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Corresponding Author:	Jeff Leips University of Maryland Baltimore County Baltimore, MD UNITED STATES
Corresponding Author's Institution:	University of Maryland Baltimore County
Corresponding Author E-Mail:	leips@umbc.edu
Order of Authors:	Shonda Campbell
	Michelle Starz-Gaiano
	Jeff Leips
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TITLE:

Assessing the Age-Specific Phagocytic Ability of Adult *Drosophila melanogaster* Hemocytes Using an In vivo Phagocytosis Assay

AUTHORS AND AFFILIATIONS:

Shonda M. Campbell, Michelle Starz-Gaiano, Jeff Leips

Department of Biology, University of Maryland Baltimore County, Baltimore, MD

campb2@umbc.edu
starz@umbc.edu
leips@umbc.edu

CORRESPONDING AUTHOR:

Jeff Leips

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SUMMARY:

This protocol describes an in vivo assay of phagocytosis used to assess and quantify the ability of young and aged *Drosophila melanogaster* hemocytes to phagocytose bacteria.

ABSTRACT:

Phagocytosis is an essential function of the innate immune response. This process is carried out by phagocytic hemocytes whose primary function is to recognize a wide range of particles and destroy microbial pathogens. As organisms age, this process begins to decline, yet little is known about the underlying mechanisms or the genetic basis of immunosenescence. Here, an injection based in vivo phagocytosis assay is used to assess age related changes in different aspects of phagocytosis, such as binding, engulfment, and degradation of internalized particles, by quantifying phagocytic events in hemocytes in adult *Drosophila. Drosophila melanogaster* has become an ideal model to investigate age related changes in innate immune function for many reasons. For one, many genetic components and functions of the innate immune response, including phagocytosis, are evolutionarily conserved between *Drosophila* and mammals. Because of that, results obtained from using this protocol are likely to be widely relevant to understanding the age related changes in immune function in a variety of organisms. Additionally, we note that this method provides quantitative estimates of hemocyte phagocytic ability, which could be useful for a variety of research topics, and need not be limited to studies of aging.

INTRODUCTION:

The innate immune system, which consists of physical and chemical barriers to infection as well as cellular components, is evolutionary conserved across multicellular organisms[1,2]. As the first line of defense, the innate immune system plays a critical role in combating invading pathogens

in all animals[1-3]. The components of the innate immune response include a wide range of cell types which are classified on the basis that they lack specificity and immunological memory[2-4]. In humans, these cell types include phagocytic monocytes and macrophages, neutrophils, and cytotoxic natural killer cells[4,5]. While having a functional immune system is imperative for host survival, it is clear that the function of immune cells declines with age, a phenomenon known as immunosenescence[5,6]. Being able to assess age related changes in the immune response, including different aspects of the process of phagocytosis, could aid in our understanding of immunosenescence. The procedure we describe here provides an effective and repeatable approach to evaluate and quantify phagocytic events by hemocytes in *Drosophila melanogaster.*

Drosophila is an ideal model for studying the immune response for many reasons. For one, there is an extensive set of genetic tools available that make it possible to easily manipulate gene expression in a tissue-dependent manner[7]. These tools include a collection of mutants, RNA interference stocks, GAL4/UAS stocks, and the *Drosophila* Genetic Reference Panel that contains 205 different inbred lines for which the entire genome sequences are catalogued[8]. The short life cycle of *Drosophila* and the large number of individuals produced allow researchers to test multiple individuals in a controlled environment, in a short period of time. This greatly improves the ability to identify subtle differences in immune responses to infection among genotypes, between sexes or across ages. Importantly, many genetic components and functions of the innate immune response, including phagocytosis, are evolutionarily conserved between *Drosophila* and mammals[1,2].

In *Drosophila,* the process of phagocytosis that follows infection is carried out by phagocytic hemocytes called plasmatocytes, which are equivalent to mammalian macrophages[9]. Hemocytes are essential for recognizing a wide range of particles and clearing microbial pathogens[9-13]. These cells express a variety of receptors that must differentiate self from non-self, and initiate signaling events needed to carry out the phagocytic process[10-15]. Once a particle is bound, it starts to be internalized by reorganization of the actin cytoskeleton and remodeling of the plasma membrane to expand around the particle, forming a phagocytic cup[11-14]. During this process, another set of signals tells the cell to internalize the particle further by closing the phagocytic cup, forming a membrane-bound phagosome[11-15]. The phagosome then undergoes a maturation process, associating with different proteins and fusing with lysosomes, forming an acidic phagolysosome[11-15]. At this point, particles can efficiently be degraded and eliminated[11-15]. *Drosophila* studies have revealed that older flies (4 weeks of age) have a reduced ability to clear an infection compared to younger flies (1-week old), likely due, at least in part, to a decline in some aspects of phagocytosis[16,17].

The method described here utilizes two separate fluorescently-labelled heat-killed *E. coli* particles, one bearing a standard fluorophore and one that is pH sensitive, to assess two different aspects of phagocytosis: the initial engulfment of particles, and the degradation of particles in the phagolysosome. In this assay, fluoro-particle fluorescence is observable when the particles are bound and engulfed by hemocytes, while pH sensitive particles fluoresce only in the low pH conditions of the phagosome. Fluorescent events can then be observed in hemocytes that localize along the dorsal vessel. We focus on hemocytes localized to the dorsal vessel, which

provide an anatomical landmark to locate hemocytes that are known to contribute to bacterial clearance, and to consistently isolate them. However, hemocytes in other parts of the body and the hemolymph are also important for clearance. Although we have not studied this cell population, our general procedure could be applicable for phagocytic assays of these cells as well. One advantage of our approach is that we can quantify phagocytic events within individual hemocytes, allowing us to detect subtle variation in phagocytic processes. Other studies that visualize fluorescent events through the cuticle[18,19] do not account for differences in the numbers of hemocytes present, which is especially important to consider in our case as total hemocyte counts are expected to change with age[17].

PROTOCOL:

1. **Collect and age *Drosophila***

1.1. To generate same aged F1 flies for testing phagocytosis, add 5-10 virgin females and 5 males of the appropriate genotypes to a vial containing fresh fly food. We use a cornmeal molasses agar based food[20], but the method should work regardless of the type of diet the flies are reared on. For this experiment we used *Hemese* (*He*)-*GAL4*; UAS-GFP flies to label hemocytes genetically.

NOTE: More flies can be used, but some lines of *D. melanogaster* may not mate or reproduce well when overcrowded, and overcrowding may have adverse effects on larval development[21] and phagocytosis.

°C

1.2. Maintain flies under the desired experimental conditions. For this experiment, maintain *He-GAL4;* UAS-GFP flies at 24 °C. Allow adult flies to mate for a week, and then remove adults. F1 flies will then be collected from these vials after eclosion for experimental use.

1.3. Collect virgin flies throughout the week, or until the desired number of flies are collected. Virgins are not required; however, mating may impact immune response. If F1 flies are to be tested as virgins, separate males and females within 8 hours of eclosion and maintain in separate vials to prevent mating. Collect enough flies to allow assessment of phagocytosis in at least 10 flies per genotype/treatment/sex per age.

1.3.1. If aging the flies to one week, collect at least 50 flies total, or 20 flies per treatment condition for each particle, either fluoro-particles or pH sensitive particles, to be injected. This will ensure a minimum of 10 flies to assay at the time of the experiment.

1.3.2. If aging flies to more than 3 weeks, collect at least 100-150 flies total, or 50-75 flies per treatment condition to ensure there are enough flies available to measure phagocytosis. When aging flies in the laboratory, we typically use insect cages maintained at 24 °C in 12:12 L:D conditions, and change the food every other day. If vials are being used instead of cages, tip flies into new vials every 3-5 days, depending on the condition of the food in the vial. The number of flies needed will depend on how late in age phagocytosis will be analyzed, and the age-specific

survival rates of that genotype in a particular environmental condition.

1.3.3. If young and aged flies will be assessed, plan accordingly so that 1-week old and aged flies will be injected on the same day. This will minimize variations in particle concentration between experiments and will ensure that the effect of age on the phagocytic measurement is not confounded with the effect of the day the assay was performed.

1.4. House the collected flies at 24 °C until they are 5-7 days old, or maintain the flies to the desired age.

2. **Prepare fluorescently labeled particles**

2.1. Reconstitute heat-killed *E. coli* fluoro-particles or pH sensitive *E. coli* particles to a stock concentration of 20 mg/mL or 1 mg/mL, respectively. Other bacteria are available for use that may be more suitable for certain experiments, but refer to the manufacturer's instructions for appropriate stock concentrations.

2.1.1. For fluoro-particles, add 990 µL of 1x PBS (pH 7.4) or preferred buffer and 10 µL of 2 mM (20%) sodium azide. Vortex to mix.

NOTE: Sodium azide is a preservative that may be omitted; however, particles prepared without sodium azide do not last as long. Fluoro-particles must be used within 24 hours and pH sensitive particles must be used within 7 days.

2.1.2. For pH sensitive particles, add 1,980 µL of 1x PBS (pH 7.4) or preferred buffer and 20 µL of 2 mM (20%) sodium azide. Vortex to mix.

2.1.3. Make multiple single-use 20 µL aliquots in 1.5 mL microcentrifuge tubes. Store fluoro-particles at -20 °C for up to a year, and pH sensitive particles at 4 °C for up to 6 months, protected from light.

2.2. On the day of injections, remove sodium azide from the particles before use. To do this, centrifuge the particles for 5 min at 15,000 x *g* at room temperature.

2.3. Remove the supernatant and wash particles twice by resuspending in 50 µL of 1x PBS or preferred buffer, and centrifuge for 5 min at 15,000 x *g*.

