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Corresponding Author:	Ashley Nazario-Toole University of Maryland, College Park College Park, MD UNITED STATES
Corresponding Author's Institution:	University of Maryland, College Park
Corresponding Author E-Mail:	anazario@umd.edu
Order of Authors:	Ashley Nazario-Toole Louisa P. Wu
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TITLE:

Assessing the Cellular Immune Response of the Fruit Fly, *Drosophila melanogaster*, using an *In Vivo* Phagocytosis Assay

AUTHORS AND AFFILIATIONS:

Ashley E Nazario-Toole^{1,2}, Louisa P Wu^{2,3}

¹Department of Biology, University of Maryland, College Park, MD, USA

²Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, USA

³Institute for Bioscience and Biotechnology Research, Rockville, MD

Corresponding author:

Ashley E Nazario-Toole (anazario@umd.edu)

Email Address of the Co-Author:

Louisa P Wu (louisa@umd.edu)

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SUMMARY:

This protocol describes an *in vivo* phagocytosis assay in adult *Drosophila melanogaster* to quantify phagocyte recognition and clearance of microbial infections.

ABSTRACT:

In all animals, innate immunity provides an immediate and robust defense against a broad spectrum of pathogens. Humoral and cellular immune responses are the main branches of innate immunity, and many of the factors regulating these responses are evolutionarily conserved between invertebrates and mammals. Phagocytosis, the central component of cellular innate immunity, is carried out by specialized blood cells of the immune system. The fruit fly, *Drosophila melanogaster*, has emerged as a powerful genetic model to investigate the molecular mechanisms and physiological impacts of phagocytosis in whole animals. Here we demonstrate an injection-based *in vivo* phagocytosis assay to quantify the particle uptake and destruction by *Drosophila* blood cells, hemocytes. The procedure allows researchers to precisely control the particle concentration and dose, making it possible to obtain highly reproducible results in a short amount of time. The experiment is quantitative, easy to perform, and can be applied to screen for host factors that influence pathogen recognition, uptake, and clearance.

INTRODUCTION:

Innate immune defenses form the first line of defense against pathogenic microbes. These responses can be functionally divided into humoral and cellular innate immunity, both of which are mediated by germline-encoded pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs)¹. Many of the signaling pathways and effector

mechanisms of innate immunity are conserved in mammals and invertebrates, such as the nematode, *Caenorhabditis elegans*, and the fruit fly, *Drosophila melanogaster*². The fruit fly has emerged as a powerful system to study host defense against infectious microorganisms³. *Drosophila* is genetically tractable, easily and inexpensively reared in laboratories, and has a short generation time. Furthermore, the fruit fly exhibits highly efficient defenses against an array of microbes, enabling the examination of host immunity against viral, bacterial, fungal, or parasitic pathogens.

Drosophila immunologists have historically utilized the forward genetic screens, genome-wide RNA-mediated interference (RNAi) screening of insect cell lines, and pre-existing mutant fly strains to examine innate immunity – leading to the identification and characterization of several evolutionarily conserved humoral immune pathways^{4,5,6,7,8}. The humoral innate immune response is, arguably, the best characterized immune defense in fruit flies. Following infection, the humoral response leads to the production and systemic release of antimicrobial peptide (AMP) molecules into the hemolymph, the blood equivalent in insects. AMPs are produced by highly conserved Toll and Imd signaling pathways. The Toll pathway is homologous to mammalian TLR/IL-1R receptor signaling, and the Imd pathway is homologous to mammalian Tumor necrosis factor-alpha signaling. In *Drosophila*, Toll signaling is induced by gram-positive bacteria, fungi, and *Drosophila X virus*^{6,9,10} and Imd signaling is induced by gram-negative bacteria^{11,12}.

Cellular immunity, comprised of encapsulation, melanization, and phagocytosis of invasive pathogens is carried out by specialized blood cells called hemocytes¹³. There are three classes of hemocytes in the fruit fly: crystal cells, lamellocytes, and plasmatocytes¹³. Crystal cells, which make up 5% of the circulating hemocytes in larvae, release proPhenoloxidase (proPO) enzymes leading to the melanization of pathogens and host tissues at wound sites. Lamellocytes, which are not normally found in healthy embryos or larvae, are adherent cells that encapsulate foreign objects. These cells are induced upon pupariation or when parasitizing wasp eggs are deposited in larvae. Phagocytic plasmatocytes, which make up 95% of circulating hemocytes in larvae and all remaining hemocytes in adults, play a role in tissue remodeling during development and, notably, serve as the main effector cell of *Drosophila* cellular immunity.

Phagocytosis is an immediate and crucial line of innate immune defense; microbes that breach the host epithelial barrier are quickly engulfed and eliminated by phagocytic blood cells (For a comprehensive review of cell biology of phagocytosis see reference¹⁴). This process is initiated when germline-encoded pattern recognition receptors (PRRs) on hemocytes recognize pathogen associated molecular patterns (PAMPs) of microbes. Once bound to their targets, PRRs initiate signaling cascades that lead to the formation of pseudopods through actin cytoskeleton remodeling. The pseudopods surround the microbe, which is subsequently engulfed and internalized into a nascent organelle, the phagosome. Microbes are destroyed as the phagosome undergoes the process of phagosome maturation when the phagosome is trafficked towards the interior of the hemocyte and acidifies through a series of interactions with lysosomes. *In vitro* and cell biology studies in mammalian primary cells have been instrumental in identifying and characterizing factors that regulate phagocytosis, such as the mammalian Fc-gamma receptor and C3b receptors^{15,16}. Nevertheless, the ability to execute large-scale screens or *in vivo* studies

are limited in mammalian systems.

