Video Article

Extraction of Hemocytes from *Drosophila melanogaster* Larvae for Microbial Infection and Analysis

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Abstract

During the pathogenic infection of *Drosophila melanogaster,* hemocytes play an important role in the immune response throughout the infection. Thus, the goal of this protocol is to develop a method to visualize the pathogen invasion in a specific immune compartment of flies, namely hemocytes. Using the method presented here, up to 3 × 10^6 live hemocytes can be obtained from 200 *Drosophila* 3rd instar larvae in 30 min for *ex vivo* infection. Alternatively, hemocytes can be infected *in vivo* through injection of 3rd instar larvae followed by hemocyte extraction up to 24 h post-infection. These infected primary cells were fixed, stained, and imaged using confocal microscopy. Then, 3D representations were generated from the images to definitively show pathogen invasion. Additionally, high-quality RNA for qRT-PCR can be obtained for the detection of pathogen mRNA following infection, and sufficient protein can be extracted from these cells for Western blot analysis. Taken together, we present a method for definite reconciliation of pathogen invasion and confirmation of infection using bacterial and viral pathogen types and an efficient method for hemocyte extraction to obtain enough live hemocytes from *Drosophila* larvae for *ex vivo* and *in vivo* infection experiments.

Video Link

The video component of this article can be found at https://www.jove.com/video/57077/

Introduction

Drosophila melanogaster is a well-established model organism for the study of innate immunity[1]. During the innate immune response, hemocytes play an important role in the response to pathogen challenge. Hemocytes are critical for encapsulating parasites, as well as having an important function in combating the pathogen through phagocytic action during fungal, viral, and bacterial infection[2,3].

In order to best understand the host's innate immune response to pathogenic microbial infection, it is important to visualize how the pathogen invades host cells during infection. This visualization contributes to an understanding of the mechanism of invasion. Together with details of pathogen intracellular localization and the cellular response, these data can provide clues about the host response to infection and the cellular organelles with which the microbe interacts. Thus, 3D model reconstruction after imaging by microscopy can be helpful to determine the precise location of pathogens in host cells. In this study, we visualized the invasion of *Coxiella burnetii (C. burnetii),* the causative agent of Q fever, a zoonotic disease that poses a serious threat to both human and animal health, into primary *Drosophila* hemocytes. Recently, it was demonstrated that *Drosophila* are susceptible to the Biosafety level 2 Nine Mile phase II (NMII) clone 4 strain of *C. burnetii* and that this strain is able to replicate in *Drosophila*[4], indicating that *Drosophila* can be used as a model organism to study *C. burnetii* pathogenesis.

Previous studies have used hemocytes to examine the host's innate immune response. Hemocytes have been used for morphological observations[5,6,7], morphometric analysis[2,8], phagocytosis analysis[2,3], qRT-PCR[2,9], immunoprecipitation[10,11], immunofluorescent analysis[10,12], immunostaining[13], immunoblotting[3,10,11] and immunohistochemistry[9,14]. Although *Drosophila* S2 cells are also available for various *in vitro* experiments, immortalization and potential pre-existing viral infection change their behavior[15,16]. The use of primary cells as opposed to an immortalized cell line, such as S2 cells, allows for the study of innate immune function in a system more representative of the whole organism. Additionally, the infection of hemocytes *in vivo*, prior to extraction, allows the cells to interact with other host proteins and tissue, an advantage over extraction of hemocytes prior to *ex vivo* infection. A number of different methods have been utilized to obtain a sufficient number of hemocytes in a short period of time to keep the hemocytes alive[8,17,18,19].

In this study, we present a method to extract hemocytes from *Drosophila* 3rd instar larvae for pathogenic microbial infection with *C. burnetii*, *Listeria monocytogenes* (*Listeria*), or Invertebrate iridescent virus 6 (IIV6). We describe the methods for both *in vivo* and *ex vivo* hemocyte infections. *In vivo*- and *ex vivo*-infected hemocytes were visualized with confocal microscopy and used to build 3D models of *C. burnetii* invasion. Additionally, using the extraction protocol, *ex vivo*-infected hemocytes were used for gene and protein expression assays. Specifically, to examine the extent of infection with IIV6 and *Listeria*, total RNA or protein was isolated from the cells for qRT-PCR or Western blot analysis. Taken together, the protocol provides methods to rapidly collect high numbers of hemocytes from 3rd instar larvae and evidence that primary

hemocytes, infected either *in vivo* or *ex vivo*, are a suitable platform for microbial pathogen infection studies and applicable downstream analyses such as microscopy, transcriptomics, and proteomics.

Protocol

1. *Ex vivo* infection

1. Medium and equipment
    1. Under sterile conditions, prepare fresh *Drosophila* Hemocyte Isolating Medium (DHIM) containing 75% Schneider's *Drosophila* medium with 25% Fetal Bovine Serum (FBS) and filter sterilize it.
    2. Layer 2-3 pieces of 10 cm x 10 cm paraffin film under a stereomicroscope.
    3. Prepare the glass capillary. Set the capillary puller heater to 55% of maximum. Pull the capillary tube to a sharp point of approximately 10 µm.
    4. Backfill the capillary with mineral oil.
    5. Assemble filled capillary tube onto the nanoinjector (**Figure 1A**), and open fused capillary tube tip by breaking off the tip with forceps (**Figure 1B**). The outer diameter of the tip should be 100 µm for easy uptake of the hemocytes.
    6. Eject as much oil as possible from the capillary tube tip, then fill with DHIM. For easy visualization of the border of up-taken hemolymph and the oil, include an air bubble between the oil and DHIM (**Figure 1B'**).