2.4. After the second wash, remove the supernatant and resuspend particles in 100 µL of 1x PBS or preferred buffer. Keep the solution in the tube and minimize exposure to light throughout the experiment. Once the sodium azide is removed, use fluoro-particles within 24 h, and use pH sensitive particles used within 5-7 days.

NOTE: In our previous experiments, we found that this concentration of particles provided countable numbers of phagocytic events and that the number of particles available for

phagocytosis by hemocytes was not limiting[17]. However, users of this protocol may want to compare results using other concentrations to ensure that there is an adequate number of particles available for phagocytosis by hemocytes in the conditions of their experiments.

2.4.1. If sodium azide was omitted, dilute the particles 1:5 in the same buffer the particles were prepared with, for injections.

2.5. Add a drop (~10 µL) of green food coloring to the particles. This makes it easier to ensure the flies have been injected.

3. **Inject the flies**

3.1. Prepare glass needles for injections.

3.1.1. Pull glass needles using a pipette puller. Set the pipette puller heater to 55 °C and the solenoid to 45. Use only the needles provided with the injector, as it is not guaranteed that other needles will work with the same precision.

3.1.2. Fill a 1 mL sterile syringe with mineral oil, and attach the 30 gauge hypodermic (G) needle, provided with the nano-injector.

3.1.3. Backfill the pulled capillary needle by inserting the 30 G needle into the blunt end of the pulled glass needle and fill with mineral oil. Slowly remove the 30 G needle, ensuring there are no air bubbles throughout the needle, as this can cause inaccurate injection volumes. The injector will not work properly without backfilling the needle.

3.1.4. Using forceps, break off the tip of the needle to create an opening to allow ejection of solution.

3.2. Assemble the nano-injector.

NOTE: Other injectors can be used. The method described below apply to the nano-injector. For other injectors, consult the user manual for instructions.

3.2.1. Set the injector to the desired volume (between 46 nL and 69 nL).

3.2.2. Remove the collet and place the sealing O-ring, the white spacer with the indentation facing up to receive the back end of the needle, and the larger O-ring onto the metal plunger, in that order. Reattach the collet without tightening it.

3.2.3. Insert the metal plunger into the blunt end of the oil-filled glass needle. Gently push the needle down, inserting it into the larger O-ring. Tighten collet until secure.

NOTE: If the metal plunger does not extend past the collet, press and hold ‘EMPTY’ until the

plunger is visible. This makes it easier to ensure the plunger is inserted into the needle.

3.2.4. Press and hold 'EMPTY' until the injector beeps. This ejects most of the mineral oil from the needle, leaving a small volume of oil to act as a barrier between the two liquids, as well as removes air bubbles.

3.2.5. Fill the needle with either fluoro-particles or pH sensitive particles by inserting the tip of the glass needle into the microcentrifuge tube containing the prepared particles.

3.2.6. Press and hold 'FILL' until the injector beeps.

3.3. Injections

3.3.1. Transfer the flies that are to be injected into an empty vial. Immobilize the flies by placing the vial in ice. CO_2 can also be used to immobilize the flies. However, note that, when using pH sensitive particles, elevated CO_2 levels can artificially acidify any buffers being used and can elevate background fluorescence.

3.3.2. Inject the flies in the sternopleural plate of the thorax (**Figure 1A**). The injection is successful if green dye is seen going into the fly (**Figure 1B**). If the fly does not turn green, make sure the needle is not clogged.

NOTE: Alternatively, flies can be injected in the abdomen, but keep the injection site consistent across all experiments.

3.3.3. Place injected flies into a new food vial, noting the time that the first and last fly was injected. To minimize experimental error due to the amount of time it takes to complete injections, complete injections in a timely manner. With practice, it should take no longer than 10 min to inject one set of flies. Lay the vial on its side until all of the flies have recovered, to prevent the flies from becoming stuck in the food.

3.3.4. Allow flies to recover for 60-90 min, depending on experimental conditions. Here, a 60-min recovery time was used. Note that this recovery time range was optimal for counting phagocytic events in the experimental conditions in the Horn et al. study[17]. However, under some conditions, this may be too long to detect subtle differences in phagocytosis between treatment groups. It may be useful to carry out time course experiments as was done previously[17] to determine the recovery time that will reveal maximal differences between control and experimental results. Whatever recovery time is chosen, keep this consistent across all experimental treatments.

3.3.5. If injecting with fluoro-particles and pH sensitive particles on the same day, use a new needle for each solution. Do not inject individual flies with both particles if they both fluoresce red, since the result would not differentiate between the two aspects of phagocytosis, particle binding/engulfment vs. particle inclusion in the phagosome.

4. **Dissecting the dorsal vessel**

4.1. After flies have recovered for 60-90 min, transfer all living flies to an empty vial and immobilize on ice.

4.2. Transfer one fly at a time onto a silicone elastomer dissection plate.

NOTE: Prepare dissection plates at least 1-week prior to use, if curing at room temperature. To do this, prepare the elastomer and pour it into a 33 mm x 10 mm Petri dish, filling the dish about halfway. Gently tap the dish on a flat surface to minimize air bubbles. Let the plates sit at room temperature, undisturbed, for at least a week.

4.2.1. Under a dissecting stereo microscope, orient the fly ventral side up.

4.2.2. Using insect pins, pin the fly onto the plate by inserting one pin through the thorax and another pin through the most posterior end of the abdomen, near the genitalia (**Figure 2A**). It may be useful to cut the pins in half before pinning the specimen so as not to obstruct the dissection. Repeat with up to 10 flies per dissection plate.

NOTE: Optional: Remove the wings and legs before pinning fly to plate. This will help prevent a bubble forming around the fly when media is added.

4.2.3. Once all flies have been pinned to the plate, add enough dissection media to cover the flies (~1 mL) using a transfer pipet.

4.2.4. Using forceps or cuticle scissors, remove the head.

4.2.5. Using cuticle scissors, make two horizontal incisions: one directly above the posterior pin in the abdomen, and another at the most anterior end of the abdomen, where the thorax and abdomen meet (**Figure 2B, C**). In **Figure 2**, the head was left intact to clarify orientation.

4.2.6. Make a vertical incision, connecting the two horizontal incisions (the three cuts resemble the letter I). This will open up the abdominal cavity (**Figure 2D**).

4.2.7. Using forceps, remove the internal organs and tissue, avoiding the dorsal vessel. If undisturbed, the transparent dorsal vessel can often be seen pulsating near the anterior end of the abdomen. Additional pins can be used to pin open newly cut ends of the cuticle (**Figure 2E, F**).

4.2.8. Using cuticle scissors, remove the thorax. Alternatively, the thorax can be removed before mounting the dissected cuticles and dorsal vessel on a microscope slide.

4.2.9. Repeat with remaining flies. dissect flies in a timely manner, to minimize possible

variations in phagocytic rate. Once all flies have been dissected, leave the cuticles with the attached dorsal vessel pinned to the plate. All of step 5 will be carried out in the dissection plate. This will prevent damaging or accidentally discarding cuticles between steps.

5. Fixation and staining

5.1. Fix dissected cuticles with attached dorsal vessel in 4% paraformaldehyde (PFA).

5.1.1. Using a new disposable transfer pipet for each step, discard the dissection media, and replace with 1 mL of 4% PFA. Throughout the fixation and staining, keep the dissections protected from light as much as possible.

5.1.2. Incubate at room temperature for 15 min with rocking at 20 rpm. Do not allow dissections to sit in fixative for more than 20 min, as this could start to damage the tissue.

5.2. Wash cuticles 2x in 1x PBS + 0.1% Tween (PBST).

5.2.1. Remove fixative and replace with 1 mL of 1x PBST.

5.2.2. Wash at room temperature for 15 min with rocking.

5.2.3. Repeat 1x.

NOTE: Dissected, fixed tissue can be stored at 4 °C, for up to 3 days, protected from light, after the first wash by replacing the wash with fresh PBST. Do not store fixed tissue if antibodies will be used. Antibodies should be used with fresh tissue for best results.

5.3. Optional: Antibody staining. Antibodies can be used to clearly visualize the dorsal vessel clearly (**Figure 3**), or to detect hemocyte specific markers. This can ensure only cells along the dorsal vessel are counted or to mark the membrane of the hemocytes.

5.3.1. Remove the second wash, add primary antibody at the appropriate dilution. Incubate overnight at 4 °C, with rocking.

5.3.2. Remove primary antibody, and wash twice with PBST for 15 min with rocking.

5.3.3. Add fluorescent secondary antibody. We recommend a green-fluorescent antibody so it does not obscure the fluorescent particles. Incubate at room temperature for 2 h, with rocking.

5.3.4. Remove secondary antibody, wash twice for 15 min each with PBST.

5.4. Stain with DAPI.

5.4.1. Remove final wash and replace with 1 mL of DAPI diluted in PBST (1:1000).

5.4.2. Stain for 20 min with rocking at room temperature.

5.4.3. Remove DAPI, and wash 2x (repeat step 5.2).

5.4.4. Replace final wash with fresh 1x PBST.

NOTE: Flies can be stored at 4 °C, for up to 3 days, protected from light, after the first wash by replacing the wash with fresh PBST.

6. **Mounting cuticles onto microscope slides and imaging**

6.1. Prepare dissected cuticles.

6.1.1. Under the dissecting stereomicroscope, cut off excess cuticle that could interfere with imaging.

6.1.2. Using forceps, transfer the cuticles with attached dorsal vessel into a 1.5 mL microcentrifuge tube containing 70% glycerol. Placing the cuticles in glycerol will help remove PBST and allows for a clearer image during imaging.

6.2. Mount cuticles onto the microscope slide.

6.2.1. Add a few drops of 70% glycerol to a microscope slide.

6.2.2. Using forceps, remove cuticles from the glycerol tube and place in glycerol on the slide.

6.2.3. Under the dissecting stereo microscope, use forceps to orient cuticles ventral side up, ensuring the dorsal vessel is visible. The darker pigment of the cuticle will be face down.