Here we present an *in vivo* assay for phagocytosis in adult fruit flies, which is based on a procedure first introduced by the laboratory of David Schneider in 2000¹⁷. The Schneider lab showed that sessile hemocytes clustered along the abdominal dorsal vessel readily phagocytose polystyrene beads and bacteria. To visualize phagocytosis, flies are injected with fluorescently labeled particles (such as *E. coli* labeled with fluorescein isothiocyanate (*E. coli*-FITC)), incubated for 30 minutes to allow hemocytes time to engulf the particles, and then injected with trypan blue, which quenches the fluorescence of particles not phagocytosed during the incubation period. Fly dorsal vessels are then imaged using an inverted fluorescent microscope. This seminal paper, using a relatively simple experiment, demonstrated that hemocytes phagocytose bacteria and latex beads, that bacterial phagocytosis can be inhibited by pre-injecting flies with latex beads, and that flies without both cellular and humoral immune responses are susceptible even to *E. coli*. The assay presented in this report builds on the work of the Schneider lab to quantify *in vivo* phagocytosis by measuring the fluorescence intensity of particles engulfed by dorsal vessel associated hemocytes.

Similar to the approach taken in mammalian systems, *Drosophila* geneticists initially used genome-wide *in vitro* RNAi screens to identify genes required for the cellular immune response^{18,19,20,21,22,23}. However, the development of the adult *in vivo* phagocytosis assay enabled follow-up experiments to be readily carried out in whole animals, thus allowing researchers to verify the biological the role of factors identified in *in vitro* studies. Such was the case with the transmembrane receptor Eater, which was first identified as a bacterial receptor in an RNAi screen using S2 cells²⁴ and then later shown to mediate *Escherichia coli* (*E. coli*), *Enterococcus faecalis*, and *Staphylococcus aureus* (*S. aureus*) phagocytosis in adults²⁵.

Our lab employed the *in vivo* phagocytosis assay in forward genetic screens and genome-wide association studies (using the *Drosophila* Genetic Reference Panel (DGRP)) to identify novel genes that regulate phagocytosis in adult hemocytes. These studies led to the characterization the receptors PGRP-SC1A and PGRP-SA²⁶, the intracellular vesicle trafficking protein Rab14²⁷, the glutamate transporter Polyphemus²⁸, and RNA-binding protein Fox-1²⁹.

We anticipate that future screens incorporating the *in vivo* phagocytosis could lead to the identification of additional genes that are important for the cellular immune response in *Drosophila*. Screens using fully-sequenced inbred lines, such as the DGRP or the *Drosophila* Synthetic Population Resource (DSPR), can identify natural variants affecting phagocytosis or hemocyte development. Furthermore, the technique could be adopted in other species of *Drosophila* or used to screen new community resources, such as the collection of 250 *Drosophila* species maintained by the National *Drosophila* Species Stock Center (NDSSC) at Cornell. These experiments can be carried out using fluorescently-labeled bacterial or fungal-wall bioparticles that are available commercially or may be performed using any number of bacterial or fungal species – provided that the microbe expresses fluorescent markers.

PROTOCOL:

1. Prepare Fluorescein particles for injection

1.1. Reconstitute 10 mg of commercially available, heat-killed bacteria particles labeled with fluorescein (see **Table of Materials**) to a stock concentration of 10 mg/mL by adding 990 μ L sterile 1x PBS and 10 μ L 50 mM sodium azide. Vortex to mix.

1.1.1 Divide into single-use 8 μ L aliquots in 0.2 mL tubes and store in a dark box at 4 °C to minimize light associated sensitivity.

NOTE: Sodium azide preservative is optional and can be omitted if 10 mg/mL stocks are made with 1 mL sterile 1x PBS, aliquoted, and stored at -20 °C.

1.2. Make a 10 mL solution of 5% food coloring in 1x PBS by mixing 500 μ L syringe filtered green food coloring and 9.5 mL sterile 1x PBS.

1.3. Wash particles before injection to remove sodium azide. Mix 42 μ L sterile 1x PBS and 8 μ L of 10 mg/mL in a 1.7 mL tube. Centrifuge at max speed for 2.5 min at room temperature.

1.3.1. Remove the supernatant, add 50 μ L 1x PBS, and centrifuge at max speed for 2.5 min at room temperature.

1.3.2. Repeat steps 1.3 and 1.3.1 2x, for a total of 3 washes.

1.3.3. After the final wash, discard the supernatant and re-suspend particles to 1.6 mg/mL in 50 μ L of 5% food coloring in 1x PBS.

1.3.4. Wrap the tube in aluminum foil to protect from light. Store at 4 °C, discard after 1 week.

2. Prepare the injection station and flies

2.1. Prepare the injection pad. To inject up to 4 genotypes of flies at the same time, use laboratory tape to divide a rectangular CO₂ fly pad into 4 sections. On the bench near the microscope, designate areas to place the vials once flies have been lined up on the pad (one for each corner of the pad).

2.2. Prepare vials of age-matched, 4-7 days-old, flies for injection. For each strain to be tested, transfer 5 males and 5 females into a fresh, labeled vial of prepared fly food and keep at 25 °C.

2.3. Prepare the pneumatic injector (see **Table of Materials**) by setting the instrument to a 100 ms (short bursts of gas pressure to expel the liquid – allowing the delivery of sub-nanoliter volumes) **TIMED** mode.