2. Hemolymph extraction
    1. Pick 3rd instar *Drosophila* larvae from the inside wall of food vials gently using forceps and place them into a 100 µm strainer (**Figure 1C**). 3rd instar larvae are found 3-6 days following fertile egg laying by an adult female.
    NOTE: These experiments used the genotype, w^{1118};P{w^{+mC}=Hml-GAL4.Δ}2,P{w^{+mC}=UAS-2xEGFP}AH2, since hemocytes from these animals express enhanced green fluorescent protein (EGFP) to aid in identification of the cells by microscopy.
    2. Pour 5 mL of sterile water over larvae and shake the strainer for 5 s. Place the strainer onto task wipe to remove the excess water (**Figure 1C'**).
    3. Transfer the larvae into a 1.5 mL microcentrifuge tube. Anesthetize them with CO_2 gas for 5 s (**Figure 1D**).
    4. Place the larvae onto paraffin film under the stereomicroscope, with dorsal-side facing up (**Figure 2A**).
    5. Place the glass capillary lightly onto the larval posterior body to hold it in place and disrupt the posterior cuticle open using fine pointed forceps (**Figure 2B**).
    6. Allow the hemolymph to flow onto the paraffin film (**Figure 2C**).
    7. Make a pool of hemolymph including hemocytes from 20-50 larvae at a time on the paraffin film.
    8. Take up pooled hemolymph using the glass capillary on the nanoinjector (**Figure 2D**).
    NOTE: There should be approximately 10-20 µL of hemolymph.
    9. Eject the hemolymph into a 1.5 mL microcentrifuge tube containing 500 µL of DHIM (**Figure 2E**).
    10. Repeat steps 1.2.4) - 1.2.9) for every batch of larvae.

3. Count the number of hemocytes.
    1. Pipette 5 µL of 0.4 % Trypan blue solution in a 0.6 mL microcentrifuge tube to stain the dead cells. Gently mix the DHIM and hemocytes in the 1.5 mL tube using a pipette, and transfer 5 µL of DHIM including hemocytes to the 0.6 mL microcentrifuge tube and mix gently.
    2. Pipette 10 µL of cells from the 1:1 Trypan blue:hemocyte mixture into the hemocytometer.
    3. Count the number of live hemocytes that are not stained with Trypan blue in each of the 4 corner fields of the hemocytomter and calculate the concentration of the hemocytes per milliliter using formula:

    $$X \text{ cells / mL} = \frac{a + b + c + d}{2} \cdot 10^4$$

    where X is the concentration of live hemocytes per milliliter; a, b, c, and d are the number of live cells (as determined by Trypan blue exclusion) in each 4 of the fields counted in the hemocytometer. Division by 2 of the total number of cells counted is due to the 1:1 dilution of the cells with Trypan blue. Cells stained with Trypan blue are considered dead.

4. *Ex vivo* Infections
    1. Determine the number of wells to be seeded with cells based on timepoints and biological replicates needed for each experiment. 5.0×10^4 hemocytes are desirable for RNA and protein purification following infection.
    2. Calculate the volume of pathogen stock to be diluted with DHIM for infection using the following formula:

    $$Y = \left(\frac{1 \text{ mL}}{\text{Pathogen titer (GE, CFU, or TCID}_{50})}\right)(\text{MOI})\left(\frac{\text{number of hemocytes}}{\text{well}}\right)\left(\frac{1000 \text{ µL}}{1 \text{ mL}}\right)$$

    where Multiplicity of infection (MOI) is the number of viral or bacteria desired per cell.
    NOTE: MOI used depends on the individual experiment and assays performed. Here, 10 genome equivalents (GE)/cell of *C. burnetii*, 10 CFU/cell of *Listeria*, or 1 $TCID_{50}$/cell of IIV6 was used.
    3. Prepare 500 µL of pathogen medium for each well of a 24-well plate by adding the proper volume of DHIM to the viral or bacterial volume in a tube.
    4. Place a 12 mm round cover glass (no. 1 thickness) in a well of a 24-well plate.
    5. Split the DHIM including the hemocytes into wells of a 24-well plate.
    6. Add 500 µL of pathogen medium to hemocytes in a well.
    7. Centrifuge the plate at 1,000 x g for 5 min.
    8. Incubate the plate for 1 h at 28 °C. Every 15 min, gently tilt the plate from back to front, then left to right for 5 s by hand.

9. After the 1 h of invasion/attachment step 1.4.8), gently pipette off the pathogen medium and wash the hemocytes with fresh DHIM, and refill it with 500 µL of fresh DHIM.
10. Incubate the infected hemocytes for the desired time. In these experiments, *C. burnetii*-or IIV6-infected hemocytes are incubated for 24 h, and *Listeria*-infected hemocytes are incubated for 1, 2, or 4 h.

2. *In vivo* infection

1. Infection
    1. Warm the *Drosophila* fruit juice agar plate at room temperature for 15 min. Plates are made as previously described[20].
        1. Add 30 g of agar to 700 mL of water and autoclave it for 40 min.
        2. Dissolve 0.5 g of methyl paraben in 10 mL of absolute ethanol.
        3. Add the methyl paraben solution to 300 mL of fruit juice concentrate.
        4. Quickly mix the juice concentrate into the autoclaved agar solution and dispense 5 mL into 10 × 35 mm Petri dishes.
        5. After the plates have cooled for 15 min, store them at 4 °C.
    2. Prepare 3rd instar larvae following steps 1.2.1) - 1.2.3).
    3. Place the yeast paste on an agar plate. Make a fine cut in the agar plate where larvae can migrate to avoid drying (**Figure 3A**). Assemble a 0.001 mm pointed tungsten needle with holding forceps using paraffin film (**Figure 3B, C**).
    4. Pipette 50 µL of high-titer mCherry expressing-*C. burnetii* (5.95×10^{9} GE/mL) onto the paraffin film under the stereo microscope and place the larvae into the pool of bacteria.
    5. Place the larvae into the pool of bacteria. Prick the larvae with a tungsten needle (**Figure 3D**). Transfer the larvae onto an agar plate (**Figure 3E**).
    6. Transfer the remaining pathogen medium onto the agar plate and seal the plate with paraffin film.
    7. Keep the larvae on the plate in moist air until the desired time post-infection (**Figure 3F**). In this experiment, *C. burnetii*-infected larvae are on the plate for 24 h.