6.2.4. Add an additional drop of glycerol, if needed. This helps prevent air bubbles and allows for a clearer image.

6.2.5. Gently place a coverslip over cuticles and seal edges with fingernail polish. Allow to dry for 10-15 min before proceeding. Image the flies immediately or store in a lightproof box at 4 °C.

6.3. Image the dorsal vessel.

6.3.1. Using a fluorescent microscope, use the structural interference system to generate optical sections of the dorsal vessel. A confocal microscope can alternatively be used which may provide additional accuracy. Obtain Z-stack images of the dorsal vessel using a 20x objective and preferred imaging software. Add a 10 μm scale bar, and properly label and save images as tiff files (**Figure 4**) or the desired format.

NOTE: The number of Z-stack images obtained can vary between dissections and depends on how well the dorsal vessel was dissected, and on the desired step size between images. Here, the step size was set to 0.49 μm. That number can range anywhere from 3 to 40 images per stack in our experience.

7. **Analyze images**

7.1. Quantify fluorescent events using ImageJ.

7.1.1. Open images and stack them: **Image → Stacks → Images to Stack**.

7.1.2. Count only the ~1 μm sized fluorescent signals within a 10 μm diameter centered on a DAPI-positive nucleus by clicking on each event from at least 10 cells per fly, from at least 10 flies per age per particle type injected: **Plugins → Analyze → Cell Counter Notice** (**Figure 5A**). The cell counter notice tool assigns a different color to every cell tracked, with a colored dot that corresponds to every fluorescent event clicked on within that cell.

7.1.3. To list the counter and stack position associated with each point, press 'm' or select **Measure** under the **Analyze** tab, or to display the number of events counted in each cell in a results table, press 'alt +y' (**Figure 5B**).

7.1.4. Transfer the cell counts to a spreadsheet to be used for statistical analysis.

7.2. Perform statistical analysis.

7.2.1. Analyze differences in phagocytic events using either fixed-effects ANOVA or mixed-model nested ANOVA. Mixed models can be used to test the main fixed effects of age and genotype and the random effect of individuals nested within each genotype[17].

NOTE: Investigators should be rigorous in testing the assumptions of any statistical procedure used to analyze the data.

REPRESENTATIVE RESULTS:

To illustrate the described injection methods, **Figure 1A** shows the injection site on *Drosophila melanogaster,* as well as how food dye allows for a visual confirmation that the fly was injected (**Figure 1B**). The addition of food dye also aids in the recognition of a clogged needle. Injections can be performed in the abdomen, but keep the injection site consistent across experiments. This will help minimize possible variations between each experiment.

To visualize the fluorescently labeled particles within resident hemocytes along the dorsal vessel, we dissected the dorsal vessel and attached abdominal cuticle. **Figure 2A-F** outlines the dissection methods.

To assess the age-specific ability of young and aged flies to carry out phagocytosis, hemocytes

along the dorsal vessel are visualized using a fluorescent microscope. To ensure that only cells along the dorsal vessel are counted, antibodies or GFP-tagged genes for certain blood cell markers or heart specific collagen, such as Hemese and Hemolectin, or Pericardin (**Figure 3**), respectively, can be used[22-24]. Fluorescently labeled *E. coli* particles are 1 μm in length, while hemocytes are 10 μm in diameter[17]. Only those fluorescent events located within a 10 μm diameter centered on a DAPI-positive nucleus are counted (**Figure 4**). To quantify fluorescent events, ImageJ software is used (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Injection site and visual verification. (**A**) Lateral side of thorax is pierced with a pulled-capillary needle. (**B**) Injections are visually verified by adding green food dye to particle solution.

Figure 2: Dorsal vessel dissection. (**A**) Pins are placed in the thorax and posterior abdomen (black arrows). (**B-C**) Two horizontal incisions (green arrows) are made at the posterior end of the abdomen (**B**), and anterior end (**C**). (**D**) A vertical incision (green arrow) is made down the middle of the abdomen, connecting the two horizontal cuts. (**E**) Optional pins (*) are used to filet open the abdominal cavity, exposing internal tissue. (**F**) Internal tissue (crop, gut, uterus, ovaries, fat bodies) is removed, exposing the dorsal vessel.

Figure 3: Ventral view of a dissected dorsal vessel from a 5-week old female injected with pH sensitive particles, stained with antibody directed against Pericardin (**A**)**.** Dotted white line outlines the lateral side of the dorsal vessel, with arrow pointing towards the anterior region. (**B**) Enlarged image of (**A**): clusters of hemocytes (blue arrow) that were actively degrading bacteria, within the first aortic chamber of the dorsal vessel. (**C**) Enlarged image of (**A**) outlining the extracellular matrix (ECM) collagen-like protein, Pericardin (green arrow), that holds the dorsal vessel in place[24].

Figure 4: Dissected dorsal vessel and associated hemocytes from a female fly injected with pH sensitive particles, or fluoro-particles. (**A**) The dorsal vessel and associated hemocytes with engulfed pH sensitive-labelled *E. coli* particles (red), or (**E**) fluoro-labelled *E. coli* particles (red), isolated from a 1-week old fly, after recovering for 60 min. (**B,F**) Magnified inset of (**A**) and (**E**) (white box), respectively, showing two individual hemocytes with countable events. (**C**) The dorsal vessel and associated hemocytes with engulfed pH sensitive-labelled *E. coli* particles(red), or (**G**) fluoro-labelled *E. coli* particles (red), isolated from a 5-week old fly, after recovering for 60 min. (**D,H**) Magnified inset of (**C**) and (**G**) (white box), respectively, showing two individual hemocytes with countable events. Dotted white line outlines the lateral side of the dorsal vessel, with arrow pointing towards the anterior region. Nuclei stained with DAPI (blue).

Figure 5: Quantifying phagocytic events within a 10 μm hemocytes using the cell counter in ImageJ. (**A**) After opening image(s) in ImageJ, the Cell Counter Notice tool can be used to keep track of phagocytic events per cell. (**B**) This tool will assign a different color to each cell selected to be counted, with each dot corresponding to a fluorescent event within that cell**.** Pressing 'alt+y' will display a table showing the number of events counted per cell.

DISCUSSION:

The protocol described here is a reliable way to quantify different aspects of phagocytosis, under controlled experimental conditions. We note that we have only tested this procedure with gram negative bacterial particles and results may differ if gram positive bacterial particles are used. Indeed, it would be interesting to compare the phagocytic responses to both gram negative and gram positive bacteria in different experimental conditions. The use of a nano-injector allows for precise control over injection volumes, ensuring each fly is injected with the same volume of particles. One limitation to the protocol comes from inconsistencies in particle preparations. Particles will aggregate once frozen, so small variations in dilution volumes, or lack of vortexing, can affect particle concentration between experiments. To minimize possible variations in particle concentrations between ages, it is beneficial to inject 1- and 5-week old flies on the same day, using the same needle and particle solution. Another potential drawback is that during dissections, the dorsal vessel and/or cuticle can easily be damaged if pins are not handled properly. To avoid disrupting the dorsal vessel, minimize the number of pins used per dissection. The advantage to this dissection method is that all fixation, washing and staining steps can be performed in the dissection plate. Because the cuticles are pinned down, this prevents the cuticles from being lost between steps.

Compared to existing methods[18,19,25-29], the described protocol has its advantages and limitations. By dissecting the dorsal vessel, we are able to visualize and quantify individual hemocytes at this location. This makes it possible to detect subtle variations in phagocytic activity between experimental groups. Other methods visualize fluorescently labeled particles by collecting hemocytes using a *Bleed/Scrape assay*[19,25-27], or through an intact ventral cuticle[18,19,28,29]; however, individual hemocytes cannot be assessed when visualized through the dorsal cuticle. The advantage of this protocol, when compared to the *Bleed/Scrape* method is that our method allows us to assess only those hemocytes associated with the dorsal vessel, and does account for circulating cells or those along the body wall, which may be functionally different. Dissecting the dorsal vessel also removes the need to include a second round of injections with a fluorescence quencher, like Trypan blue[19,26]. This is because any particles not bound to or engulfed by a cell will be washed away during wash steps. Conversely, alternative methods may be easier to perform because they do not require dissections. While dissecting the dorsal vessel is easy to learn, this step adds a level of complexity that may not be feasible in some experimental designs.

Although the described use of this in vivo phagocytosis assay is to assess and quantify phagocytic events between different ages, this protocol is highly adaptable and can be used to analyze different aspects of phagocytosis between genotype, sex, or tissue type. With phagocytosis being of central importance for most multicellular animals, understanding how this process declines with age could lead to better therapeutic treatments for the aging population. This approach offers long-term potential for elucidating aspects of age-related changes in the immune response, with special focus on phagocytosis.

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The authors have nothing to disclose.

