SCV) of *Coxiella*. Recently, the development of an axenic media allows for growth in the absence of host cells (Omsland, 2012). For experiments that only use wild-type bacteria or bacteria that do not have a growth phenotype in cells (e.g., *Coxiella*

expressing mCherry), we use *Coxiella* isolated from Vero cells infected for 28 days (Cockrell et al., 2008). We find the yield from Vero cells to be significantly higher than axenic media, enabling the same stock to be used for months to years. While *Coxiella* stocks stored at -80°C in 10% DMSO appear to be stable for multiple freeze/thaws, it is a good idea to make single-use aliquots to eliminate the possibility of viability loss.

Working with eukaryotic cells—HeLa cells should be handled at BSL2 level using aseptic techniques. As with all eukaryotic cells, it is critical that HeLa cells are cultured under conditions that maintain optimum health. For example, cells should be split every 2–3 days, kept sub-confluent, and used for a limited number of passages (typically 20) as HeLa cells acquire genetic alterations the longer they are kept under continuous culture. Stocks and cultures should be checked regularly for mycoplasma infection. A sudden loss in transfection or knockdown efficiency can often be traced to the cell health.

Timing of live cell imaging—Live cell imaging experiments are often limited by the frequency with which imaging can be performed for a given number of fields or stage positions. It is imperative that the speed of the microscope and its peripherals is sufficient to accomplish the required imaging between the desired timepoints. This will often limit the number of fields or cells that can be imaged, and can require multiple experiments to image a statistically significant number of PVs. A strict measurement of pulse time and dead time is required to enable comparisons between experiments.

Anticipated Results

This assay has proven to be sensitive and precise. In HeLa cells over a 30 minute time course, we have measured up to a 2 fold change in fluorescence at 3 days post infection. Importantly, we found a statistically significant 20% change in fusion between control and experimental conditions, indicating this assay can be used to measure subtle changes in PV-endosome fusion.

Time Considerations

The significant variables in total experimental time include siRNA knockdown and *Coxiella* infection. Our typical experiment, examining trafficking at 3 days post infection, is five days long: siRNA transfection at day 1, *Coxiella* infection at day 2, replating onto Ibidi μ -Slides on day 4, and live cell imaging on day 5. In every experimental run of the basic protocol, nine to ten PVs should be imaged. A single run will take approximately 45 minutes and thus over a half day, accounting for live-cell setup and equilibration, 40–60 PVs can be measured. With properly staggered infected cells, a complete set of three experiments, with controls and experimental conditions, can be completed within two weeks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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