2.4. Prepare the microscope slides. Cut 1.5-inch strips of electrical tape, fold into a loop with the adhesive side out, and place onto a labeled microscope slide.

3. Prepare glass capillary needles

3.1. Pull glass needles (thin wall glass capillaries) using a needle puller.

3.1.1. Hold the needle under the microscope with a micrometer and break the tip using #5 fine point stainless steel tweezers. 100 μm tips are sufficient to pierce the fly's cuticle while minimizing wounding.

3.1.2. Measure the volume of liquid that will be injected into each fly. Load the needle with sterile 5% food coloring in 1x PBS and expel the liquid onto a drop of mineral oil on a 0.01 mm stage micrometer.

NOTE: If the liquid droplet is spherical, the volume in picoliters is calculated as $(\text{size})^3/1910$.³⁰ A needle with a 100 μm diameter will eject ~2 nL in 100 ms.

4. Inject flies

4.1. Pipette 10 μL of 1.6 mg/mL particles onto a small square of parafilm.

4.1.2. Pull the liquid into the needle and mount in the injector nozzle (see **Table of Materials**).

4.1.3. Anesthetize flies with CO_2 and line them up in their designated area on the flypad, with the ventral side up and the heads oriented towards the front of the pad. Place vials in corresponding areas on the bench.

4.1.4. Inject flies at the upper corner of the abdomen with 5, 100 ms pumps of liquid (~10 nL total).

4.1.5. Transfer the injected flies to the appropriate vials, note the time on the vial. Keep at 25 $^\circ\text{C}$.

4.2. Load a new needle with 0.4% Trypan Blue Solution.

4.3. Set the pneumatic injector to **GATED**, which allows a constant flow of air to push the liquid out of the needle.

4.4. Anesthetize flies after they have rested for 30 min and inject with Trypan Blue until the abdomen is full and distended.

NOTE: When examining phagosome maturation with particles labeled with a pH-sensitive dye, allow flies to rest for 1 h and do not inject with trypan blue before mounting flies.

4.5. Mount flies on microscope slides with electrical tape, ventral side down. Push the wings to the side of the fly and secure them to the tape. Also, gently push the head into the tape to ensure that the fly will not move.

4.6. Immediately move to step 5.

5. Imaging flies

5.1. Image flies, one at a time, at 25x or 32x magnification using an inverted fluorescence microscope attached to a digital camera and computer (see **Table of Materials**). Focus on the dorsal vessel of the fly using computer software for the digital camera.

5.1.2. Record the exposure time and magnification between experiments.

NOTE: Losing track of genotypes is a potential source of error when photographing multiple strains in a single sitting. To avoid mislabeling flies, make a note of the image number of the first and last fly photograph for each genotype.

6. Quantifying and normalizing the fluorescence

6.1. Open the software and open one image at a time.

6.1.1. Measure the fluorescence intensity of the dorsal vessel. Draw a polygon around the dorsal vessel. Select **Measure** and record the fluorescence intensity inside the polygon.

6.1.2. Determine the background fluorescence intensity. Copy the first polygon and move it to an area adjacent to the dorsal vessel of the fly. Select **Measure** and record fluorescence intensity of the background area.

6.1.3. Normalize the dorsal vessel fluorescence by the background fluorescence:

Dorsal vessel \div background.

6.1.4. Calculate the average normalized dorsal vessel fluorescence intensity of all flies in a strain.

6.1.5. Repeat the experiment 2 more times.

6.1.6. Use a Student's unpaired *t*-test to compare the mean relative fluorescence intensities of control flies and test flies from the three experiments. Calculate the effect size using the formula: Cohen's $d = (M_1 - M_2) \div SD_{\text{pooled}}$, where M_1 is the mean of genotype 1 and M_2 is the mean of genotype 2 &

$$SD_{\text{pooled}} = \sqrt{(SD1^2 + SD2^2) \div 2},$$

where *SD1* is the standard deviation of genotype 1 and *SD2* is the standard deviation of genotype 2.

REPRESENTATIVE RESULTS:

A schematic of the *in vivo* phagocytosis assay using fluorescein-labeled particles is shown in **Figure 1A**. Flies are mounted ventral side down on a piece of electrical tape and the first two segments of the abdomen, where the dorsal vessel is located, is clearly visible (**Figure 1B**). Key sources of experimental error arise at the injection and imaging steps of the procedure (**Figure 1C**). Using the same needle to inject multiple flies may cause it to become clogged with fly tissue or particles (top left panel in **Figure 1C**). Because *Drosophila* auto-fluoresces under GFP light, flies injected with a clogged needle will fluoresce faintly but lack the clear, punctate fluorescence of particles internalized by hemocytes. Another potential source of experimental error can occur during the Trypan Blue injections. Thirty minutes after the first injection with fluorescently-labeled particles, flies receive a second injection with Trypan Blue, which quenches the fluorescence of extracellular, un-phagocytosed particles. Relative fluorescence intensity for each fly is calculated by dividing the fluorescence of the dorsal vessel by that of an equally sized adjacent area on the abdomen. Flies that do not receive enough Trypan Blue fluoresce brightly throughout their entire abdomen (bottom left panel of **Figure 1C**). This bright fluorescence can reduce the ratio of the dorsal vessel to background fluorescence, thus decreasing the true fluorescence intensity ratio of the animal. Finally, flies should be photographed while immobilized by CO₂, as actively moving flies produce blurry images that cannot be quantified (top and bottom, right panels of **Figure 1C**).