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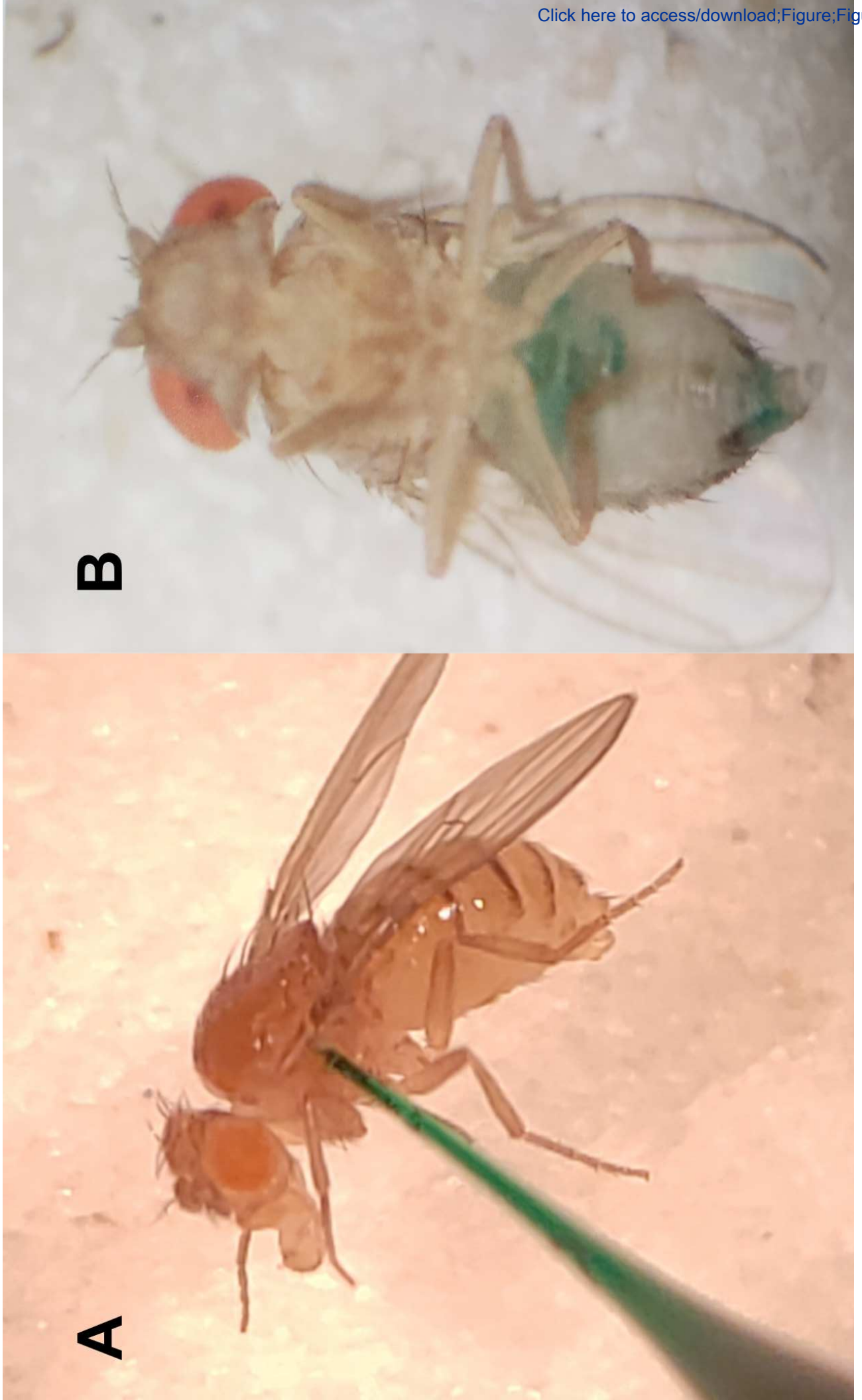
Figure

Click here to access/download;Figure;Figure 1.eps



Figure

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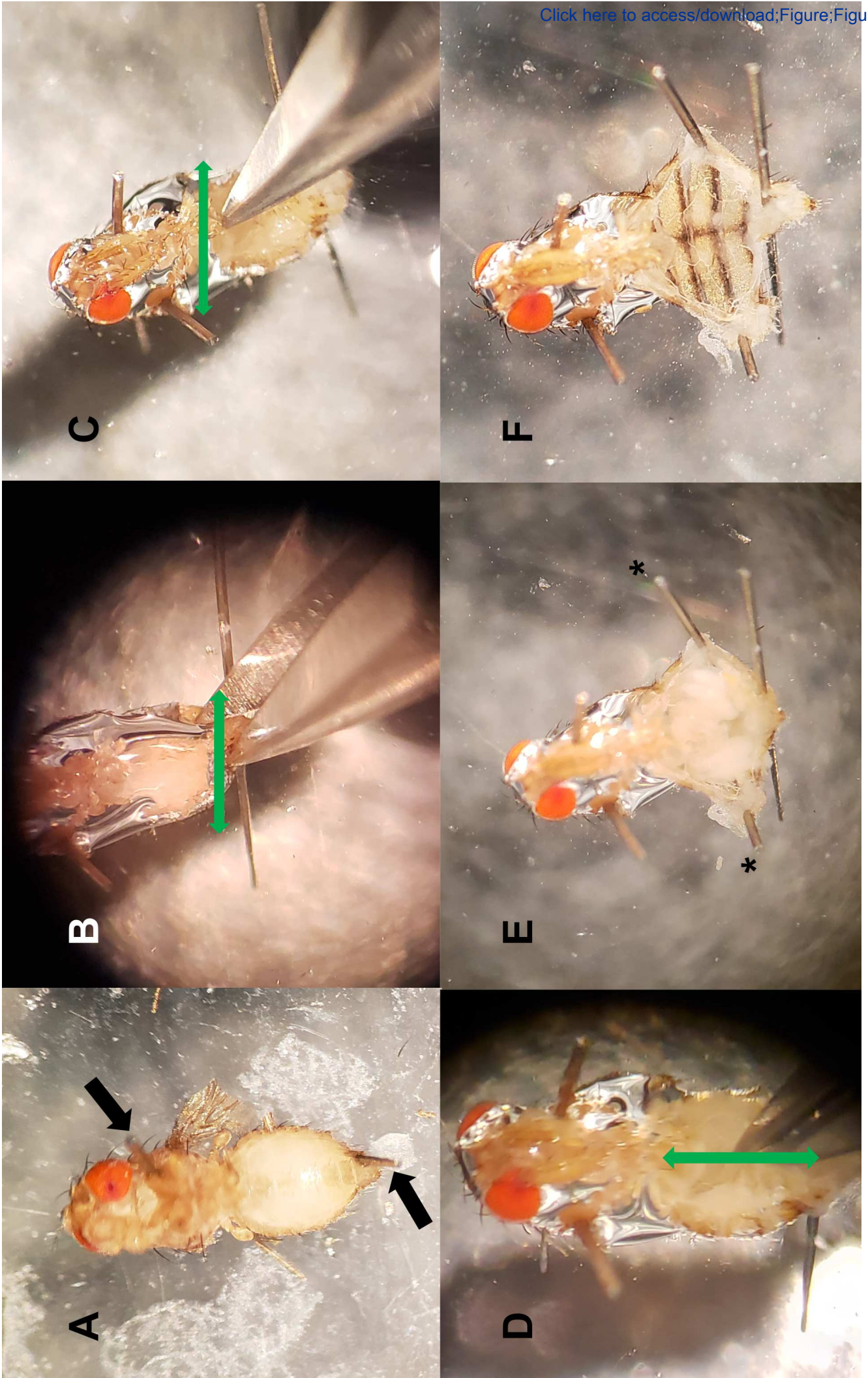

