



METHOD ARTICLE

REVISÉ Detecting proliferation of adult hemocytes in *Drosophila* by BrdU incorporation and PH3 expression in response to bacterial infection [version 2; referees: 3 approved]

Previously titled: Detecting proliferation of adult hemocytes in *Drosophilaby* BrdU incorporation

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Abstract

Drosophila and mammalian hematopoiesis share several similarities that range from primitive and definitive phases of hematopoiesis to the battery of transcription factors and signaling molecules that execute this process. The similarities in blood cell development across these divergent taxa along with the rich genetic tools available in fruitfly makes it a popular invertebrate model to study blood cell development both during normal and aberrant scenarios. The larval system is the most extensively studied till date. Several studies have shown that these hemocytes just like mammalian counterpart proliferate and get routinely regenerated upon infection. However, employing the same protocol it was concluded that blood cell proliferation although abundant in larval stages is absent in adult fruitfly.

The current protocol describes the strategies that can be employed to document the hemocyte proliferation in adulthood. The fact that a subset of blood cells tucked away in the hematopoietic hub are not locked in senescence, rather they still harbour the proliferative capacity to tide over challenges was successfully demonstrated by this protocol. Although we have adopted bacterial infection as a bait to evoke this proliferative capacity of the hemocytes, we envision that it can also efficiently characterize the proliferative responses of hemocytes in cancerous conditions like leukemia and solid tumors as well as scenarios of environmental and metabolic stresses during adulthood.

Keywords

Adult *Drosophila*, Hematopoiesis, Proliferation, Macrophage, Infection



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REVISED Amendments from Version 1

The submission has been updated to incorporate the Reviewers' comments and suggestions.

The previous title is modified from "Detecting proliferation of adult hemocytes in *Drosophila* by BrdU incorporation" to "Detecting proliferation of adult hemocytes in *Drosophila* by BrdU incorporation and PH3 expression in response to bacterial infection"

The two new aspects added to the previous Title are 'Infection' and 'PH3 expression'. This has been done keeping in mind a suggestion from one of the Reviewers and also to include the new assay that we have done in response to Reviewers' comments.

As far as the text is concerned:

1. In the revised version we have added the PH3 assay: the strategy, experiment and outcome. Details regarding the experimental evidence has now been incorporated to endorse mitotically active (PH3⁺) hemocytes in response to bacterial challenges in adult fly. The discussion has also been strengthened.

Following are the changes in the Figures of revised manuscript based on Reviewers' input or suggestions:

- Figure 1: C and D and Figure 3: B-B" from the earlier version are removed.
- Figure 2: Panel J-P' is added in the revised version to show the adult fly bleeding procedure.
- Figure 4: B-B'" is added to this figure to show the mock infection result.
- Since we have performed PH3 labelling of adult hemocyte post infection, we now have included a complete new figure (Figure 6) to demonstrate the response.

See referee reports

Introduction

Drosophila hematopoiesis generates three kinds of mature differentiated blood cell types: plasmatocytes, crystal cells, and lamellocytes which are responsible for phagocytosis, melanization and encapsulation respectively (Crozatier & Meister, 2007; Evans *et al.*, 2003; Lebestky *et al.*, 2000; Meister, 2004; Parsons & Foley, 2013). Amongst the three, plasmatocytes are the major cell type which acts as "professional macrophages" and consists of ~95% of total hemocyte population. Detail investigations on the behaviour of *Drosophila* macrophages revealed that they are multitaskers (Brückner *et al.*, 2004; Bunt *et al.*, 2010; Dimarcq *et al.*, 1997; Evans *et al.*, 2010; Franc *et al.*, 1996; Tucker *et al.*, 2011; Wood *et al.*, 2006). In all developmental stages they are actively involved in tissue morphogenesis (by secreting ECM proteins), engulfment of apoptotic cells, incessant surveillance against foreign infection and wound healing (Wood & Jacinto, 2007). During immune surveillance, along with phagocytosis of pathogen, the hemocytes also secrete antimicrobial peptide (AMP) to combat infection (Dimarcq *et al.*, 1997). Hemocytes play a crucial role in inter-organ communication by secreting cytokine-like Unpaired (Upd) to activate JAK/STAT signalling as well as Spatzle (Spz), a pro-inflammatory cytokine to activate Toll signalling in fat body for AMP production (Agaïsse *et al.*, 2003; Kounatidis & Ligoxygakis, 2012; Shia *et al.*, 2009). The embryonic hemocytes after egg hatching divide and populate the larval hemolymph. These macrophages switch

to a proliferative state in the larval circulation and increase their number (Holz *et al.*, 2003; Leitão & Sucena, 2015; Makhijani *et al.*, 2011). In addition to the above population, larval hemocytes migrate into specific immunological sites present in the dorsal and lateral side of larval cuticle (Makhijani *et al.*, 2011; Makhijani *et al.*, 2017; Márkus *et al.*, 2009). The circulating hemocytes are guided by peripheral nervous system to home into the hematopoietic pockets where activin- β signaling regulates their proliferation (Makhijani *et al.*, 2011; Makhijani *et al.*, 2017). This proliferative property of plasmatocytes, apart from helping them to increase their number, facilitates their transdifferentiation to lamellocytes during wasp infection (Anderl *et al.*, 2016).