The Zuker collection is a panel of ethyl methanesulfonate (EMS) treated flies with autosomal mutations.³¹ Originally established as a community resource in 2004, the lines have been used to carry out forward and reverse genetic screens. Our lab identified a line from the Zuker collection that is unable to phagocytose gram-negative (*E. coli* K-12 strain) and gram-positive bacteria (*S. aureus* Wood strain without protein A) (**Figure 2**). This mutant, called *argus*, shows almost no dorsal vessel fluorescence in the *in vivo* phagocytosis assay. When compared to the Zuker isogenic background strain, *cn bw*, *argus* shows significantly less uptake of *E. coli* (*p*-value = 0.019; Cohen's *d* = 1.677) and *S. aureus* (*p*-value = 0.0083; Cohen's *d* = 1.672). *Argus* bacterial phagocytosis is also significantly impaired, *E. coli* (*p*-value = 0.045; Cohen's *d* = 0.850) and *S. aureus* (*p*-value = 0.0136; Cohen's *d* = 1.422) when compared to another common laboratory control strain, Canton-S.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview and representative images of *in vivo* phagocytosis assay. **A.** Schematic of the *in vivo* phagocytosis assay using fluorescein-labeled particles. Two injections, 30 minutes apart, are used to visualize particle recognition and uptake by dorsal vessel-associated blood cells. **B.** White light image showing a fly mounted on tape, with clearly visible anterior abdominal

segments. **C.** Example *in-vivo* phagocytosis images. (*top left*) A fly injected with a clogged needle has no particle fluorescence. (*bottom left*) A fly with high extracellular fluorescence because of insufficient trypan blue. (*top right*) An immobilize fly with no extracellular fluorescence – ideal image. (*bottom right*) A fly moving as CO₂ anesthesia wears off creates a blurry image. Scale bar, 0.2 mm.

Figure 2: Phagocytosis of bacteria in mutant and control flies. **A.** Representative images of phagocytosis bacteria labeled with fluorescein dye; *E. coli* (top panels) and *S. aureus* (bottom panels). **B.** The fluorescence intensity ratio of flies injected with fluorescein-*E. coli* particles. **C.** The fluorescence intensity ratio of flies injected with fluorescein-*S. aureus* particles. n = 3 experiments for each type of bacteria, with 6-8 flies per experiment. Error bars, \pm SEM. Statistical analysis = Two-tailed *t*-test. Scale bar, 0.2 mm.

DISCUSSION:

Commercially available, fluorescently labeled particles are used to assess phagocytosis in general (0.2 μ m carboxylate-modified microspheres) or phagocytosis of microbes (fluorescently-labeled heat- or chemically killed bacteria or yeast). To assess phagosome maturation, researchers can select particles labeled with a pH-sensitive dye that fluoresces when pH decreases from neutral to acidic, as in the phagolysosome. Alternatively, to examine the initial steps of phagocytosis, pathogen recognition and uptake, researchers should choose particles labeled with fluorescent tags that fluoresce inside and outside of cells.

Carefully controlled experimental conditions are key to ensure the *in vivo* phagocytosis assay is carried out in a reproducible manner. First, *Drosophila* undergoes cellular immunosenescence³², so flies used in the experiment should be age-matched, optimally within a range of 4-7 days old. Second, the period between injections should be consistent. Phagocytosis occurs rapidly, within minutes of the host cell sensing the microbe¹⁴. Over the next hour, phagosome maturation takes place, leading to the acidification of the phagosome and destruction of the internalized microbe. Designating a set interval to wait between injections reduces experimental variability and allows comparisons to be made between strains and experiment conducted on different days. We recommend waiting thirty minutes when using fluorescein particles and one hour for the *in vivo* phagosome maturation assay using particles labeled with pH-sensitive fluorescent dye.

Using the same needle to inject multiple strains ensures that all flies receive the same dose of particles and experimental variability can arise if glass needles break or become clogged. So, setting up the injection station and flies beforehand is key to ensuring that the *in vivo* phagocytosis assay is carried out quickly and efficiently. Before starting the experiment, measure the volume of liquid expelled by several needles and retain a set of needles of the same size. Thus, if one needle breaks between injections, it can be replaced immediately. Furthermore, clogged needles can lead to false negative results, making it difficult to distinguish between flies whose hemocytes are unable to phagocytose microbes and flies that did not receive fluorescent particles. Dye solutions made with green food coloring, make it easier to track which flies have been injected and to identify issues with clogged needles. Ideally, the experiment should be

performed multiple times, with least 6-8 flies per strain in each experiment, and the results of experiments compared to identify outliers. Finally, enough Trypan Blue should be administered to fully quench the fluorescence of extracellular particles so that the background fluorescence is comparable between flies (**Figure 1C**).

When imaging flies, care should also be taken to obtain clear, consistent images of the dorsal vessel. Black electrical tape is used to create a dark background and flies should be mounted ventral side down, with their wings and heads secured to the adhesive side of the tape. If possible, flies should remain immobilized under CO₂ while images are taken. Finally, to limit the possibility of experimental bias, we recommend using a blinded experimental setup; where vials and strains are coded by one researcher and the experiment is carried out and quantified by another researcher.

The *in vivo* phagocytosis assay is a useful tool to identify global defects in phagocytosis. However, it does not distinguish between hemocyte phagocytosis efficiency and differences in hemocyte number or location, due to age-related decline or developmental defects.³² Moreover, the procedure does not provide information on differences in the size or phagocytic capacity of individual hemocytes. Thus, once flies with reduced phagocytosis have been identified using the *in vivo* phagocytosis assay, researchers should carry out follow-up studies, perhaps using alternative particles, in order to discriminate between immune-related phagocytosis defects and general phagocytic defects. Ultimately, determining the molecular mechanisms that underlie phagocytic defects in mutant strains requires an examination of phagocytosis in single hemocytes. This can be accomplished using, for example, confocal microscopy, fluorescence activated cell sorting or magnetic bead isolation of adult hemocytes^{27,28,29,32,33,34}.

