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Measuring pH of the *Coxiella burnetii* Parasitophorous Vacuole

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Abstract

Coxiella burnetii is the causative agent of human Q fever, a zoonotic disease that can cause a debilitating, flu-like illness in acute cases, or a life-threatening endocarditis in chronic patients. An obligate intracellular bacterial pathogen, *Coxiella* survives and multiplies in a large lysosome-like vacuole known as the *Coxiella* parasitophorous vacuole (CPV). A unique characteristic of the CPV is the acidic environment (pH ~5.0), which is required to activate *Coxiella* metabolism and the *Coxiella* type 4 secretion system (T4SS), a major virulence factor required for intracellular survival. Further, inhibiting or depleting vacuolar ATPase, a host cell protein that regulates lysosomal pH, inhibits intracellular *Coxiella* growth. Together, these data suggest that CPV pH is an important limiting factor for *Coxiella* growth and virulence. This unit describes a method to determine CPV pH using live cell microscopy of a pH–sensitive fluorophore conjugated to dextran. This technique is useful to measure changes in CPV pH during infection or in response to drug treatment.

Keywords

Coxiella burnetii; parasitophorous vacuole; pH; quantitative microscopy

INTRODUCTION

Following internalization by the host cell, *Coxiella* resides in a tight-fitting nascent phagosome that progresses through the default endocytic pathway (Heinzen, Scidmore, Rockey, & Hackstadt, 1996). At about one to two days post-infection, the CPV expands through fusion with late endosomes, lysosomes, and autophagosomes (Voth & Heinzen, 2007), acquiring late endosomal proteins CD63 and Rab7 (Berón, Gutierrez, Rabinovitch, & Colombo, 2002; Ghigo et al., 2002), lysosomal-associated membrane protein 1 (Lamp1) (Heinzen et al., 1996), and autophagic proteins LC3 and Rab24 (Berón et al., 2002; Gutierrez et al., 2005). A key characteristic of the CPV lumen is that it is acidic in nature, with a pH of ~5.0 (Akporiaye, Rowatt, Aragon, & Baca, 1983; Grieshaber, Swanson, & Hackstadt, 2002; Hackstadt & Williams, 1981; Maurin, Benoliel, Bongrand, & Rault, 1992). When the CPV pH becomes more basic by treatment with lysomotropic amines or the vacuolar ATPase inhibitor bafilomycin A, *Coxiella* growth is significantly blocked (Hackstadt & Williams, 1981; Heinzen et al., 1996). *Coxiella* glucose and glutamate transport and catabolism is most active between pH 2.0 and 5.5 (Hackstadt & Williams, 1981), and a recently developed axenic medium supports *Coxiella* growth at pH 4.75 (Omsland et al., 2009). In addition to activating bacterial metabolism, CPV acidification

triggers the *Coxiella* type 4 secretion system (T4SS) (Newton, McDonough, & Roy, 2013), a major bacterial virulence factor which translocates *Coxiella* effector proteins into the host cell cytosol where they manipulate host cellular processes. Given the importance of CPV acidification, accurately measuring CPV pH is essential to better understand development of the *Coxiella* intracellular niche.

This unit describes a ratiometric fluorescent microscopy approach to measure CPV pH using pH-sensitive and pH-insensitive fluorophores conjugated to dextran. Dextran is internalized by host cells through fluid phase endocytosis and delivered to the CPV by CPV-endosome fusion (Heinzen et al., 1996), providing an efficient method of labeling the CPV lumen. The Basic Protocol describes labeling and imaging of *Coxiella*-infected cells with fluorescent dextrans, including experimental samples and standards for the pH calibration curve. Data analysis is described in Support Protocol 1, with set up of the live cell imaging chamber in Support Protocol 2.