Figure

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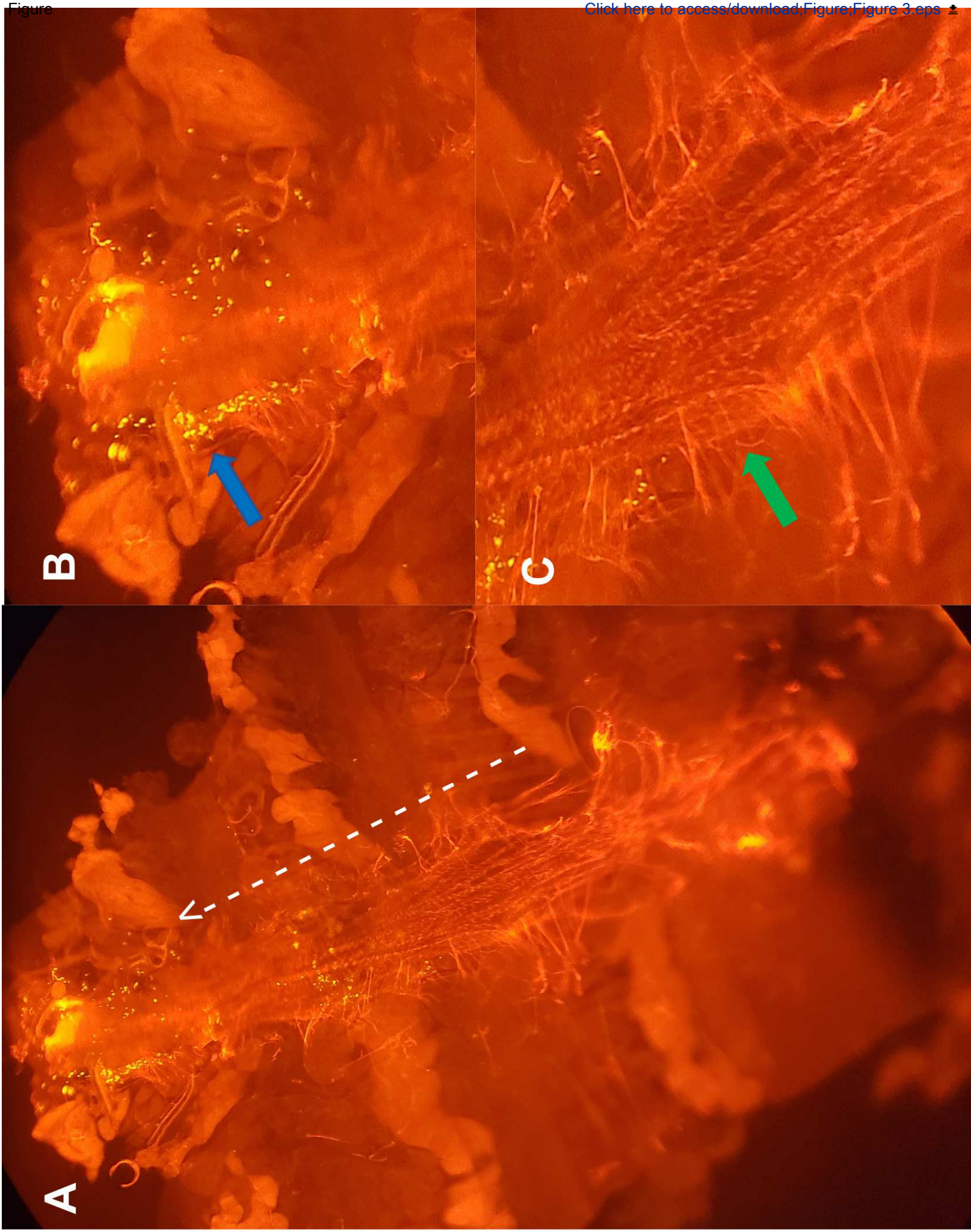
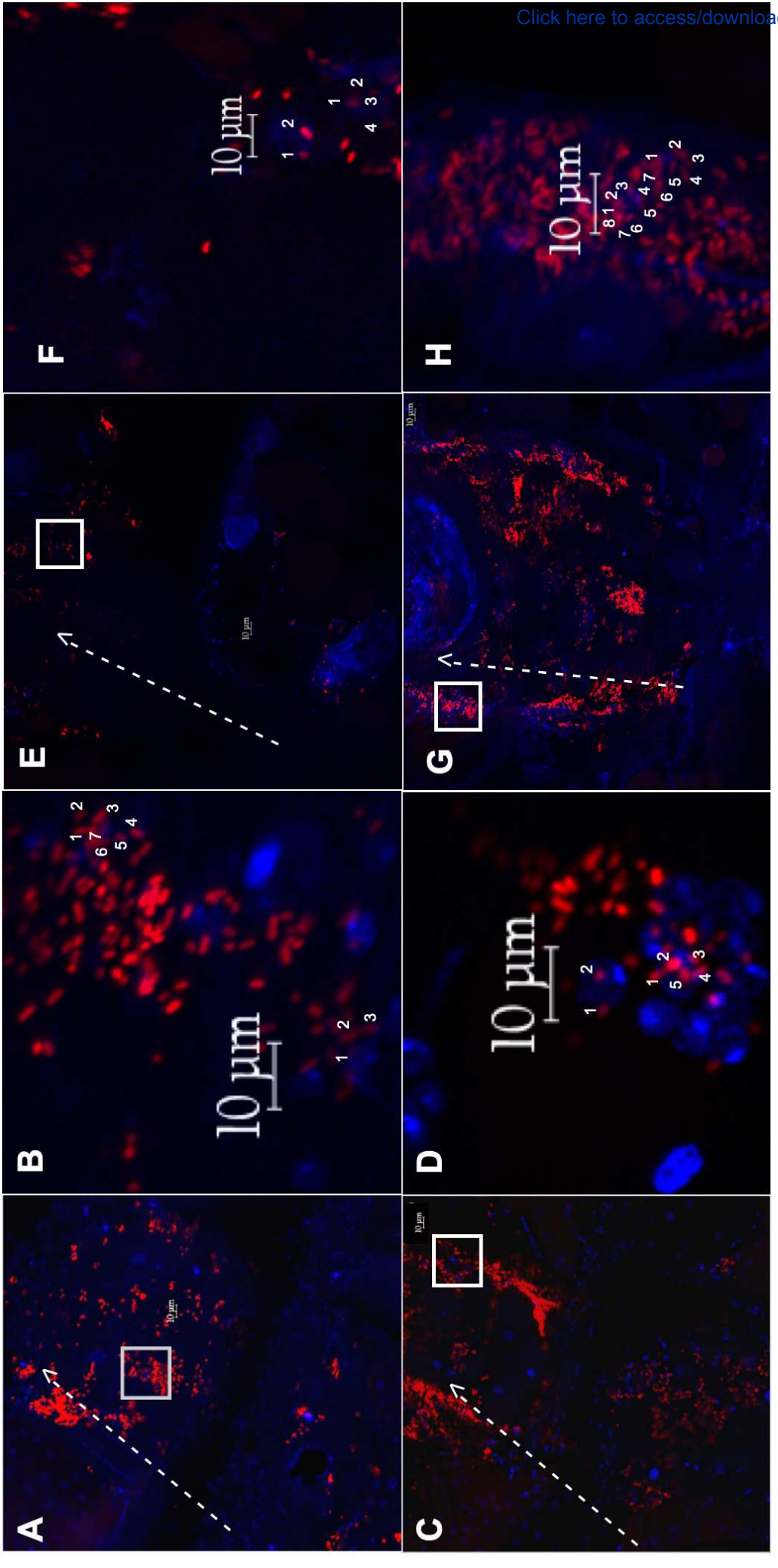
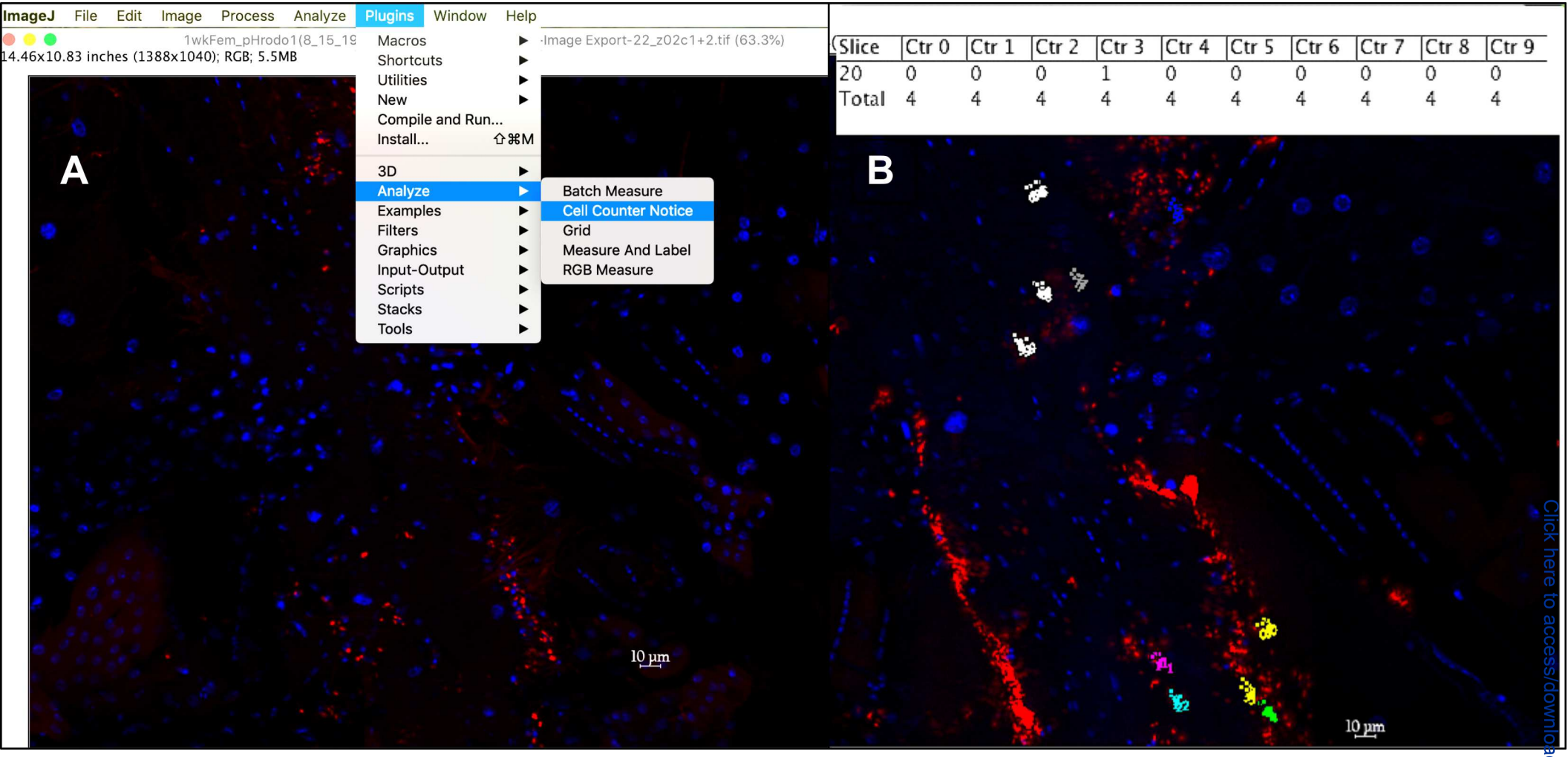


Figure

Click here to access/download;Figure;Figure4.eps





0.10 mm Insect pins	Fine Science Tools	26002-10	Here: pins are cut in half, and the sharp end is used
1 mL sterile syringes	Becton Dickinson	309602	Filled with mineral oil to load needle
15% Fetal Bovine Serum (FBS)	Gibco	16000-044	for dissection media
16 % Paraformaldehyde	Electron Microscopy Sciences	15710	EM-grade, 4% working, diluted in 1X PBS
1x Phosphate buffered saline (PBS)	Sigma	P3813	
3 mL Trasnfer Pipet	Falcon	357524	
3.5" Glass Capillaries	Drummond	3-000-203-G/X	1.14mm O.D X 3.5" length X 0.53" I.D
35x10 mm Petri dishes	Becton Dickinson	351008	Used as dissection plate, filled half way with Sylgard
6x penicillin/streptomycin	Life Technologies	15140-122	for dissection media
70% Glycerol	Sigma	G9012	
Analog Vortex mixer	VWR	58816-121	
Biological point forceps, Dumont No. 5	Fine Science Tools	11295-10	
DAPI (4',6-diamidino-2-phenylindole)	Life Technologies	D1306	Diluted 1:1000 in 1x PBST
Drosophila strain			w[*]; P{w[+mC]=He-GAL4.Z}85, P{w[+mC]=UAS-GFP.nls}8
E. coli (K-12 strain) BioParticles™, Alexa Fluor™ 594 conjugate	Life Technologies	E23370	
Glass slides	Premiere	D17026102	
Live cell imaging solution	Life Technologies	A14291DJ	preferred buffer for particle preparation and dilutions
Mineral oil	Mpbio	194836	
Name	**Company**	**Catalog Number**	**Comments**
Nanoject II automatic nanoliter injector	Drummond	3-000-204	