Overall, *Drosophila* has proven to be an effective model to study innate immunity in live animals. Here we have provided an overview of the *in vivo* phagocytosis assay, a method to study the global phagocytosis and the cellular immune response in adult flies. The procedure can be applied to screen for new genes that may be important for host defense, validate genes identified in large-scale *in vitro* RNAi screens, and further characterize mutants with defects in the humoral, gut or antiviral immune responses. It is easy to set-up and can yield a large amount of reliable data about multiple strains of flies in a short amount of time.

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DISCLOSURES:

The authors have nothing to disclose.

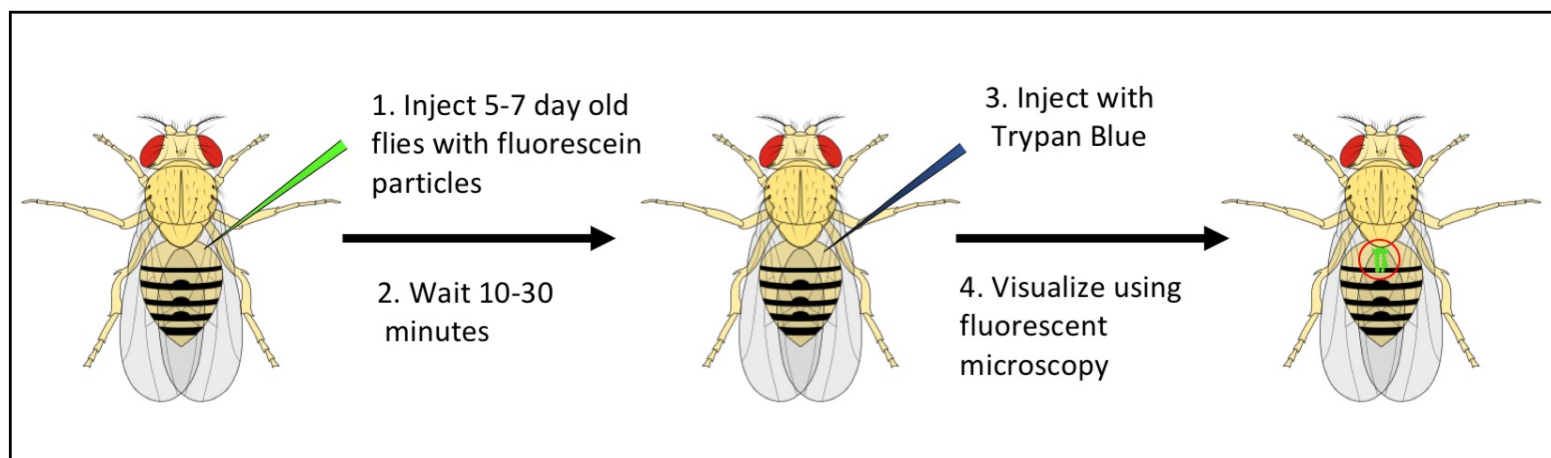
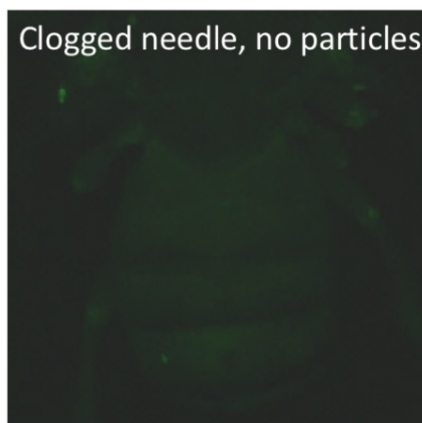
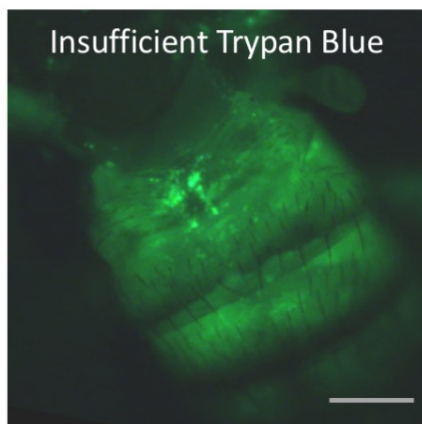
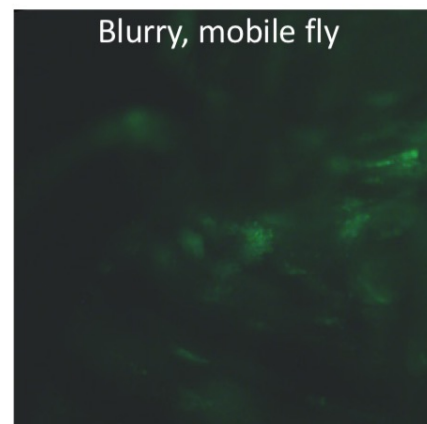
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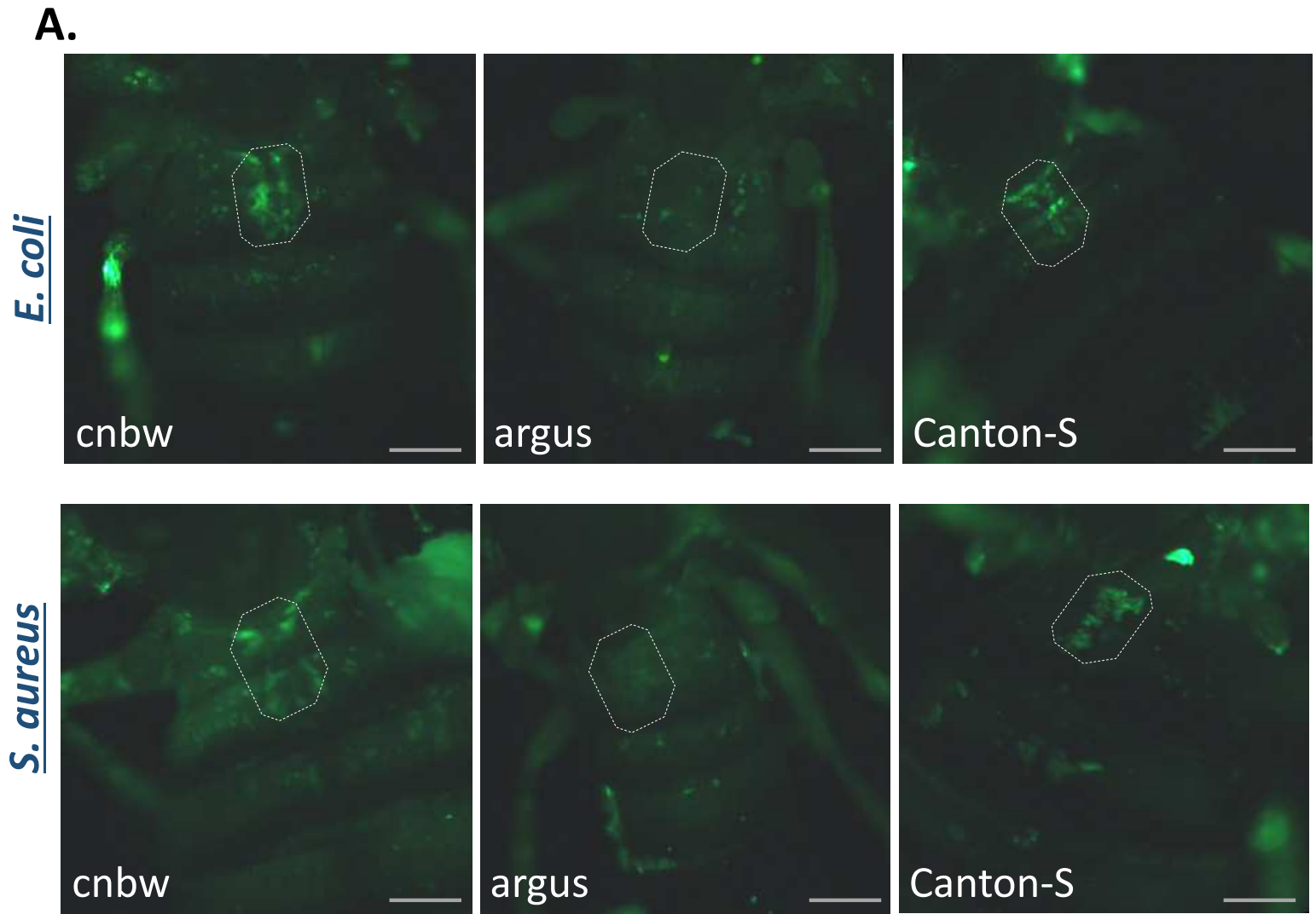
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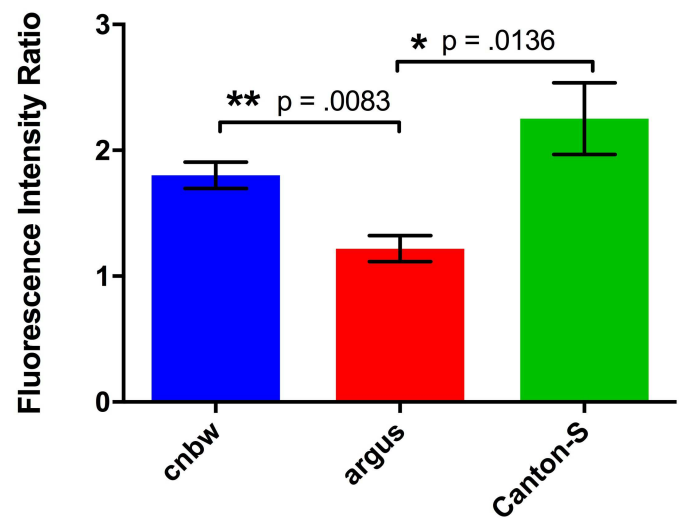
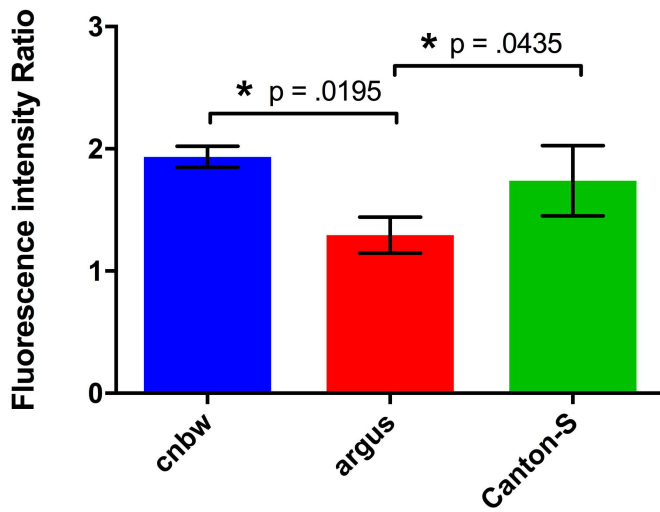
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488