BASIC PROTOCOL

IMAGING OF FLUORESCENT DEXTRAN IN THE CPV

Oregon Green, a fluorinated analog of fluorescein (Sun, Gee, Klaubert, & Haugland, 1997), has a pK_a of 4.7, making it more suitable than fluorescein (pK_a of 6.4) to measure the acidic pH of the CPV (Johnson, Ostrowski, Jaumouillé, & Grinstein, 2016). Once delivered to the CPV lumen via fusion with dextran-loaded endosomes, Oregon Green fluorescent intensity will vary depending on the CPV pH. In contrast, Alexa647 fluorescent intensity is pH-insensitive (Chen, Cheng, Behlke, & Tsourkas, 2008). The Oregon Green/Alexa647 fluorescent intensity ratio is compared to a standard curve to calculate the pH of individual CPVs. The standard curve is prepared by treating infected cells with the ionophores monensin and nigericin, which allows the intracellular pH (including the CPV) to equilibrate with an extracellular buffer of known pH. The effect of drug treatment, host gene knock down, protein overexpression, or bacterial mutation on the CPV pH can be measured to determine a role in the establishment or maintenance of CPV pH.

In this protocol, wild-type *Coxiella* expressing mCherry red fluorescent protein (mCherry-*Coxiella*) is used to infect HeLa cells, allowing for efficient identification of bacteria-containing CPVs. The protocol is also applicable to other cell types, although infection and dextran labeling conditions may need to be optimized. This protocol uses Leica DMI 6000B inverted microscope with 63× oil immersion objective and the TOKAI HIT INU series stage top incubator for environmental control. However, other microscope systems can also be used as long as required filters are available.

Materials

Dextran working solution (see recipe)

Complete RPMI-1640 culture medium (with 10% fetal bovine serum)

HeLa cells infected with mCherry-*Coxiella* and plated on ibidi µ-Slides (Support Protocols 1 and 3, Winfree & Gilk, 2017, *UNIT 6C.2*)

Phosphate-buffered saline (PBS; HyClone, GE Healthcare Life Sciences, cat. no. SH3025601), sterile

Ionophore working solution (see recipe)

pH standards (see recipe)

Water bath

Sterile biosafety cabinet

CO_2 incubator for tissue culture

200-µl pipette and sterile tips

TOKAI HIT INU series stage top incubator (see Support Protocol 1)

Leica DMI 6000B inverted microscope with 63X oil immersion objective lens

Preparing the samples for microscopy

1. Warm the complete RPMI 1640 culture medium and dextran working solution to 37°C in a water bath.
2. In a sterile biosafety cabinet, remove medium from each channel of the µ-Slide with a 200-µl pipette, and replace with 50 µl of the dextran working solution.

   Consult the manufacturer's instructions and Support Protocol 3 of Winfree and Gilk, 2017 (UNIT 6C.2) for details on working with ibidi µ-Slides. We typically use one µ-Slide for the standard curve, and a second µ-Slide for experimentals.

   Stagger the labeling start time for multiple µ-slides (e.g., standards and experimentals) to avoid overloading cells with dextran. We typically use a 4-hr stagger between the standard and experimental samples. For example, dextran is added to standard samples at 8 a.m. and experimentals at noon. Noon to 4 p.m. is utilized to image the standard curve, with imaging of the experimentals at 4 p.m.
3. Incubate the µ-Slide for 4 hr in 37°C, 5% CO_2 tissue culture incubator.
4. Wash each channel of the µ-Slide at least four times, each time with PBS.

   To avoid additional internalization and increased variability between samples, it is crucial to wash off all the extracellular dextran. With a 200-µl pipette, carefully remove the medium from the far reservoir. Then add 120 µl of PBS to the near reservoir. Repeat four times, or until all the bluish-green dextran has been removed.
5. After the final wash, replace PBS with 120 µl pre-warmed RPMI 1640 culture medium in each channel.

This will prevent flexing of μ-slides that occurs with temperature fluctuations, which can affect stability of imaging.

Imaging the standard samples

6. Replace the culture medium with 120 μl ionophore working solution (pre-warmed) in the first channel of the μ-Slide.

7. Incubate in the live cell chamber attached to the microscope for 5 min.

8. Replace the ionophore working solution in the first channel with 120 μl of pH standard 7.0 (pre-warmed) and incubate for 5 min.

9. Using the red filter of the microscope, identify CPVs by focusing on mCherry-*Coxiella.*

   It is critical not to use green (Oregon Green) or far red (Alexa647) filters at this point, as prolonged exposure in these channels will bleach the fluorescent signal and affect final measurements.

10. Switch to the green filter and empirically determine the exposure setting. Repeat for two more CPVs to determine the best exposure settings for Oregon Green.