2. Hemolymph extraction and plating of hemocytes
    1. Prepare the medium and equipment following step 1.1).
    2. Place a 12 mm round cover glass (no. 1 thickness) in a well of a 24-well plate. Pipette 500 µL of DHIM into the well.
    3. Extract the hemolymph from the infected larvae following steps 1.2.4) - 1.2.8).
    4. Eject the hemolymph into the well following step 2.2.2).
    5. Repeat 2.2.3) and 2.2.4) for multiple batches of larvae.
    6. Centrifuge the plate at 1,000 x g for 5 min.

3. Visualization

1. Fixing and staining
    1. After allowing the hemocytes to settle on the round cover glass in the well, gently remove the medium from each well.
    2. Gently add 200 µL of 4% paraformaldehyde (PFA) to each well of settled hemocytes. Incubate the hemocytes for 20 min at room temperature.
    3. Remove the 4% PFA and gently add 200 µL of PBS containing 0.1% Triton X-100 and 1% Bovine Serum Albumin (BSA) to each well. Incubate the hemocytes for 10 min at room temperature.
    4. Remove the PBS and gently add 200 µL of 1× 4',6-diamidino-2-phenylindole (DAPI) to each well. Incubate the hemocytes for 10 min in the dark at room temperature.
    5. Remove the DAPI solution and gently add PBS to each well. Incubate the hemocytes for 5 min at room temperature.
    6. Drop 10 µL of the antifade mounting medium on a glass microscope slide.
    7. After removing the PBS from each well, remove the cover glass from the 24-well plate using fine-pointed forceps. Gently place the cover glass onto the antifade mounting medium on the glass slide, with the hemocytes facing down.
    8. Allow the slide to dry by placing it in the dark overnight.

2. Confocal Imaging
    1. Configure the confocal microscope for three color imaging of DAPI, EGFP, and mCherry. Use the following setting: DAPI excitation (ex) 405 nm, emission (em) 415-480 nm; EGFP ex 488 nm, em 493-564 nm; mCherry ex 587 nm, em 597-700 nm.
    2. Place the sample on the microscope and focus on the sample using a 63X/1.4 numerical aperture (NA) objective. Locate desired hemocytes in the field of view for imaging.
    3. Adjust laser power and detector gains to achieve appropriate exposure of the sample. Check multiple z-planes to ensure the exposure level is appropriate for the entire sample thickness.
    4. Find the top and bottom position on the z-axis of a whole hemocyte. Set these positions as the start and end positions for z-sectioning.
    5. Only use scanning zoom to image the area containing the hemocyte. Zoom factors of 3X are often used.
    6. Collect the image series at appropriate resolution such as 1024 x 1024 pixels in the x-y plane, and 0.3 µm spacing in the z dimension.

3. 3D model reconstruction
    NOTE: Open source software exists that performs many of the functions described below for 3D model reconstruction.
    1. Import the z-sectioned image series file into the software associated with the confocal microscope for 3D model reconstruction.
    2. Select a cell showing co-localization of nuclei stained with DAPI and mCherry expressing *C. burnetii* in an EGFP expressing hemocyte. Crop the image series to contain only the single cell.

3. Select the 3D Viewer option which reconstructs the 3D model using the software's pre-packaged algorithm. Choose the desired type of 3D representation among Blend, Surface, and Mixed options. In this method, hemocytes, nuclei, and *C. burnetii* are shown using surface models.
4. Observe the 3D reconstructed cell from various viewing positions by holding the mouse button and dragging the cursor around the screen. Adjust the cell orientation and position of the modeled light source to optimize the image. Other options for Opacity, Minimum and Maximum Threshold, Specular, Ambient, Shineness, and Gamma exist to optimize the image.
5. Take cross-sections through the model using clipping and sectioning commands to visualize the interior contents of the hemocyte.

4. Application for gene and/or protein analysis

1. Following the infection with IIV6 and *Listeria*, lyse cells for subsequent qRT-PCR or Western blot analysis as previously described[21] and following manufacturer's instructions.
   NOTE: Refer the **Table of Materials** for the primers for qRT-PCR and the antibodies for Western blot.
2. Analyze PCR products by agarose gel electrophoresis, as previously described[22], to ensure the proper length of the amplified product.

Representative Results

To collect live hemocytes for *ex vivo* infection, up to 3×10^6 hemocytes were extracted from 200 *Drosophila* 3rd instar larvae. To develop our method, a number of different techniques were attempted. Individual larval dissection would take up to 1.5 h, and an average of ~8000 cells were obtained using this method[18], most of which were not alive by the end of collection. Next, we tried to extract hemolymph, which contained the hemocytes, from 20 larvae at a time using a glass capillary tube[19], but the capillary became clogged with cuticle material and the hemolymph was not able to be efficiently taken up by the glass capillary. Finally, we mechanically disrupted the cuticle of 20-50 larvae per batch with fine forceps[12], and made a pool of hemolymph for easy uptake. This allowed easy collection of a large amount of hemocytes from a large number of larvae (**Table 1**).