Narrow Polystyrene Super Bulk *Drosophila* Vials	Genesee	32-116SB	Size: 25 X 95 mm
Nutating Mixer	Fisher Scientific	88-861-043	Speed used: 20 rpm
pHrodo™ Red *E. coli* BioParticles™ Conjugate for Phagocytosis	Life Technologies	P35361	
Schneider's *Drosophila* cell culture media (1x)	Gibco	21720-024	Dissection media, combine: Schneiders, FBS, and pen/strep; filter sterilize
Sodium azide	Sigma-Aldrich	S2002	2mM (or 20%) working
Spring scissors	Fine Science Tools	15000-00	
Sylgard 184 Silicone elastomer	Electron Microscopy Sciences	24236-10	Prepare according to provided protocol
Tween 20	Sigma	P1379	For PBS + 0.1% tween
Vertical Pipette Puller Model 700C	David Kopf Instruments	812368	Heater: 55℃ Solenoid: 45
Zeiss AxioImager.Z1 fluorescent microscope	Zeiss		Here: Apotome structural interference system with Zeiss Zen imaging software

December 12, 2019

Dear Editor,

Thank you for inviting us to submit a revised draft of our manuscript entitled, "Assessing the Age-Specific Phagocytic Ability of Adult *Drosophila melanogaster* Hemocytes, Using an *In vivo* Phagocytosis Assay" to JoVE. We sincerely appreciate the time and effort you and each of your reviewers have dedicated to providing insightful feedback on ways to strengthen our paper. We have modified the paper in response to the extensive and insightful comments, and hope our edits and the responses we provide below satisfactorily address all the issues and concerns you and the reviewers have noted. We have copied each point provided by the reviewers and embedded our answers below each one.

Editorial Comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have proofread the manuscript and corrected the spelling and grammar.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Response: Done.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: pHrodo, McCormick, Zeiss, Apotome

Response: We have revised the manuscript to remove commercial language.

Protocol:

4. For sections 3.3, 4, and 5, do you want to film the entire section? Please highlight all steps you wish to use. Please ensure the total highlighted length is no more than 2.75 pages, including headers and spacing.

Response: We have revised the manuscript to highlight only the steps we want to be filmed.

5. For each protocol step/substep, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: Done.

Table of Materials:

6. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Response: We have revised the Table of Materials, ensuring every material is included and has all appropriate information.

Reviewer 1 Comments:

Manuscript Summary:
1. Authors give description of a protocol for an in vivo assay of phagocytosis to quantify the ability of plasmatocytes of Drosophila melanogaster to phagocytose bacteria, namely heat-killed E. coli. I consider this as an interesting technique and it is worthy of publishing in JoVe. I have only minor comments.

Minor Concerns:

2. There may be alterations in the total amount of dorsal vein associated plasmatocytes between experimental groups. Therefore, there may be more bacteria / cell to be engulfed in certain groups. Should this be taken into consideration when analyzing the results?

Response: This is a good point. We have clarified in the manuscript that the concentrations suggested provide countable events in the experimental conditions we used. Concentrations may need to be adjusted depending on the experimental conditions used and we have added a discussion of this to the manuscript (beginning line 151)

3. Introduction lines 59-61: "These cells express a variety of receptors that must differentiate self from non-self, and initiate signaling events needed to carry out the phagocytic process" at least Ramet et al., 2001 Immunity, Ramet et al., 2002 Nature, Kocks et al., 2005 Cell, Kurucz et al 2007 Current Biology) needs to be cited.

Response: Done.

4. Introduction lines 61-63: "Once a particle is bound, it is internalized by reorganization of the actin cytoskeleton and remodeling of the plasma membrane to expand around the particle, forming a phagocytic cup." Also Ramet et al., 2001 Immunity should be cited.

Response: Done.

5. Methods: Use either "min" or "minutes" (see for example rows 125 and 127)

Response: The use of the word "minutes" has been revised and replaced to "mins" throughout the protocol section, for consistency.

6. Line 172: Recovery time after injection. To me, 60-90 minutes seems long time for phagocytosis to happen. I suggest 30-60 minutes (if the time is too long it may be difficult to identify subtle differences).

Response: This is an excellent point. The recovery time of 60-90 minutes we used was chosen based on the findings from a time course study performed by Horn et al., 2014, where phagocytic events / cell was counted at 5, 30, 60, and 90 minutes, in 1-week and 5-week old flies. However, we do agree that, for different experimental conditions, a 60-90-minute recovery time may be too long. In such cases, time courses studies can be performed to determine an optimal recovery time. We have added a discussion of this issue to the manuscript (beginning line 213).

7. Discussion line 345: "Compared to existing methods, the described protocol has its advantages and limitations. " Lacks references. Authors should cite at least Elrod-Erickson et al., 2000 Current Biology

Response: We thank the reviewer for pointing this out and have included appropriate references.

Reviewer 2 Comments:

Manuscript Summary:

1. In this manuscript the authors provide an experimental method to assess in vivo phagocytosis in Drosophila adult flies of different ages. This method allows a single hemocyte resolution quantification of the phagocytic activity, which could be of a particular interest while studying the phagocytosis process. In addition, the method analyses bacteria engulfment of dorsal vessel-associated hemocyte only.

Major/minor Concerns:

2. In the manuscript the authors provide a visual example of only pHrodo infected flies. It may be useful to give also an example of Bioparticles injection, to show what it should normally look like.

Response: This is a good suggestion and so we have added an image showing the bioparticles (Figure 4).

3. Point 4. It could be useful to give a short guideline on how the dissection plate has to be prepared, in case for example a specific concentration of the Sylgard is needed. If no specific

concentration is better than another, an explanation is not required and the text can be left like this.

Response: Sylgard (brand name) is prepared according to the provided protocol, and the prepared concentration seems to work just fine. However, we did add a brief description of how the dissection plates are made for clarity beginning line 229.

4. Point 6. It is not really clear to me the moving from 6.1.2 to 6.2 (why do I need to place the dorsal vessels in a microcentrifuge tube, for then moving it into a microscope slide? And yet, how do I get them on the microscope slide from the centrifuge tube? Explain better, and if needed, corresponding illustrating images could be added.

Response: Transferring the cuticles to a microcentrifuge tube containing 70% glycerol, before placing the cuticles onto a slide, is to help provide the clearest image. The glycerol also helps to further remove excess tissue that may have been left behind during wash steps. We have added additional detail to the manuscript explaining the need for this step beginning line 300.

5. Point 7.1.2. "Count only the ~1 um sized fluorescent signals within a 10 um diameter centered on a DAPI-positive nucleus…" I think it might be a little limiting to count red signals this way, you might miss some phagocytic events. For example, in Fig. 4B-higher cell, why not counting the red signal between point 6 and 7 (the one touching the scale bar)? If the hemocyte is spread, that bacteria could totally be inside the phagocyte…Maybe using HmlGal4,UAS-GFP flies (followed by confocal colocalization) could help? This could also help to better discriminate phagocytic events in single hemocytes in case of clusters.

Response: We agree with the reviewer's comment that it may limit the available cells that can be counted. We have had success generating optical sections through structural interference, which is faster and less expensive than using a confocal, but also agree that using a confocal may provide increased accuracy. The use of the Hml line as well would also be useful to study phagocytosis in that genotype. However, we designed the method to be usable with any genotype. With that being said, we have clarified that use of the confocal microscope if available would provide more detail and increase the number of possible cells that could be counted (line 316).

Commented [MS1]: My feeling on this is that we are looking only in the center of the cell, when the outer cortex and nucleus is in focus, so likely we miss events above or below this, but we aim to have a fast method and it would provide an accurate relative number of events- not the absolute number. Not sure if that is worth discussing anywhere though.

6. Another point that is less clear to me is the following. PHrodo particles usually fluoresces when internalized by the phagocyte. Therefore, what about the red signals that do not seem to be in proximity of a cell DAPI-positive nucleus (and therefore not count in the analysis)? Is it background?

Response: pHrodo particles that are not engulfed by a cell should not fluoresce and should be washed away in the washing steps. Any visible fluorescent particle then is assumed to be internalized. Any red signals not associated with DAPI are not counted.

Commented [MS2]: We could say that these could be background or events in cells that are out of focus. It is worth saying here that we see a very low background, typically.

7. Line 315, Figure 3 legend. Mention in the figure legend how old is the fly.

Response: Done.

8. Figure 5B-table, not clear to me how to read it. Are the coloured dots representative for the phagocytic events in each cell analyzed (for a total of 9 cells?)? And what are the corresponding numbers on the table?

Response: Each color represents an individual cell that was quantified, with each dot corresponding to a single fluorescent event within that cell. Once fluorescent events from 10 cells have been quantified, a table can be displayed showing the number of events that were counted within each cell. The manuscript has been revised for clarity (Line 330).

9. Line 337. "The protocol described here is a reliable way to quantify different aspects of phagocytosis…". What do the authors means by different aspects? Which are those different aspects that a researcher can investigate by using this method? Might be nice to just write few of them.

Response: The manuscript has been revised to make clear what different aspects we are investigating, in the abstract and beginning line 75.

10. Lines 351-354. What is the main importance of assessing only phagocytosis activity of dorsal vessel-associated hemocytes? Maybe the authors could add this short explanation in the text. Also not clear to me whether for "hemocytes associated with dorsal vessel" the authors refer to hemocytes outside the heart only, or also hemocytes that are present inside the heart.