    As Oregon Green is most fluorescent at pH 7.0, the exposure settings should be determined for the pH 7.0 standard and kept constant for the remainder of the experiment.

11. Switch to the far red filter and determine exposure settings for Alexa647, as described in step 10. These settings should be used for the remainder of the experiment.

12. Switch to red filter and identify a new CPV based on mCherry-*C.burnetii.* Image both the green and far red channels using the exposure settings determined in steps 10 and 11.

13. Repeat step 12 for at least 30 total CPVs in the first channel of the μ-slide.

    CPVs used to determine exposure settings should not be used for measurements in the standard curve.

14. Move over to the next channel of the μ-Slide, and repeat steps 6–8, 12, and 13 for pH standard 6.0.

15. Repeat for all the channels of the μ-slide using pH standard 5.5 for the third channel, pH standard 5 for the fourth channel, pH standard 4.5 for the fifth channel and pH standard 4 for the sixth channel.

16. Carefully save all the images with appropriate annotation for analysis purpose (Support Protocol 2).

    Keep images organized by pH standards used. Separate folders for each pH standard will simplify the image analysis process.

Imaging the experimental samples

17. Label infected HeLa cells in the second μ-Slide with dextran as in steps 1 to 5.

    Use the second μ-Slide with mCherry-Coxiella-infected HeLa cells for experimental samples (e.g., control and treatment conditions).

18. Perform steps 12 and 13 for each channel, using the capture settings used for the pH standards.

19. Carefully save all the images with appropriate annotation for analysis purpose (Support Protocol 2).

SUPPORT PROTOCOL 1

SET UP OF TOKAI HIT INU STAGE TOP INCUBATOR FOR LIVE-CELL MICROSCOPY

During live-cell microscopy, cells are maintained in their normal growth conditions, including 37°C, 5% CO_2, and ~75% humidity using specially designed chambers attached to the stage of the microscope. The chamber is connected to a control unit, where the investigator can set the desired temperature and percentage of CO_2. This protocol describes imaging cells in μ-Slides using a chamber slide adapter in a TOKAI HIT INU series stage top incubator connected to a TOKAI HIT INU control unit. The lid of the live-cell chamber and the stage are heated to avoid condensation. To precisely maintain the tissue culture media at 37°C, a metal lid with a sterile feedback sensor is placed on top of the μ-Slide, and the feedback sensor is immersed in the media. An objective heater is used to minimize heat dispersion through the objective. CO_2 is supplied from commercial CO_2 tank connected to the control unit, and incubator humidity is maintained by adding deionized sterile water to the designated water channel around the edge of the incubator. The water channel is also heated to facilitate humidification.

Materials

Sterile deionized water

70% ethanol

Leica Microsystems 3P immersion oil Type F

TOKAI HIT INU series stage top incubator and TOKAI HIT INU temperature and gas-mixer control unit (Fujinomiya, Japan)

μ-Slide $VI^{0.1}$ (Ibidi, cat. no. 80666)

TOKAI HIT CSG1–200F sensor lid for chamber slides (Fujinomiya, Japan)

Set up of stage top incubator for equilibration

1. Place the TOKAI HIT INU series stage top incubator on the stage of the Leica DMI 6000B microscope.

2. Removing the lid of the incubator chamber, add 15 ml sterile deionized water to the designated water bath.

   The water level should be below the CO_2 supply channel opening.

3. Install objective heater on 63× oil immersion objective lens and heat to 37°C by connecting the wire of the objective heater to the control unit.

4. Install the appropriate adapter for chamber slides in the incubator chamber.

5. Place a μ-Slide with sterile deionized water in the channels in the chamber slide adapter.

   This μ-Slide is used for equilibration of the chamber, and can be saved and used in future experiments.

6. Remove the plastic lid of the μ-Slide and install the TOKAI HIT CSG1–200F sensor lid for chamber slides on the μ-Slide, ensuring its stability using the two flat springs of the chamber slide adapter. Place the sensor wire into one of the reservoirs of the ibidi μ-Slide.

   Sterilize the feedback sensor wire of the sensor lid by wiping with 70% ethanol before placing it on the μ-Slide. While placing the sensor lid, ensure that the feedback sensor wire is immersed in the water.