Interestingly, a separate source of larval hemocyte arises from the *Drosophila* blood-forming organ: lymph gland. Within this organ, the hemocytes proliferate and increase the organ size during larval development (Jung *et al.*, 2005). At the time of pupation, the mature lymph gland ruptures and releases the hemocytes into circulation (Grigorian *et al.*, 2011; Jung *et al.*, 2005; Lebestky *et al.*, 2000). Although proliferation in hemocytes is in abundance in immature stages, it is not detectable in the adult fruit fly. This observation led to the proposition that adult hemocytes are in a state of senescence (Honti *et al.*, 2014).

The identification and characterization of Hematopoietic Hub in *Drosophila* (Ghosh *et al.*, 2015; Ramond *et al.*, 2015) clearly revealed that the process of new blood cell formation continues even in the adult stage and it seems to be relevant for combating microbial infection. The adult hematopoietic hub, positioned at the dorsal side of the heart tube houses hemocytes nested in an intricate network of extracellular matrix proteins like Pericardin and Laminin A. Hemocytes from both embryonic and larval lineages home into this dorsal abdominal site. In addition to differentiated cells homing in, several hematopoietic progenitor cells arrive in the hub, which subsequently employs Notch signaling to differentiate into mature plasmatocytes and crystal cells. Moreover, the study also demonstrated that the adult macrophages which were thought to be in senescence can still proliferate in response to a high dose of infection (Ghosh *et al.*, 2015). Here, we describe a detailed step-by-step protocol to assay the proliferative capacity of adult hemocytes by BrdU incorporation and PH3 expression in response to high dose of bacterial infection.

Methods

Reagents

- 1X Phosphate buffered saline (1X PBS)
- 5-bromo-2'-deoxyuridine (BrdU) (Sigma Cat. No. B5002)
- Paraformaldehyde (Sigma Cat. No. P6148)
- Sodium Deoxycholate (Sigma Cat. No. D6750)
- TritonX 100 (Merck Cat. No. MB031)
- Bovine serum albumin (BSA) (Sigma Cat. No. A7906)
- Sodium azide (Sigma Cat. No. S2002)
- Hydrochloric acid (HCL) (Merck Cat. No 101834)

- Colchicine (Sigma Cat. No. C9754)
- Phospho Histone H3 (Cell Signalling, Cat. No. 9713, RRID: AB_823532)
- Monoclonal Anti-Green Fluorescent Protein (GFP) antibody produced in mouse clone GFP-20, ascites fluid (Sigma Cat. No. G6539, RRID: AB_259941)
- Rat monoclonal anti-BrdU antibody [BU1/75 (ICR1)], (Abcam Cat. No ab6326, RRID: AB_305426)
- FITC conjugated Goat anti-mouse antibody (Jackson Immuno Research Cat. No. 115-095-166, RRID AB_2338601)
- Cy3 conjugated Donkey anti-rat antibody (Jackson Immuno Research Cat. No. 712-165-153, RRID: AB_2340667)
- Cy3 conjugated Donkey anti-rabbit antibody (Jackson Immuno Research Cat. No.711-165-152)
- Vectashield (Vector Laboratories, Cat. No. H-100)
- *HmlGal4.UAS GFP* flies (BDSC Cat# 30140, RRID: BDSC_30140)
- *Escherichia coli* (DH5 α) expressing RFP (pFPV25.1 RFP plasmid)

Equipment

- Fly food vials
- Dissecting microscope (Carl Zeiss Stemi 2000)
- Orbital shaker (New Brunswick Scientific Excella E5 Platform Shaker)
- Glass slide (Blue Star Micro Slides PIC-1, size: 75X25mm, thickness: 1.35mm)
- Cover glass (Blue Star Microscopic Cover Glass, Size: 24X24mm)
- Glass capillaries (Sutter Instruments)
- Fine surgical Forceps (Local surgical store)
- Fine surgical Scissors (Local surgical store)
- Dissecting needles
- Pipetman
- Pipette tips
- Moist chamber
- Nail polish
- 4°C Refrigerator
- Confocal microscope (Carl Zeiss LSM 780 & Leica SP8)
- Imaging software ([Image J](#), RRID:SCR003070, [Photoshop CS3](#), RRID: SCR002078 and [Bitplane Imaris 64X](#), RRID: SCR007370)

BrdU feeding of adult fly. To assay the cell proliferation in adult hemocytes, a synchronized collection of freshly eclosed

adult flies is collected. The feeding protocol is adapted and further modified from the study ([Micchelli & Perrimon, 2006](#)). (A minimum number of 40-adult flies per set of experiment is used).