Response: We assess phagocytic events in hemocytes localized to the dorsal vessel in part for convenience as the dorsal vessel provides an anatomical landmark to locate sessile blood cells that are known to contribute to bacterial clearance, and because this tissue is easy to isolate and stain. We assume that this is a subset of the total number of hemocytes in the adult. However, we realize that circulating blood cells and those in other parts of the body are also important for clearance, but these may have different capacities for phagocytosis. Counting sessile blood cells in the dorsal vessel allows us to standardize the type of cell studied and minimizes the potential variance that might be imposed if we measured phagocytosis in different cell types (and treated them as if they were one population). That said, the procedure should be applicable for both circulating and sessile blood cells. We have added a small section to the manuscript to explain this beginning on line 79.

Commented [JL3]: Michelle - I did my best with this but if you can add more (or delete my nonsense) please do. And that goes for what I added to the ms as well

Commented [MS4]: I think what you have here is good- I think the main reason we did it this way is a large number are easily purified this way. when this all started, I thought it was a random subset pumped through the heart but now I'm not sure if they are specialized, and I'm not even sure if they are all inside or some are on the outside of the vessel. Not sure why it would matter if since they seem to all behave the same in this region.

11. Figure 3: write on the image the definition of the corresponding colour(s).

Response: The corresponding colors are now defined in the figure legend.

12. Figure 4 and 5: same comment as in Figure 3 (for ex: E. coli in red, DAPI writ
ten in blue).

Response: See response, above.

Reviewer 3 Comments:

Manuscript Summary:

1. Description of a phagocytosis assay with standardized and commercially available reagents and tools.

Major Concerns:

2. None

Minor Concerns:

3. The reproducibility and the applicability of the method depends on the availability of standardized products and infrastructure. I am afraid that in the long run the method will be applied by a narrow segment of the laboratories.

Response: Yes, unfortunately the equipment needed to carry out this method is somewhat restrictive. Our hope is that this method will be useful for those that are able to use it.

Reviewer 4 Comments:

Manuscript Summary:

1. The article submitted by Campbell et al. presents a method that allows studying the phagocytic capacity of Drosophila plasmatocytes associated with the dorsal vessel as a function of age. Overall, the article is interesting and well written, and provides a useful methodology to assess age-related changes in one aspect of innate immune function, which is phagocytosis. My detailed suggestions for improvement of the protocol are outlined below.

Major Concerns:

2. Title: I think the authors should mention somewhere in the title that their protocol refers to assessing the function of hemocytes. In Drosophila, other cell types such as glial cells are also phagocytic.

Response: This is a good point. The title has been modified as suggested.

3. Summary (lines 15-16): similarly here, the authors could emphasize that they are looking at hemocytes and not other cell types. I also recommend editing "to phagocytose a bacterial infection" to "to phagocytose bacteria".

Response: We have modified the manuscript as requested.

Introduction:

Commented [MS5]: I'm not sure it is that restrictive. The most prohibitive thing is the structural interference microscope module and the injector. We could suggest that people could use a GFP-expressing bacteria instead of a commercially available one, although there is no phrodo equivalent, and I am not sure that can be heat-killed and still work. But the point is, that there may be less-restrictive adaptations that we can suggest. Could it be done with a syringe-based injector? I'm not sure an epifluorescent microscope would work that well, but it might be okay if people went up in mag to 63X. Maybe Shonda has a sense of how things looked when she wasn't using the apoptome. In any case, I agree with your response but, I would say it is "somewhat restrictive" and maybe we can add to discussion about less restrictive possibilities.

4. Line 57: Please, clarify here that you are looking in the context of infection. Glial cells for instance can also phagocytose debris during development.

Response: We have modified this section to make clear that the method is designed to be used in the context of infection (Starting at line 59).

5. Lines 70-71: It is probably worth emphasizing here that you are looking at Gram- bacteria. Have you done this with S. aureus bioparticles? Maybe this could be addressed somehow in the discussion.

Response: This is an interesting point. We have not tested the method with gram + bacteria. We have added a small section to the discussion addressing this issue beginning on line 405.

6. Lines 74-76: Do the authors know whether differences in phagocytic capacity have been observed between hemocytes along the dorsal vessel and the circulating hemocytes? If such age-dependent differences exist, I recommend to the authors to include this information.

Response: It is possible that there are differences between circulating and DV-resident hemocytes, but this has not yet been studied. However, we only assess those hemocytes associated with the dorsal vessel. Reviewer 1 had a similar comment. We discuss this issue in a newly added section beginning line 79 and again at line 426.

Protocol 1

7. Line 82: Please, indicate the genotype of the flies. In order for a protocol to be reproducible, all experimental details need to be provided. Also, why there is a need to perform a cross? Could this assay be done in wild type flies?

Commented [MS6]: I don't think we really explain this in the text. Do you want to justify further.

Response: The genotype of the flies used in this protocol has been listed in the Table of Materials. The manuscript has been revised to better explain how flies are set up for the phagocytosis experiment beginning on line 95.

8. Line 83: What does exactly "freshly prepared food vial" mean? I think it will be more informative if the authors provide the type of food they use and the actual composition of the food (Bloomigton formulation vs molasses food etc.).

Response: This has also been revised as part of the response to the previous comment beginning on line 94.

9. Line 85: What is the meaning of experimental conditions here? Do they refer to the crosses or the rearing conditions? Please, clarify.

Response: We have revised this section to clarify what we meant as part of the edited section beginning on line 94.

10. Lines 87-88: Please, provide the exact genotypes for experimental flies. It is important for the reader to be able to collect the right flies after a cross, especially if parental stocks are over a balancer.

Response: See response to reviewer 4's comment #7: the genotype of the flies used in this protocol has been listed in the Table of Materials.

11. Line 93: Please, indicate the aging conditions: temperature, how often they are being flipped etc.

Response: Temperature will depend on the experimental conditions being tested. In general, and in the case of this protocol, flies are kept at 24℃, which is mentioned in 1.5. For flies that are to be aged to 1-week, there is no need to transfer the flies to new food vials throughout the week. For flies that are to be aged to 1+ weeks, the protocol has been revised to (1.3.2, beginning Line 113) clarify how to maintain those flies.

12. Lines 109-110: Please, indicate how often the flies are being flipped in fresh vials during the aging process.

Response: See response to #11 above.

Protocol 2

13. Line 131: in which buffer is the dilution made? PBS? Please, clarify.

Response: The dilution would be made in the same solution the particles were prepared in. We have modified the manuscript to clarify (beginning line 158).

Protocol 3:

14. I think that it would be particularly helpful if the authors could show in the video how the injector is mounted with the needle and the solution aspired prior injection in the fly. This is a meticulous task and a visualization of the process could help.

Response: We have requested this procedure to be filmed.

15. Line 136: Please give a reference for the glass needles. What is their diameter and length for instance? Please, also include this information in the reagents table.

Response: The glass needles have now been referenced in the Table of Materials.

16. Line 137: Please provide the heating conditions of the capillary puller. Also, what type (brand) of capillary puller you used? You can list this in the reagents/materials table.

Response: The capillary puller and heating conditions have been added to the Table of Materials, as well as to the protocol (3.1.1).

17. Line 138: Does 30G mean 30-gauge hypodermic needle? Please, specify.

Response: Correct. We have modified the manuscript now to spell this out (Line 168).

18. Lines 155-156: Is the Bioparticles solution in a microcentrifuge tube or is pipetted as a drop placed on a parafilm and aspired under the scope? Please, specify.

Response: Once the particles are prepared, they were aliquoted into microcentrifuge tubes. The particles remain in the microcentrifuge tubes throughout the protocol. Transferring the particles to parafilm could expose them to direct light longer than is recommended. The manuscript has been revised for clarity (Line 149).

19. Line 182: Is the vial polystyrene, polypropylene or glass? What diameter it is?

Response: We have added this information to Table 1.

20. Line 184: It would be very helpful to have a section that describes how to prepare the dissection plates. I assume it is a petri dish containing sylgard, but the size is not specified? Could you please include this information?

Response: The size of the petri dished is now noted in the Table of Materials. The Sylgard silicone elastomer is prepared according to its provided protocol. Plates are filled no more than half-way. the manuscript has been revised to clarify this part of the procedure (Line 229)

21. Line 191: What is dissection media and what volume precisely?

Response: The dissection media is listed in the Table of Materials, and the volume needed for each plate is now stated in the protocol (4.2.3).

22. Line 193: Could you please specify the size of the forceps used?

Response: The size of the forceps, included the exact catalog number, is now provided in the Table of Materials.

23. Line 196: Similarly, please specify size of the scissors.

Response: See response, above.

24. Lines 204-205: Are these full size pins or pins cut in half? From the image I can't say, but if several full-length pins are used, I believe it should be mentioned that they would need to be placed under a certain angle and not at 90 degrees as this could "block" the forceps access to the tissue that is being dissected tissue (e.g. dorsal vessel).

Response: The length of the pins depends on personal preference. We, personally, prefer to cut the pins in half. If full-sized pins are used, inserting the pin at an angle is ideal, as it will not block access to the fly. We have added a sentence about this on line 237.

25. Lines 215-216: is this step done in the dissecting plate? If yes, it is important to say what diameter the plate is. Is 1mL sufficient to cover the plate surface?

Response: The protocol states (in 4.2.9) to leave flies pinned to plate, that all of step 5 will be performed in the plate. The size of the plate is now provided in the Table of Materials. The manuscript has been revised (Line 242) to clarify that sufficient volume (~1mL in this case) is needed to cover the surface of the plate. If other size plates are used the volume needs to be adjusted to cover the specimen.