7. Connect the wire of the sensor lid to the designated port (refer to manufacturer's instructions).

8. Replace the lid of the incubator chamber.

Set up of the control unit

9. On the control unit, press the main power button and turn on the "Mix Gas Power," "Sample Temp," and "Lens Power" switches.

10. Set the "Top Heater," "Bath Heater," "Stage Heater," "Lens Heater," and "Sample Temp" gauges to 37°C, and set "CO_2 concentrator" gauge to 5%.

11. Wait for at least 1 hr for the incubator chamber to equilibrate.

Installation of sample slide

12. Carefully remove the lid of the incubator chamber.

13. Remove the flat springs of the chamber slide adapter, and remove the sensor lid.

    While removing the sensor lid, ensure not to touch the feedback sensor wire to avoid contamination.

14. Remove the μ-Slide with water.

15. Add a drop of Leica Microsystems 3P immersion oil Type F on the objective lens.

16. Place the μ-Slide with infected HeLa cells in the chamber slide adapter.

17. Replace the sensor lid and carefully immerse the feedback sensor wire into the culture medium in one of the near reservoirs of the μ-Slide. Ensure the stability of the sensor lid with the two flat springs.

18. Replace the lid of the incubator chamber, and proceed to the Basic Protocol.

SUPPORT PROTOCOL 2

ANALYSIS OF THE IMAGE DATASETS

Images acquired at steps 16 and 19 of the Basic Protocol are analyzed using the open-source imaging analysis software FIJI (Schindelin et al., 2012), and plotted with the graphing software Prism (GraphPad). FIJI software can open and analyze most imaging files via Bio-Formats. Moreover, FIJI is supported by both Windows and Macintosh platforms.

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) or another spreadsheet software

Prism (GraphPad) or other graphing software

Measurement of fluorescent intensities of the CPVs in FIJI

1. Open dataset in FIJI a “File” : “Open” → “Bioformats” → “Dataset file name.”

   This will open “Bioformats Series Options” window. Select the images you want to analyze. Click OK.

2. 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.

   50 pixels is an average of the smallest and largest CPVs that are found in HeLa cells with these infection conditions at three days post infection. With different cell lines, and with different infection conditions the pixel diameter for background subtraction need to be adjusted.

3. Open “Analyze” → “Set Measurements . . . ” and select “Mean gray value.”

4. Draw a “region of interest” (ROI) around the CPV in green channel.

   Use “oval,” “polygon selections.” or “freehand selections” tool to draw an ROI around the CPV. Use different drawing tools based on the shape of the CPV. Extreme precision in drawing the ROI is not required, as the fluorescent intensity of Oregon Green will be normalized to Alexa647 fluorescence within the ROI.

5. Open “Analyze” → “Tools” and select “ROI Manager . . . ” Add the selection to the “ROI Manager” by selecting “Add” in the “ROI Manager” window.

   “ROI Manager” saves the “ROI” for future use. If accidently removed, the ROI can be recovered by clicking on the selection in the “ROI Manager.”

6. In the Oregon Green channel, record the fluorescent intensity of the ROI by pressing the “M” key.

   This generates a results window. The values in the “Mean” column is the average fluorescent intensity of all the pixels in the CPV.

7. Switch to the Alexa647 channel by scrolling the bottom bar to the right. Bring back the same ROI (if not already there) and press ‘M’ key to record the fluorescent intensity of the Alexa647 in the same CPV.

Determination of ratio of fluorescent intensities in Excel

8. Record the mean fluorescent intensities of the Oregon Green and the Alexa647 in separate columns in Excel, preferably side by side.

9. Repeat steps 4–8 for both standard and experimental CPVs.

10. In Excel, calculate the ratio of mean intensities of Oregon Green and Alexa647 for each CPV by dividing Oregon Green intensity by Alexa647 intensity.

Generation of standard curve and determination of the pH of experimental CPVs in Prism

11. Copy mean intensity ratio values of standards from Excel to “Prism” and arrange in columns: Open “Prism” → Select “Column” from the sidebar → Check “enter replicate values stacked into column.”

    For example, label Group A as pH 7.0, Group B as pH 6.0, etc.

12. Calculate column averages, standard deviation and standard errors: Within the data sheet under Analysis on the toolbar, click → “Analyze” → “Column Analyses” → double click “Column Statistics” → in the “Parameters: Column Statistics” dialog box check “Mean, SD, SEM.” Click “Ok.”