1. 3rd-day old adult flies (+;*hmlGal4-UASGFP*;) are transferred from normal food to cornmeal yeast fly food (2ml volume) which is supplemented with 200 μ l of 6mg/ml BrdU. (*BrdU solution freshly prepared in PBS*).
2. The flies are then reared for two days in the BrdU containing food. [Incorporation of Bromodeoxyuridine (BrdU), a thymidine analog is an established assay for determining cell proliferation in different organisms. Replicating cells during their S phase of the cell cycle readily incorporates BrdU instead of thymidine. (*Rearing flies in BrdU food generates a pool of BrdU in the system that increases the chance of incorporation in cells especially the ones that undergo rare cell division*). The BrdU incorporation can be detected by commercially available anti-BrdU antibody].
3. Post two days of rearing in the BrdU supplemented food, flies are infected with *E.coli* by following the procedure as mentioned below.

Bacterial infection of the adult fly.

1. The sharp end of the capillary is dipped in a colony of *Escherichia coli* expressing RFP (pFPV25.1 RFP plasmid) ([Figure 1B](#)).
2. Using this fine glass capillary, lateral side of the thorax of a pre-anesthetized adult fly is pierced ([Figure 1A–D](#)).
3. Infected flies are re-transferred in BrdU supplemented food and reared for five days before dissection. In between two intermittent flips are required in fresh BrdU food. [*Post infection BrdU feeding is important to maintain the level of BrdU, which increases the chance of incorporation in the slowly dividing hemocytes in response to bacterial infection. Two consecutive flips in fresh BrdU food ensures a constant source of BrdU and maintains healthy fly culture*]
4. The flies are dissected and immuno-stained using anti-GFP (to mark the hemocytes: *hml-GFP*), and anti-BrdU antibody to visualize the replicating cells. Co-localization of GFP and anti-BrdU antibody expression will ensure that the replicating cell is indeed a hemocyte.

As a control experiment, a mock infection was performed. The thorax of adult flies are pierced with fine glass capillary dipped in sterile 1X PBS (similar to point 2) and the flies are then subjected to all the steps similar to the experimental flies.

Adult fly dissection for Hematopoietic Hub isolation. BrdU fed adult flies are anesthetized and dissected carefully using fine scissors and forceps in ice-cold 1X PBS (pH 7.2).