26. Line 217: rocking at what speed? Please, specify.

Response: The manuscript has been revised to state the rocking speed (Line 268), and the rocker used has been added to the Table of Materials.

27. Lines 225-227: Please specify what antibodies you used precisely.

Response: We have added this information to the Representative results and Figure 3.

28. Line 228: Incubation is for how long? Please, specify.

Response: 5.3.1 now includes instructions to incubate overnight at 4C.

29. Line 232: Washes are for how long? Please, specify.

Response: 5.2 now specifies the details of the washes.

30. Line 234: In what buffer the dilution was made? I assume either PBS or PBST?

Response: DAPI is diluted in PBST. The manuscript has been revised to clarify this.

31. Line 249: How do you orient the cuticles? Using forceps and under the stereoscope? Please provide more detail.

Response: The manuscript has been revised for clarification (Line 306).

32. Lines 259-260: How many stacks do you acquire in general? Do you cover the entire organ from top to bottom? Can you provide an estimate of Z-stack number?

Response: The anterior portion of the dorsal vessel is focused, first. However, it is easy to disrupt that portion of the dorsal vessel while removing the thorax. In that case, continue down the dorsal vessel moving away from the thorax, focusing on the next compartment containing fluorescent events. The number of stacks acquired depends on how well the dorsal vessel was

Commented [MS7]: I don't know what this means. When you say "down the dorsal vessel", do you mean away from the thorax? Or focusing down away from coverslip??

dissected, and on the desired step size between images. That number can range anywhere from 3 to 40 images per stack. We have added this detail to Line 320.

33. Line 263: Please, provide reference for the software.

Response: The software is now referenced in the Table of Materials.

34. Lines 291-295: This part of the results could probably go in the discussion.

Response: We agree, and have done so.

Results:

35. In results it looks like only pHRodo examples are shown. As the protocol is also for Bioparticles, it would be nice to show an example.

Response: Another reviewer also commented on this. We have now added a figure that shows the bioparticles (Figure 4).

36. Line 312: is all FB removed? The FB in the dorsal cuticle seems to be present. Please, specify.

Response: Ideally all of the fat body should be removed. In the Figure 2, some fat body is still present as noted. To prevent damaging the cuticle by trying to further remove FB's, excess tissue will be washed away and/or loosened up during wash steps, making it easier to remove before mounting.

Commented [MS8]: Not sure what this means. If its fixed it shouldn't wash away. Maybe just say that this can vary with age and that if not all fb can be removed without damaging the cuticle, it is left in place.

Minor Concerns:

37. Line 308: Replace place with placed.

Response: Done.

38. Line 318: Do the authors mean image(s) instead of imaged?

Response: The text has been corrected.

39. Line 389: Please correct the reference. Authors' names are cited twice.

Response: Done.

Reviewer 5 Comments:

Manuscript summary:

1. This protocol describes a method for measuring phagocytic ability in the dorsal vessel of Drosophila. This is a potentially challenging technique that could be of utility to multiple researchers and the protocol is clearly described.

Major concerns:

2. None

Minor comments:

3. The protocol is presented in the context of immunosenescence, but the procedure is much more generally applicable to any scientific question for which measuring phagocytic capacity is useful. I recognize that the authoring laboratory works on immunosenesence, but the authors may wish to emphasize the generality of the technique.

Response: We agree, this protocol can be applied to many scientific questions regarding phagocytosis. The manuscript has been revised to state as such. (line 31)

4. This protocol will not measure the phagocytic capacity of circulating hemocytes, only of sessile hemocytes around the dorsal vessel. This is presented as an advantage in Discussion (beginning Line 351). It should be clearly stated in the Introduction as well, that the technique is focused on the hemocytes adhering to the dorsal vessel and will not yield a measure of total phagocytic capacity.

Response: Another reviewer had a similar comment. We have added a new section beginning on Line 78 to clarify this point.

5. The protocol explains how the Bioparticles and pHrodo differentiate between internalization and formation of the phagolysosome on step 3.3.3.5. This is a crucial conceptual point and should be emphasized on lines 72-73.

Response: We agree, and have revised the introduction.

6. Line 26: Drosophila is not an ideal model for measuring age-related changes in immunity "because" the genetic components and immune responses are shared with mammals. I see the point that the authors are trying to make, but the causal relationship stated is not correct. A minor re-wording will fix this.

Response: Agreed, and revised (see Line 48).

7. Line 35: Delete "Known as"

Response: Done.

8. Line 54: The use of "Lastly" makes the evolutionary conservation to mammals seem less important than the other factors listed.

Response: We have revised this section (and see Line 56).

9. Protocol step 1.1: Note that overcrowding may also have adverse effects on developing larvae, which may then manifest as altered phagocytic capacity.

Response: This is a good point. We have added this detail (Line 96).

10. Protocol step 1.2: It appears that the parents are being saved when they are transferred to new food vials. Why? They are not used later in the protocol.

Response: Parental flies are transferred as a way to continue future F1 collections, but this is not required. The protocol was revised for clarification (beginning Line 100).

11. Protocol step 1.3: Specify that the eclosing experimental flies are the progeny of the adults in step 1.1.2. Why do the flies need to be virgins? That does not seem to be obligatory for measuring phagocytosis. If the flies do need to be virgin, emphasize that the flies need to be collected and separated by sex within 8 hours of eclosion.

Response: This is a good point. We have revised this section to clarify (beginning on Line 101).

12. Protocol step 1.3.1: Be explicit that "each particle" being injected is the Bioparticle or the pHrodo labeled bacteria.

Response: We have now made this clear throughout the manuscript.

13. Protocol step 2.1.3: How long with the particles last if sodium azide is not added?

Response: We have revised the manuscript to include that information (beginning Line 150).

14. Protocol step 3.1.1: Describe the settings for pulling the needles, and the properties of the pulled needle including diameter. This also applies to step 3.3.1.4.

Response: Done.

15. Protocol step 3.3.1: Can other methods of anesthesia, such as FlyNap be used? Or will these also damage the assay?

Response: We have not tried to use other methods of anesthesia so we cannot really comment on this.

16. Protocol step 3.3.2: The Note states that flies can be injected in other sites "such as" the abdomen, but really the abdomen is the ONLY other site where injection could be performed.

Injection into the head would be inadvisable and injection into the dorsal plate of the thorax would destroy the assay. A small tweak in writing would clarify this.

Response: Agreed. That statement has been revised (line 203).

17. Protocol steps 3.3.3 and 3.3.4: It seems like timing should be critical in this assay. Why is only the time of the final injection recorded in step 3.3.3? In step 3.3.4, phagocytosis and formation of the phagolysosome will be occurring during the 60-90 minute "recovery" window. It therefore seems possible that a fly harvested after 60 minutes will appear to have different phagocytic capacity than a fly harvested at 90 minutes. Can the timing really be this liberal or should it be more precise?

Response: Another reviewer had a similar concern. Step 3.3.3 has been revised. Timing is critical. With that being said, injections for one set of flies (ex: 1-week old flies being injected with pHrodo) should be completed in a timely manner. With practice, it should take no longer than 5-10 minutes to inject 20-25 flies. In reference to step 3.3.4, the recovery time of 60-90 minutes was chosen based on the findings from a time course study performed by Horn et al., 2014, where phagocytic events / cell was counted at 5, 30, 60, and 90 minutes, in 1-week and 5-week old flies. However, we do agree that, for some lines, a 60-90-minute recovery time may be too long to detect subtle differences. In such cases, time courses studies can be performed to determine an optimal recovery time. Also, it's important to be mindful of how many flies a single individual can dissect in a timely manner. 20-25 flies is not feasible, so having multiple people performing dissections will also help minimize any differences due to timing. We have added some discussion about optimizing the timing for different experimental conditions (beginning Line 213).

18. Protocol step 4.2: Define what a dissection plate is.

Response: Done.

19. Protocol step 4.2.2: Be explicit that the pins being used are the insect pins listed on the materials and reagents.

Response: Done.

20. Protocol step 4.2.4: Consider rephrasing "This step is optional, as the head can be removed later…" to "The head can alternatively be removed later…". Consider moving the Note from step 4.4.2.5 to this step.

Response: Done.

21. Protocol step 5.3: Is it permissible to describe when and why it might be desirable to do antibody staining? It is not inherently obvious that it should be necessary for this procedure.

Response: Agreed, and revised.

22. Protocol step 7.1.2: The use of a 10 um diameter around the nucleus is because this is the size of a typical cell, but this approach seems to assume that all cells are circular in the plane of vision. Might it be more precise to use a membrane marker and only look at particles that are inside the membrane boundary? How does this technique distinguish particles that have been internalized from particles that might be adherent to the outside of the cells?

Response: Particles that are bound to the membrane fall within the 10 um diameter based on our earlier study in which we did use a membrane marker (Horn et al. 2014). We believe this may result in undercounting events, but prefer a somewhat conservative approach. Horn et al. also showed the cells were consistently circular and that bound bacteria were either washed away or were predominantly already internalized by the hemocyte at the time of fixation. That said, we added to the protocol that a membrane marker might be useful (line 278)

23. Protocol step 7.1.3: "excel spread sheet" should be "Microsoft Excel or other spreadsheet"

Response: Done.

24. Protocol step 7.2.1.1: The transformation described is not guaranteed to satisfy the assumptions of ANOVA. Perhaps the authors should suggest that investigators are rigorous in testing those assumptions.

Response: This is an excellent point. We have revised this statement (Line 343).

25. Line 294: "discarded" may not be the best word to use here.

Response: Changed to "accidentally discarded" (Line 260).

26. Line 357: Consider replacing "similar" with "alternative".

Response: Done.

27. Figure 3 legend: Move the statement about the dotted white line to the description of panel A.

Response: Done.

28. Why not put all images in the same orientation, so the dotted white lines in Figures 3 and 4 points in the same direction?

Response: This is a good suggestion. Done.