    This will create a “Col Stats of ...” sheet in the “Results” section.

13. In the “Col Stats” sheet, copy mean values, standard error of mean values (SEM), and number of values (N) for each standard.

14. From the drop-down menus, click “File” → “New” → “New Data Table (+ Graph)” → select “XY” from the sidebar → under Enter/import data: Y: select “Enter and plot error values already calculated elsewhere” → select “Mean, SEM, N” from the “Enter:” drop down menu.

15. Paste Mean, SEM, and N values generated in the “Col Stats” sheet under “Group A” and arrange in rows according to the pH standards in the “X” column. Put “pH” as “X title”.

For example, in the first row, in the “X” column, type 7.0, and in group A, paste mean, SEM, and N values for pH 7 (see step 13) in respective columns. In the second row, type 6.0 in the “X” column and paste mean, SEM, and N values for pH 6 in respective columns and so on.

16. To interpolate the experimental samples to the standard curve, paste the ratio of mean intensities for the experimental samples, calculated at step 10, in the “Mean” column of group A of the same data sheet as in step 15.

17. Plot a non-linear regression curve (Boltzmann Sigmoidal) of the standards and interpolate ratio of mean intensities of the experimentals: Click “Analyze” → “XY analyses” → “Interpolate a standard curve” → OK → Select “Sigmoidal, 4PL, X is log(concentration)” → Click “More” → In Nonlinear Regression parameters dialog box → Select classic equations → Boltzmann sigmoidal → Check “Interpolate experimentals from standard curve” → OK.

    This will create a “Nonlin fit of . . . ” sheet in “Results” section containing a “Interpolated X values” sheet. These are the CPV pH values of the experimental samples.

18. Finally, copy the CPV pH values of the experimental samples and paste in a new data sheet arranged in columns, with each column being a condition (e.g., control or treatment). Perform column analyses as in step 12 to determine mean differences of the CPV pH between different conditions used.

REAGENTS AND SOLUTIONS

Use deionized, distilled water, unless otherwise noted.

Dextran solutions—Prepare a stock solution of Oregon Green-dextran (ThermoFisher Scientific, cat. no. D7171) by dissolving to a final concentration of 2 mg/ml in sterile phosphate-buffered saline (PBS; HyClone, GE Healthcare Sciences, cat. no. SH3025601). Prepare a stock solution of Alexa647-dextran (ThermoFisher Scientific, cat. no. D22914) by dissolving to a final concentration of 2 mg/ml in sterile PBS. To remove insoluble particles from dextran solutions, centrifuge for 2 min at 12,000 × *g*, 4°C. Transfer the supernatant into new sterile 1.5-ml microcentrifuge tubes as 100-µl aliquots, and store up to 6 months at −20°C protected from light. Immediately before use, prepare a dextran working solution by diluting both Oregon Green-dextran and Alexa647-dextran in warm culture medium to a final concentration of 0.5 mg/ml each, and vortex well. Centrifuge for 2 min at 12,000 × *g*, 25°C, to pellet insoluble particles, and transfer to a new tube.

Ionophore solutions—Prepare a stock solution of nigericin sodium salt (Sigma Aldrich, cat. no. N7143) by dissolving in methanol to a final concentration of 10 mM. Prepare a stock solution of monensin sodium salt (Sigma Aldrich, cat. no. M5273) in methanol to a final concentration of 10 mM. Store ionophore stock solutions up to 3 years at −20°C. Immediately before use, dilute both ionophores in warm culture medium to a final concentration of 10 µM each, and vortex well.

pH standards—Citrate or phosphate buffers of varying pH containing 10 μM of both nigericin sodium salt and monensin sodium salt are used as pH standards in this protocol. Refer to the *APPENDIX B* of Promega Buffers for Biochemical Reactions found at https://www.promega.com/resources/product-guides-and-selectors/protocols-and-applications-guide/buffers-for-biochemical-reactions/ to prepare citrate buffers of pH 4, 4.5, 5, and 5.5 and phosphate buffers of pH 6 and pH 7. Filter sterilize the pH buffers and store at room temperature. Immediately before use, add 10 μM of nigericin (10 μM final) and monensin (10 μM final) to all pH buffers to prepare pH standards.