To indicate that the extracted hemocytes were alive and suitable for the infection, a Trypan blue exclusion assay was performed to calculate the percentage of live hemocytes extracted using the method presented here (**Figure 6A**). In addition, we compared our method for hemocyte extraction with a previously published method where the goal was to develop a mechanical disruption method to isolate and differentiate between circulating and resident hemocytes from individual *Drosophila* larva[23]. Upon comparison between the two methods from 10 independent experimental isolations, we observed that cell viability was slightly greater using the method presented here (**Figure 6A**); however, the method from Petraki *et al*. yielded close to twice as many hemocytes from every 10 dissected larvae (**Figure 6B**). A reason for the increased number of hemocytes from the Petraki *et al*. method is that this method collects resident (sessile) hemocytes, as well as circulating hemocytes. To confirm that the cells extracted with the method presented here were in fact hemocytes, we utilized a fly line containing transgenes for the hemolectin (Hml) promoter driving GAL4 in circulating hemocytes that binds the upstream activator sequence (UAS) to activate enhanced green fluorescent protein (EGFP) transcription and expression in the hemocytes. Hemocytes from *hml-GAL4>UAS-EGFP* larvae were extracted onto chambered coverglass slides, fixed, permeabilized and stained with DAPI as previously described[21]. **Figure 7** shows that most DAPI-positive cells are also EGFP-positive. Quantification of co-expression was performed from multiple images, from which we calculated that 86±9% of cells isolated were hemocytes.

The protocol in innovative 3D models of pathogen invasion into *Drosophila* hemocytes extracted from 3rd instar larvae following *ex vivo* or *in vivo* *C. burnetii* infection. We were able to image *C. burnetii* infection since the bacteria express mCherry. After infected hemocytes were fixed and stained, they were imaged for DAPI, EGFP, and mCherry using confocal microscopy. Hemocyte infection rates, as determined by the percentage of EGFP-positive cells that also exhibited mCherry signal, for both *ex vivo* and *in vivo* *C. burnetii* infections was nearly 100%. This was expected since an MOI of 10 GE/cell was used for *ex vivo* infection, which should result in infection of 100% of cells[24]. Regarding the *in vivo* infections, since larvae are placed in a high-titer droplet of mCherry expressing-*C. burnetii* (5.95×10^9 GE/mL), the number of bacteria is roughly 10,000-fold higher than the number of hemocytes per larva. Therefore, a 100% infection rate is expected for the *in vivo* infections.

Next, Z-sections were collected through the hemocyte to visualize the entire cell in 3D, and to confirm the presence of *C. burnetii* in the interior of the cell. **Figure 4** shows transparent cross-sections of a hemocyte (in green) with *C. burnetii* (in red) seen in the interior of the cell. The images were also reconstructed into a 3D model (**Figure 5**) representing the surfaces of the hemocyte and *C. burnetii*, again showing *C. burnetii* in the interior of the cross-sectioned hemocytes. Interestingly, *in vivo*-infected hemocytes exhibited greater cytoplasmic extensions and were flatter in nature, while *ex vivo*-infected hemocytes were more spherical (**Figure 5**). This could indicate a greater population of lamellocytes in the *in vivo*-infected population and plasmatocytes in the *ex vivo* population[7]. While lamellocyte differentiation is generally induced during parasitic wasp infection[25], wounding of the *Drosophila* larvae is also sufficient to induce lamellocyte differentiation[26]. Finally, while not utilized in the experiments presented here, there are GFP-expressing forms of *Listeria*[27] and IIV6[28] that could be used to generate 3D models of hemocyte infection. Instead, *Listeria*- and IIV6-infected hemocytes were used for Western blotting and qRT-PCR experiments.

Using the method presented here, infections are performed both *ex vivo* and *in vivo* for imaging. In addition, pathogen mRNA and protein analysis followed *ex vivo* infections. Specifically, extracted hemocytes were infected with IIV6 and were applied to qRT-PCR analysis. It showed significantly higher levels of IIV6-193R, a viral gene that encodes for a putative inhibitor of apoptosis[29], between mock- and IIV6-infected cells (**Figure 8A**). Electrophoresis of the amplified products confirmed the presence of a band for the IIV6-193R gene product in the infected sample, but not the mock-infected sample (**Figure 8B**). Amplified bands for the RpII endogenous control were found in all samples, and IIV6 infection in S2 cells were performed as a positive control. Since IIV6 infections were performed at an MOI of 1 $TCID_{50}$/cell, approximately 50% of the cells were expected to be infected, based on the Poisson distribution[24].

Total protein from mock- or *Listeria*-infected hemocytes was collected at 1, 2, and 4 h post-infection and protein concentration was determined by Bicinchoninic acid (BCA) assay. 100% of the cells were expected to be infected *ex vivo* since the MOI was 10 CFU/cell[24]. Western blotting confirms the presence of *Listeria*-derived protein products in the infected hemocytes, with high levels achieved by 4 h post-infection (**Figure 9**). *Listeria* inoculum is used as a positive control for the detection of *Listeria*-specific bands. The presence of actin is shown in the hemocyte samples to confirm levels of protein loading. Taken together, these results indicate that the hemocytes extracted using the method presented here were suitable for both viral and bacterial infection experiments.