A.**B.****Visualizing the Dorsal Vessel****C.****Injection Issues****Clogged needle, no particles****Insufficient Trypan Blue****Imaging Issues****Clear, anesthetized fly****Blurry, mobile fly**



B. Phagocytosis of *E. coli* bioparticles

C. Phagocytosis of *S. aureus* bioparticles



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2µm Red Fluorescent Carboxylate Modified FluoSpheres	Invitrogen	F8810	Fluorescently-labeled latex beads to test general phagocytic capacity of phagocytes. (~580/~605 nm) Inject a 1:20 dilution in PBS with 5% dye.
5430-10 PicoNozzle Kit	World Precision Instruments	5430-10	Holder for 1.0mm pipette Killed <i>E. coli</i> labeled with Alexa Fluor 488. Use to test phagocyte recognition and uptake of gram-negative bacteria. (~495/~519 nm)
<i>E. coli</i> (K-12 Strain) BioParticles, Alexa Fluor 488 conjugate	Invitrogen	E13231	Killed <i>E. coli</i> labeled with Alexa Fluor 594. Use to test phagocyte recognition and uptake of gram-negative bacteria. (~590/~617 nm)
<i>E. coli</i> (K-12 Strain) BioParticles, Alexa Fluor 594 conjugate	Invitrogen	E23370	Killed <i>E. coli</i> labeled with FITC (Fluorescein). Use to test phagocyte recognition and uptake of gram-negative bacteria. (~494/~518 nm)
<i>E. coli</i> (K-12 Strain) BioParticles, Fluorescein conjugate	Invitrogen	E2861	Killed <i>E. coli</i> labeled with Texas Red. Use to test phagocyte recognition and uptake of gram-negative bacteria. (~595/~615 nm)
<i>E. coli</i> (K-12 Strain) BioParticles, Texas Red conjugate	Invitrogen	E2863	Killed <i>E. coli</i> labeled with Texas Red. Use to test phagocyte recognition and uptake of gram-negative bacteria. (~595/~615 nm)
<i>E. coli</i> (K-12 Strain) BioParticles, Texas Red conjugate	Invitrogen	E2863	
Needle Pipette Puller	David Kopf Instruments	Model 725	

pHrodo Red <i>E. coli</i> BioParticles Conjugate for Phagocytosis	Invitrogen	P35361	Killed <i>E. coli</i> labeled with pHrodo Red. Use to test phagocyte recognition, uptake, and phagosome maturation of gram-negative bacteria. (~560/~585 nm). No need to quench with Trypan Blue.
pHrodo Red <i>S. aureus</i> BioParticles Conjugate for Phagocytosis	Invitrogen	A10010	Killed <i>S. aureus</i> labeled with pHrodo Red. Use to test phagocyte recognition, uptake, and phagosome maturation of gram-positive bacteria. (~560/~585 nm). No need to quench with Trypan Blue.
Pneumatic PicoPump PV820	World Precision Instruments	SYS-PV820	The World Precision Instruments Pneumatic PicoPump PV820 uses differential pressures to hold liquid in the glass needle between injections. The user manually controls short bursts of gas pressure to expel the liquid – allowing delivery of sub-nanoliter volumes. The amount of liquid delivered depends on two main variables – the size of the glass needle opening and the amount of time injection pressure is applied. set the instrument to 100 ms “TIMED” mode. Killed <i>S. aureus</i> labeled with Alexa Fluor 488. Use to test phagocyte recognition and uptake of gram-positive bacteria. (~495/~519 nm)
<i>S. aureus</i> (Wood Strain without protein A) BioParticles, Alexa Fluor 488 conjugate	Invitrogen	S23371	

<i>S. aureus</i> (Wood Strain without protein A) BioParticles, Alexa Fluor 594 conjugate	Invitrogen	S23372	Killed <i>S. aureus</i> labeled with Alexa Fluor 594. Use to test phagocyte recognition and uptake of gram-positive bacteria. (~590/~617 nm)
<i>S. aureus</i> (Wood Strain without protein A) BioParticles, Fluorescein conjugate	Invitrogen	E2851	Killed <i>S. aureus</i> labeled with FITC (Fluorescein). Use to test phagocyte recognition and uptake of gram-positive bacteria. (~494/~518 nm)
Thin Wall Glass Capillaries	World Precision Instruments	TW100F-3	Needles for injection. OD = 1.0 mm Used to quench extracellular fluorescence of Fluorescein, Alexa Fluor, or Texas Red labeled particles.
Trypan Blue Solution (0.4%)	Sigma	T8154	
ZEISS SteREO Microscope (Discovery.V8)	Zeiss	SteREO Discovery.V8	Inverted fluorescence microscope for imaging flies. Use a digital camera (example: AxioCam HC camera) and the accompanying software (example: AxioVision 4.7 software) to take pictures.
Zymosan A (<i>Saccharomyces cerevisiae</i>) BioParticles, Alexa Fluor 488 conjugate	Invitrogen	Z23373	Killed labeled with Alexa Fluor 488. Use to test phagocyte recognition and uptake of yeast. (~495/~519 nm)
Zymosan A (<i>Saccharomyces cerevisiae</i>) BioParticles, Alexa Fluor 594 conjugate	Invitrogen	Z23374	Killed labeled with Alexa Fluor 594. Use to test phagocyte recognition and uptake of yeast. (~590/~617 nm)
Zymosan A (<i>Saccharomyces cerevisiae</i>) BioParticles, Fluorescein conjugate	Invitrogen	Z2841	Killed labeled with FITC (Fluorescein). Use to test phagocyte recognition and uptake of yeast. (~494/~518 nm)
Zymosan A (<i>Saccharomyces cerevisiae</i>) BioParticles, Texas Red	Invitrogen	Z2843	Killed labeled with Texas Red. Use to test phagocyte recognition and uptake of yeast. (~595/~615 nm)