COMMENTARY

Background Information

Following internalization by the host cell, the nascent *Coxiella*-containing phagosome matures through the host endocytic pathway, eventually fusing with host endosomes, autophagosomes, and lysosomes to form a large, fusogenic, lysosome-like vacuole (Heinzen et al., 1996). Despite the presence of active lysosomal hydrolases, *Coxiella* thrives in the acidic environment of the mature CPV (Heinzen et al., 1996; Howe, Shannon, Winfree, Dorward, & Heinzen, 2010). CPV acidification is at least partially due to the presence of vacuolar ATPase (V-ATPase) on the CPV membrane (Heinzen et al., 1996). V-ATPase is a multimeric protein responsible for lysosomal acidification, and either inhibiting V-ATPase activity or depleting V-ATPase protein using siRNA blocked *Coxiella* growth (Heinzen et al., 1996; McDonough et al., 2013). This suggests that V-ATPase-mediated CPV acidification is essential for bacterial survival within the host cell.

Early studies suggested that the CPV pH is between 4.5 and 5.3 (Akporiaye et al., 1983; Grieshaber et al., 2002; Maurin et al., 1992). *Coxiella* metabolism is most active between pH 4.5 and 4.9 in an axenic medium (Omsland, Cockrell, Fischer, & Heinzen, 2008), and CPV acidification is necessary for *Coxiella* T4SS-mediated secretion of bacterial proteins into the host cytoplasm (Newton et al., 2013). A recent study in CHO (Chinese Hamster Ovary) cells suggests CPVs exist in two heterogeneous populations, depending on CPV membrane integrity (Mansilla Pareja, Bongiovanni, Lafont, & Colombo, 2017). While the average CPV pH of the two populations was 5.68, intact CPVs had an average pH of 5.2, while a second population of damaged CPVs were more alkaline (>pH 6) (Mansilla Pareja et al., 2017). A recent study in HeLa epithelial cells and cholesterol-free fibroblasts found the average CPV pH to be 5.2, with acidification to pH 4.5 upon elevated cholesterol in the CPV (Mulye, Samanta, Winfree, Heinzen, & Gilk, 2017). Therefore, it appears that CPVs could have a wide range of luminal pH, though it is not clear how differences in technique and cell type contribute to this range of measurements. Careful studies are needed to determine how CPV pH changes during infection in different cell types, and elucidate both host and bacterial factors involved in establishing and maintaining CPV pH.

Given the importance of CPV pH to *Coxiella* pathogenesis and the wide range of published measurements, it is critical to accurately measure CPV pH during infection. One method is dual wavelength ratiometric measurement of either fluorescein or Oregon Green, a fluorescein derivative. This approach relies on a single fluorophore for both pH-sensitive and pH-stable fluorescence; the major drawback of using a single fluorophore is that

the reference (pH-insensitive) wavelength can be hampered by poor signal or underlying pH-sensitivity (Canton & Grinstein, 2015). Several studies used fluorescein to measure CPV pH; however, the higher pK_a of fluorescein (6.4) makes it less accurate at acidic pH compared to Oregon Green (pK_a 4.7). To measure CPV pH, the fluorophore is conjugated to dextran and delivered to the CPV by fluid-phase endosomes. CPVs are then imaged at both the pH-sensitive (490 nm) and a pH-insensitive (440 nm) excitation wave-lengths, with a common emission wavelength of 520 nm. To determine individual CPV pH, the 490/440 fluorescent intensity ratio is compared to a calibration curve. The calibration curve can be generated in buffers of known pH; however, this overlooks cellular factors which could potentially affect intracellular fluorescence. A preferred method is to treat cells with ionophores, followed by bathing the cells in pH buffers prior to imaging. Dual wavelength imaging of Lysosensor yellow/blue DND-160 dye has also been used to measure CPV pH, though in this case the fluorophore is membrane-permeable and not restricted to endosomes or the CPV.