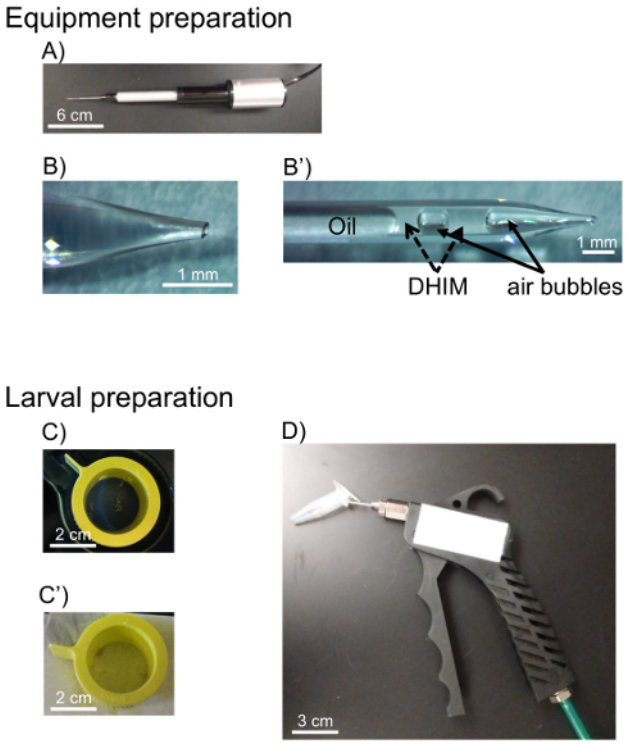

Figure 1: Outline of equipment and materials used for hemocyte extraction. Equipment was first prepared prior to dissection. A) The pulled glass capillary was inserted into the nanoinjector after being back-filled with mineral oil. B) The fused tip of the glass capillary was broken with forceps to create a 100 µm outer diameter. B') DHIM was taken up by the capillary to avoid cell contamination and air bubbles were introduced to make a clear distinction between oil and DHIM. C) Larvae are picked from food vials and placed into a cell strainer. C') Larvae are washed in sterile water and water is removed with a task wipe, D) Larvae are transferred into a microcentrifuge tube and anesthetized with CO_2 gas. Please click here to view a larger version of this figure.

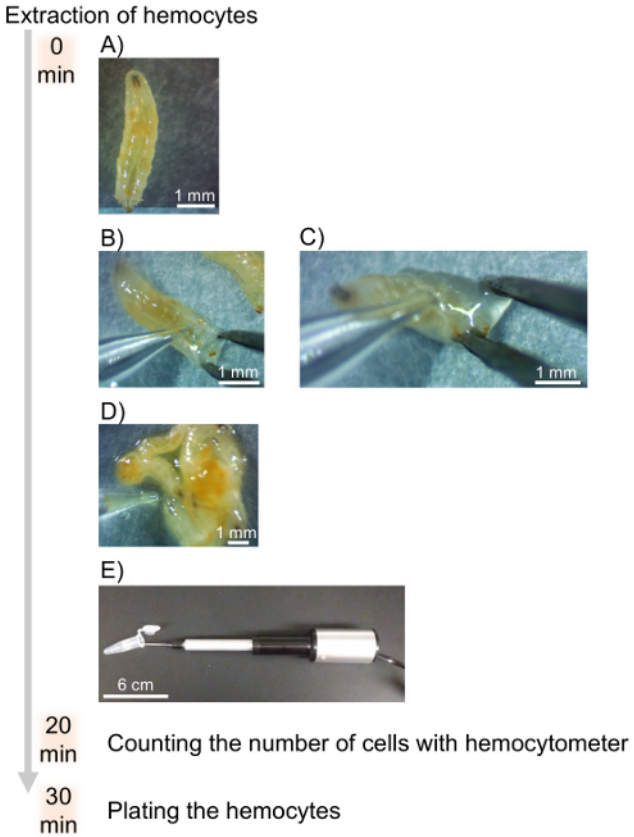

Figure 2: Timeline and flow chart for the extraction of hemocytes. A) Larvae are placed on their dorsal side prior to opening the cuticle. B) The cuticle is disrupted with fine pointed forceps and the capillary needle. C) The hemolymph is bled onto paraffin film. D) Pools of hemolymph from 20-50 larvae are taken up with the glass capillary and nanoinjector. E) The hemolymph and hemocytes are transferred into a microcentrifuge tube containing 500 µL DHIM to be counted with a hemocytometer. Please click here to view a larger version of this figure.