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Author(s):	Ashley E. Nazario-Toole and Louisa P. Wu

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CORRESPONDING AUTHOR

Name:	Ashley E. Nazario-Toole	
Department:	Cell Biology and Molecular Genetics	
Institution:	University of Maryland, College Park	
Title:	Assistant Clinical Professor (FIRE: The First-Year Innovation & Research Experience)	
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[Please see the protocol, representative results, and figure legends these changes.](#)

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

[The numbered steps of the protocol should now only contain action items. The discussion that was previously included in the protocol has now been primarily moved to the first paragraph of the Discussion section.](#)

8. Everything in the protocol (except for the introductory ethics statement) should be in a

numbered step (in the imperative tense and with no more than 4 sentences), numbered header, or a 'Note'. Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.

[These paragraphs have been either removed or moved to the Discussion section.](#)

9. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

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10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

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[Completed](#)

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

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13. Figure 1: Please describe panel C in the figure legend.

[A description for panel C has been added \(lines 450-454\).](#)

14. Figure 2: Please define error bars in the figure legend.

["Error bars, \$\pm\$ SEM" has been added to the Figure 2 legend.](#)

15. Figures: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

[0.2 mm scale bars have been added to each figure. .](#)

16. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

[Completed](#)

17. References: Please do not abbreviate journal titles.

[All journals are now listed by their full names.](#)

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors of this manuscript demonstrated in vivo phagocytosis assay to quantify particle uptake in *Drosophila*. The explanation of protocol is enough to follow their assay. This method is useful to identify phagocytosis-related genes. I have minor points that can be revised.

Major Concerns:

N/A

Minor Concerns:

1. In introduction, the distinction between what David Schneider et al did and what the authors did is not clear (Schneider et al: They developed the method to visualize in vivo phagocytosis in adult flies. The authors: They used or evolved Schneider's method to quantify in vivo phagocytosis by measuring the intensity of fluorescence derived from engulfed particles. They enabled us to identify phagocytosis-related genes with mutant flies or RNAi lines). It's difficult to understand it if we didn't read previous paper written by Schneider et al. More detailed explanation is needed.

[We thank the reviewer for their time and thoughtful suggestion. We've updated the language in the paragraph referenced above \(lines 96-109\) and we've added the following sentence to the end of the paragraph:](#)

["The assay presented in this report builds on the work of the Schnieder lab to quantify *in vivo* phagocytosis by measuring the fluorescence intensity of particles engulfed by dorsal vessel associated hemocytes."](#)

2. In Protocol, description in step 1.3.2 is maybe wrong. I think that 1.6 should be changed to 1.3.1.

[Thank you. We've corrected this error \(line 169\).](#)

Reviewer #2:

Manuscript Summary:

This manuscript describes a procedure that is commonly used to assess the phagocytic ability of blood cells in *Drosophila*. The procedure described is important as phagocytic function is a critical component of the innate immune response in all organisms, and *Drosophila* have proven to be useful in particular for identifying genes influencing this process. Given the large number of researchers working on *Drosophila* immunity, and the difficulty of assessing cellular immune function this JoVE article will be extremely useful. The paper is well-written, provides extensive background on phagocytosis and provides an up to date review of other studies that have used this, and similar

procedures, to assess phagocytic function in *Drosophila*. The methods described are clear and easy to follow. As such, I am in the unusual position of not having many suggestions to improve the manuscript.

Major Concerns:
None

Minor Concerns:

I do have one suggestion that I think would improve the paper. Lines 291 -294 report significance levels with no real information on sample sizes or effect sizes of the differences between samples. I know that the authors suggest using t-tests and comparing 3 samples, but I wonder what this sample size suggestion was based on. The sample size that might be appropriate will depend on the differences in normalized fluorescence levels between the control and test flies. It would be nice to know what size of an effect the authors measured that resulted in the level of significance reported in this section. I suppose this could be done by adding a figure showing the data from the experiments or simply reporting the effect sizes. Maybe adding some information about what the typical differences in fluorescence that are seen in these types of experiments would be also be useful to include to help researchers plan their experiments. Other than this, I think the authors should be congratulated on putting together such a tight manuscript on their first submission.

We thank the reviewer for their assistance with the manuscript. We determined the effect sizes for each of the comparisons listed in lines 426-48 by calculating Cohen's d (see lines 393-396 for the formula used). With the exception of one test, *E. coli* phagocytosis in *argus* versus Canton-S, all of our effect sizes were over 1. Therefore, carrying out 3 experiments with 18 flies total (6 flies tested for each genotype per experiment) yields sufficient power to detect differences in the phagocytic capacity of different fly strains. However, the large effect sizes we report in this paper may be limited to strains with extreme and nearly complete defects in phagocytosis such as *argus*.