The method described in this protocol uses a second pH-insensitive fluorophore such as rhodamine or Alexa647 (Drecktrah, Knodler, Ireland, & Steele-Mortimer, 2006; Johnson et al., 2016). Using two separate fluorophores avoids overlap between the excitation wave-lengths and potential pH-sensitivity of the reference wavelength. Similar to the single fluorophore method, an *in situ* calibration curve using ionophore-treated cells bathed in pH buffers is generated based on the Oregon Green/Alexa647 fluorescent intensity ratio. To avoid differential bleaching of the two fluorophores, the shortest exposure times possible must be used. In addition, because the Oregon Green and Alexa647 are not conjugated to the same dextran, this approach assumes equal trafficking of the two dextrans to the CPV over a period of four hours.

Critical Parameters

Refer to Winfree and Gilk (2017; *UNIT 6C.2*) for information on working with HeLa cells and *Coxiella burnetii*.

Anticipated Results

In infected HeLa cells and mouse embryonic fibroblast cells, we found the CPV pH to be 5.2 under control conditions and statistically significant differences between control and experimental conditions (Mulye et al., 2017). Moreover, upon drug treatment the CPV pH decreased from 5.2 to 4.5 within 30 min post-treatment and stayed stable for 2 hr. Despite the range of CPV pH that is observed in control cells, this protocol can be used to determine subtle changes in CPV pH. Oregon Green fluorescence should change noticeably between pH 7 to pH 4 (e.g., Fig. 6C.3.1A). When the Oregon Green/Alexa647 ratio is plotted against pH, a standard curve similar to Figure 6C.3.1B should be obtained.

Time Considerations

A typical experiment examining pH at three days post infection would include plating and infecting HeLa cells on day 0, replating onto μ-Slides on day 2, and dextran labeling and live cell imaging on day 3. Dextran labeling of standard and experimental samples are staggered by 4 hr. In our experience, imaging the standards (six channels) takes about three hours,

while imaging the experimental samples with two control conditions and two treatment conditions takes about an hour. A set of three experiments, with staggered infected cells, standards, and experimental conditions can be completed within three weeks.

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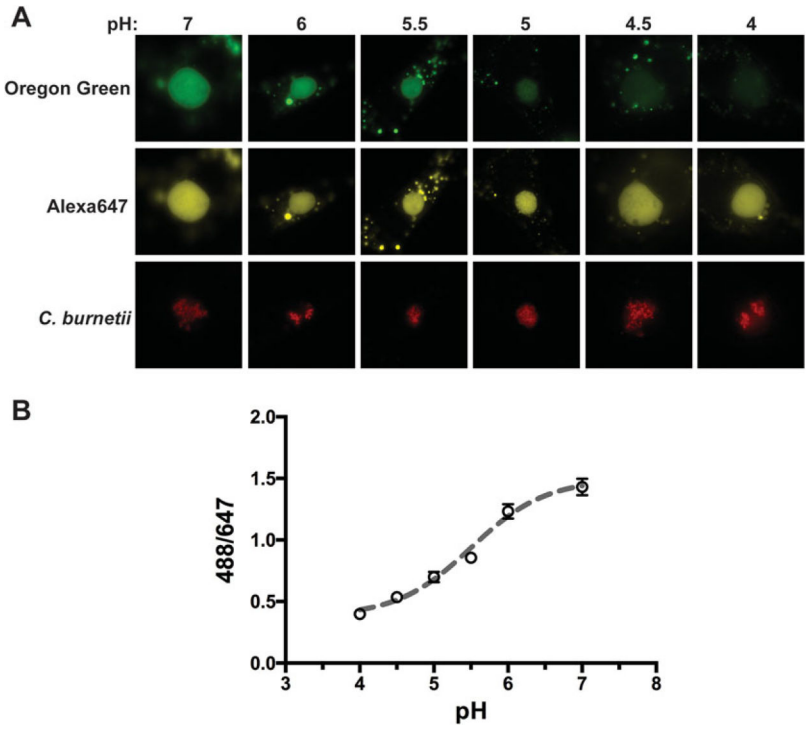

Figure 6C.3.1.

(**A**) Representative images of CPVs labeled with Oregon Green and Alexa647 conjugated dextrans, treated with ionophores, and bathed in buffers of known pH. Note the decrease in Oregon Green fluorescence as the pH becomes more acidic, while Alexa647 remains stable. (**B**) Calibration curve prepared by plotting Oregon Green (488)/Alexa647 fluorescent intensity ratio to pH.