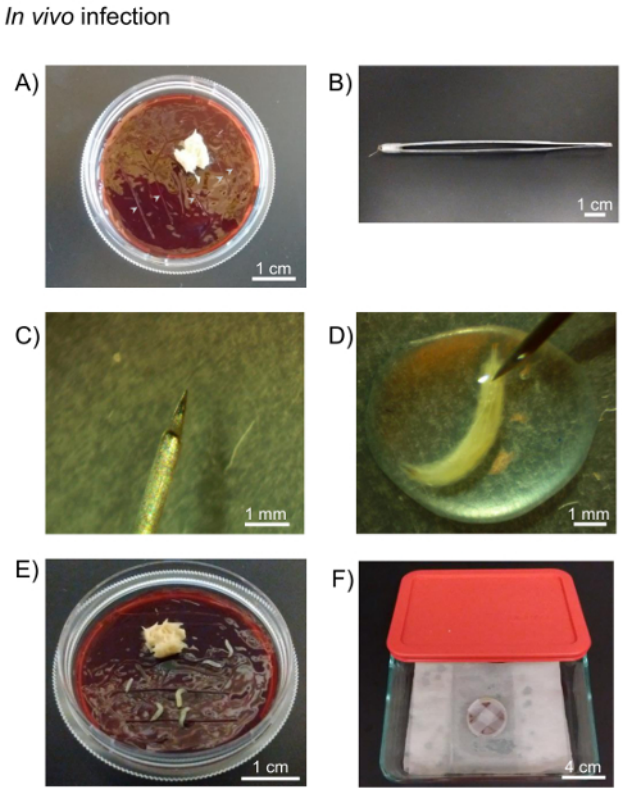

Figure 3: ***In vivo* infection.** A) *Drosophila* fruit juice agar plates and yeast paste are used for incubation of *in vivo* infected larvae. Cuts are made in the agar plate to facilitate larvae longevity (grey arrows). B) A 0.001 mm pointed tungsten needle is attached to forceps using paraffin film. C) The tip of 0.001 mm pointed tungsten needle. D) The larva is pricked with tungsten needle in the pathogen pool on the paraffin film under stereo microscope. E) The pricked larvae are placed onto the agar plate. F) The plate is sealed with paraffin film and kept on moist paper towels in the container until appropriate time. Please click here to view a larger version of this figure.

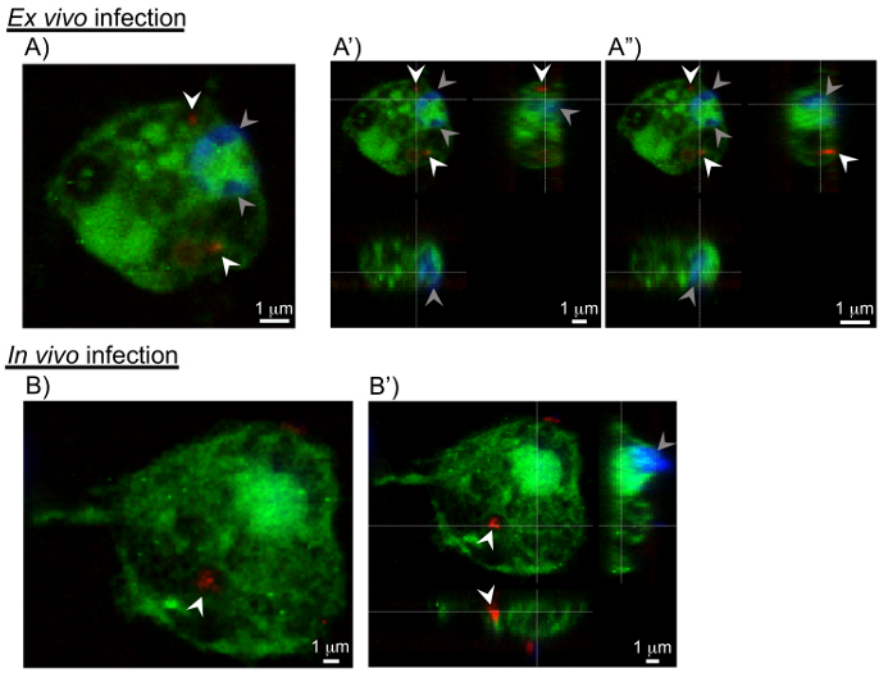

Figure 4: Cross-sections of pathogen invasion into the hemocytes. Sections extracted from 3D confocal scanning of pathogen-infected hemocytes. A, B) The xy-sections shows *C. burnetii* (in red marked with white arrowheads) in the interior of the hemocyte. The grey arrowheads show the nuclei of hemocytes. A', A", B') Views including yz- and xz-section images show the invasion of *C. burnetii* into the hemocytes from 2 additional viewing points. Please click here to view a larger version of this figure.

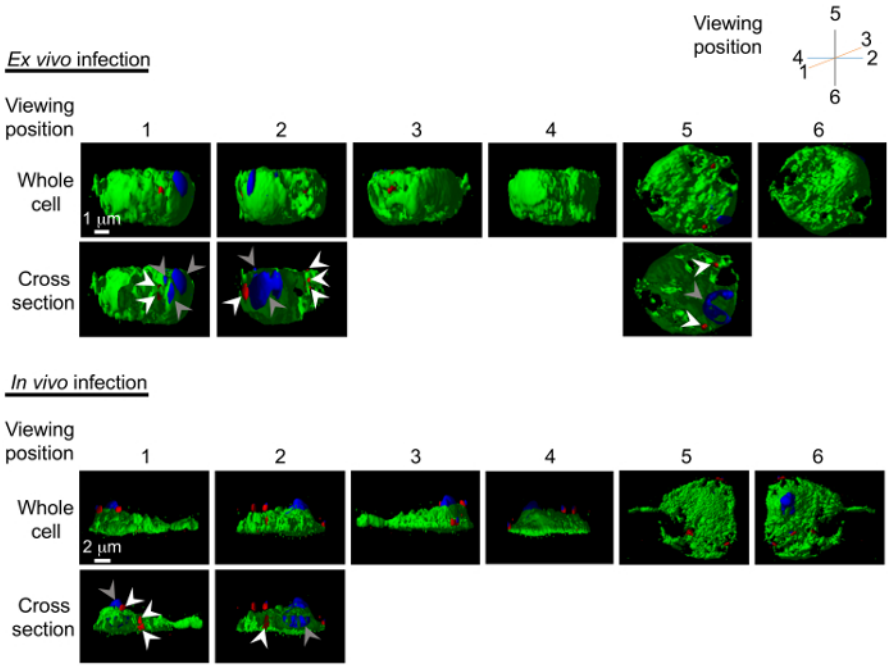

Figure 5: 3D models of pathogen invasion into the hemocytes. Reconstructed 3D models of *C. burnetii* invasion into the hemocytes are generated following *ex vivo* and *in vivo* infection. Model can be freely rotated using the software; here 6 viewing points are shown with the hemocyte in green, *C. burnetii* in red (white arrowheads), and nucleus in blue (grey arrowheads). The cross-section images showing the interior of pathogen-invaded cells are also shown. Please click here to view a larger version of this figure.

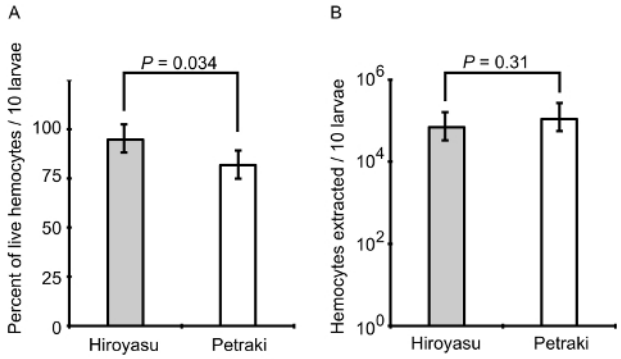

Figure 6: The percentage and population of live hemocytes. Hemocytes were extracted from groups of 10 3rd instar larvae using the method of Petraki et al. and the method presented here. A) The percentage of the live hemocytes was calculated for each technique by Trypan blue exclusion assay. B) The total population of hemocytes was compared between the two techniques. The bar graphs represent the mean ± standard deviation from N = 10 biological replicates per method of extraction and assay type. P-values are indicated from a two-tailed Student's T-test assuming unequal variance. Please click here to view a larger version of this figure.

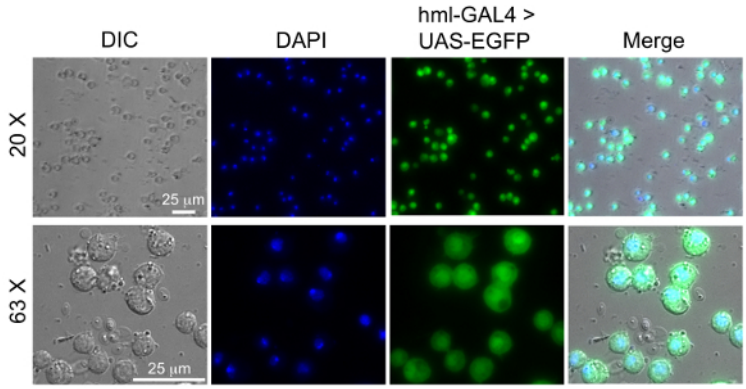

Figure 7: Images of extracted hemocytes using the hemolectin driver. Hemocytes were extracted from 80 larvae of w^{1118};P{w[+mC]=Hml-GAL4.Δ}2, P{w[+mC]=UAS-2xEGFP}AH2. The promoter for Hml drives GAL4 in circulating hemocytes, which binds UAS to activate EGFP transcription and expression. The hemocytes were fixed and stained with DAPI as previously described[20]. Hemocytes are mounted on chambered coverslips and imaged by differential interference contract (DIC) and fluorescence microscopy. The blue channel shows the nucleus stained with DAPI and the green channel shows the hemocytes expressing EGFP. Please click here to view a larger version of this figure.

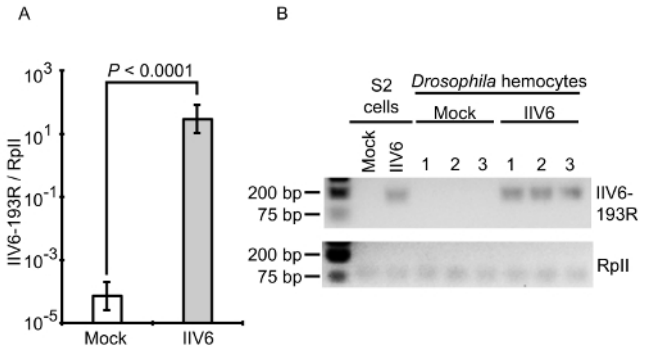

Figure 8: qRT-PCR and gel electrophoresis. The expression of the IIV6-193R gene was observed by qRT-PCR only in the IIV6-infected hemocytes. A) 193R gene expression was normalized to the internal control, RpII, and presented as a ratio. The cycle number for the mock-infected hemocytes was arbitrarily set to a maximum cycle number of 40 for analysis since 193R was not detected in these samples. Data represents the mean ± standard deviation from N = 3 biological replicates per group. The P-value indicates a two-tailed Student's T-test assuming unequal variance. B) The qRT-PCR products are shown by agarose gel electrophoresis. Please click here to view a larger version of this figure.

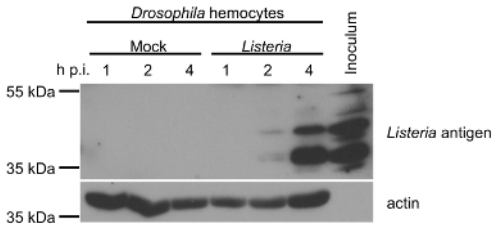

Figure 9: Western Blot Analysis. The expression of *Listeria* antigens and actin in mock- and *Listeria*-infected hemocytes from 3rd instar *Drosophila* larvae at 1, 2, and 4 h post-infection (p.i.) are determined by Western blotting. Total protein concentrations were determined by the Bicinchoninic acid (BCA) assay to normalize total amount of protein loaded in each lane of the gel. Inoculum used to infect the hemocytes was used as a positive control sample. Please click here to view a larger version of this figure.

Method	Average number of larvae for hemocyte extraction	Average number of total hemocytes
hemolymph capillary extraction (this method)	192.44 (SD: 86.05)	4.56 x 10^5 cells (SD: 5.83 x 10^5)
	(Range: 70 - 390, N = 25 trials)	(Range:1.20 x 10^5 - 2.95 x 10^6 cells)
individual larvae dissection	26.67 (SD: 11.55)	8.33 x 10^3 cells (SD: 6.66 x 10^3)
	(Range: 20 - 40, N = 10 trials)	(Range: 4.00 x 10^3 - 1.60 x 10^4 cells)
larval capillary extraction*	N/A*	N/A*
*hemocytes were unable to be extracted using this method due to clogging of the capillary needle		

Table 1: Number of dissected larvae and extracted hemocytes using different techniques. The average numbers of dissected larvae and extracted hemocytes were compared between the method presented here and other methods. Our method resulted in collection of higher numbers of larvae and hemocytes. The larval capillary extraction method was also attempted, but hemocytes were unable to be extracted due to clogging of the capillary tip.

Discussion

To better understand how host cells become infected, it is important to clarify the localization of pathogen in the cells, especially when experimenting on previously untested pathogen and cell type combinations[4]. While studying the cellular response cascade following infection can indicate productive pathogen invasion, the combination of imaging and cellular response data is essential to demonstrate pathogen invasion and infection. While reports showing 2D images of pathogen invasion into the host cells tends to indicate productive infection, some questions may remain regarding the timing of initial pathogen invasion in host cells. Thus, 3D models reconstructed from z-section scanning of 2D images can address these questions and indicate pathogen location in the host cells (**Figures 4** and **5**). However, a limitation of using primary hemocytes is their longevity in cell culture. A previous report states that hemocyte survival in cell culture media is only 8 h[18], and in our infection protocol, we were able to perform assays up to 24 h, but not longer. Therefore, it was not possible to observe high levels of *C. burnetii* replication or the formation of the parasitophorous vacuole which does not begin to form until 2 days post-infection and is not observably large until 4-6 days post-infection[30].

For many experiments where the endpoint assays utilize protein or RNA for analysis, large numbers of cells are usually required to produce sufficient material for interrogation. For example, here, we use endpoint analyses such as Western blotting and qRT-PCR to probe the extent of pathogen infection in primary hemocytes derived from 3rd instar *Drosophila* larvae. Thus, the method presented here focuses on rapid larval dissection and the collection of the hemolymph containing sufficient live hemocytes for *ex vivo* pathogen infection. This method introduces the use of a nanoinjector to take up the hemolymph and hemocytes quickly. Placing the hemocytes in DHIM containing 25% FBS is important for hemocyte survival. Additionally, for the *ex vivo* infections presented here, a large number of hemocytes are needed. To avoid melanization[31,32,33], dissection and hemocyte collection must occur rapidly. While other methods for hemocyte extraction exist[18,19,23], the duration of these methods to collect a sufficiently large number of hemocytes for *ex vivo* infection was too long. The 100 µm fine tip is beneficial for the uptake of hemolymph without taking up other organs that may lead to clogging of the capillary needle. The use of a nanoinjector may also be automated when it is attached to a micromanipulator stage and foot pedal, allowing the user to focus on quick dissection of the *Drosophila* larvae and decrease the time that the hemocytes are without surrounding larval tissue or cell culture media. Nevertheless, the use of a pipette is also available to take up the hemolymph for transfer to DHIM. In addition, our method utilizes CO_2 gas to anesthetize larvae prior to dissection; the use of a cold block with paraffin film covering is another viable method[23]. Finally, dissection of larvae in a drop of DHIM during the release of the hemolymph may reduce the number of hemocytes that are lost from sticking to the paraffin film but will increase time needed for uptake by the capillary needle.

A common immune response in *Drosophila* to injury, which occurs during the opening and dissection of the larval cuticle, is melanization[31,32,33]. During the extraction of hemocytes, we would observe melanization of the hemocytes in the DHIM as early as 10 min following extraction. As melanization is a rapid process, these cells would be excluded from the pathogen infection experiments. Additionally, the anti-coagulant phenylthiourea can be added to DHIM to inhibit the phenoloxidase-activating system and melanization during wounding[9]. Nevertheless, as melanization and apoptosis are innate immune responses of *Drosophila*, their levels can be quantified following hemocyte extraction and stimulation using the methods described here.

While recovering large amounts of protein or RNA material is a requirement for techniques such as Western blot and qRT-PCR, new techniques, such as RNAseq require much less input material, as little as 10 pg of high-quality total RNA from a single cell[34]. Experiments such as these raise interesting experimental questions, and since *Drosophila* hemocytes are a heterogeneous population containing crystal cells, plasmatocytes, and lamellocytes[7], one could begin to interrogate the transcriptional response of each cell type[35]. For example, Kurucz et al., have developed antibodies that could be used to isolate the *Drosophila* hemocyte subsets that could be used for transcriptional or proteomic profiling following various stimuli. Additionally, the recent development of single cell RNAseq technology could define transcriptomes of each cell type without the use of antibodies to initially separate each cell type[36,37,38,39]. Furthermore, one could ask questions regarding gene regulation in the hemocyte subsets that may help us understand how human blood cell lineages originated and evolved from ancient organisms through comparative genomics efforts. Tackling problems such as these requires the combination of a wide range of experimental techniques, and the technique described here may be useful for such efforts when the isolation of a large number of hemocytes from invertebrate larvae is required.

Here, we suggest the combination of 3D models with numerical, biochemical data for confirmation of infection. In future studies, we can use the methods described here to observe immune responses and the mechanism of pathogen invasion into host cells by co-staining host proteins for microscopy analysis.

Disclosures

The authors declare that they have no competing financial interests.

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