1. The flies are positioned dorsal side down in a drop of PBS placed on a clean glass slide ([Figure 2A](#)).

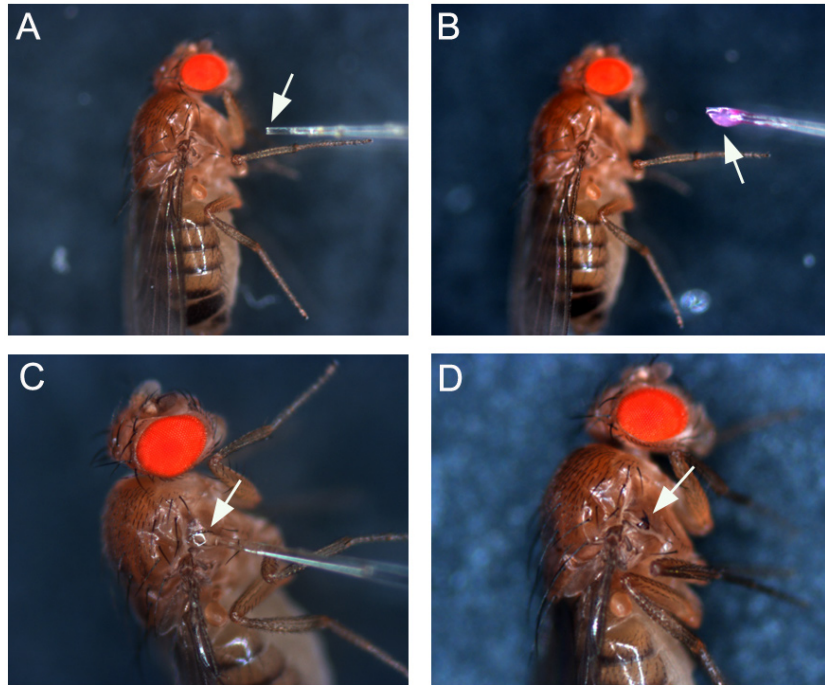
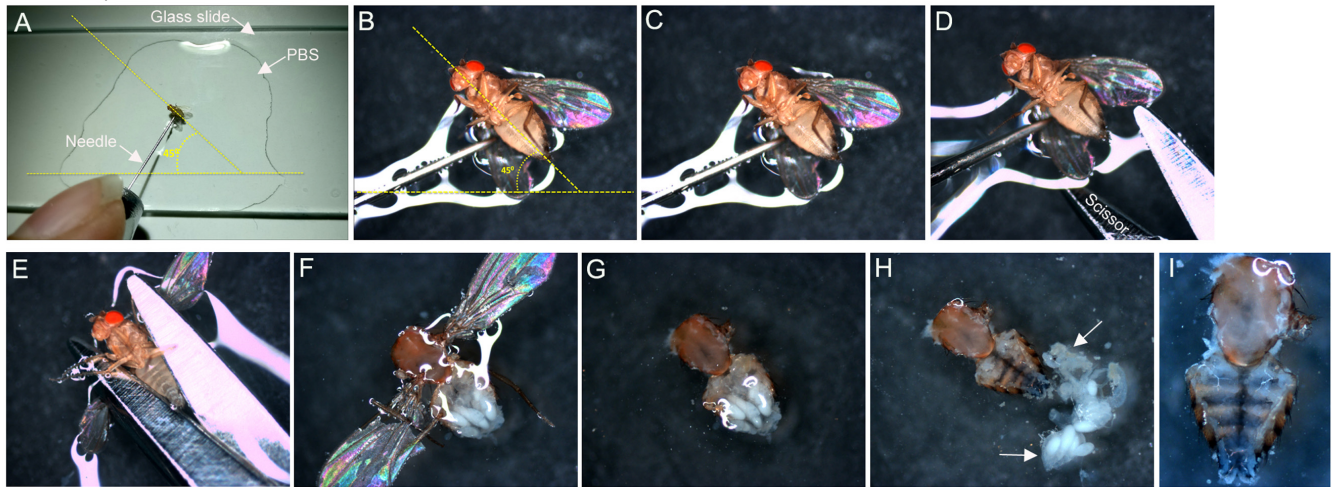


Figure 1. Infection procedure in adult *Drosophila*. (A) A fine glass capillary with a pointed tip (arrow) is used to infect an anesthetized adult fly. (B) The image shows a colony of RFP expressing *E.coli* picked up at the tip of the capillary (arrow). (C) The lateral side of the thoracic region is pierced with the fine capillary. Arrow shows the position at which the capillary is inserted in the thorax. (D) 24 hour post infection; the site of insertion in the thorax can be detected by the presence of a black melanised spot.

2. Using two needles, the wings are stretched apart so that the dorsal abdomen comes in contact with the PBS surface (Figure 2B–C). It is very important to ensure that dorsal half of the abdomen is submerged in PBS. [This step is crucial as the wings are hydrophobic in nature, therefore, they obstruct the dorsal abdomen of the fly from dipping into the PBS].
 3. A clean scissor, rinsed in alcohol, is used for dissecting the fly from ventral side (Figure 2D–E) (Dissection is done from the ventral side as the hematopoietic hub is positioned on the dorsal side of the abdomen).
 4. The fly is placed at an angle of 45°. In order to hold the fly in that position, a dissecting needle from one side is placed on the stretched wing (Figure 2A–C) and a scissor is aligned parallel to the fly (as shown in Figure 2D). A fine incision is then made from the posterior tip of the fly abdomen ventrally (i.e. anus and vaginal plate) and continued up to the head (Figure 2E). While doing so, care should be taken to ensure that the pale non-pigmented ventral part of the abdomen is detached from rest of the fly.
 5. During this procedure, the head automatically gets disengaged from the rest of the body. The dissected fly body part consists of the dorsal side of the thorax and abdomen (Figure 2F–G).
 6. The wings are then removed with help of a sharp scissor. While the thorax is retained for holding the tissue for the entire duration of the staining procedure. [This ensures that the hub hemocytes in the abdomen remain undisturbed].
 7. The tissues present inside the abdominal cavity (like gut, ovaries, malpighian tubules) are gently removed while the dorsal abdominal diaphragm is kept untouched (Figure 2H). This dorsal abdominal diaphragm composed of fat body layers, which are tightly connected with alary muscles, heart muscle, and pericardial cells. All of these in some way contribute to maintain the structural integrity of the Hub. [Therefore, in this entire process of dissection, it has to be ensured that the sharp ends of needles and scissors should not poke the dorsal side of the abdomen. Otherwise, this might dismantle the Hematopoietic Hubs, thereby interfering with the analyses].
 8. The samples are now ready for immunostaining (Figure 2I).
- Immunostaining of adult hematopoietic hub.** Please note all incubation and washings are done at room temperature unless otherwise mentioned.

Dissection procedure



Bleeding procedure

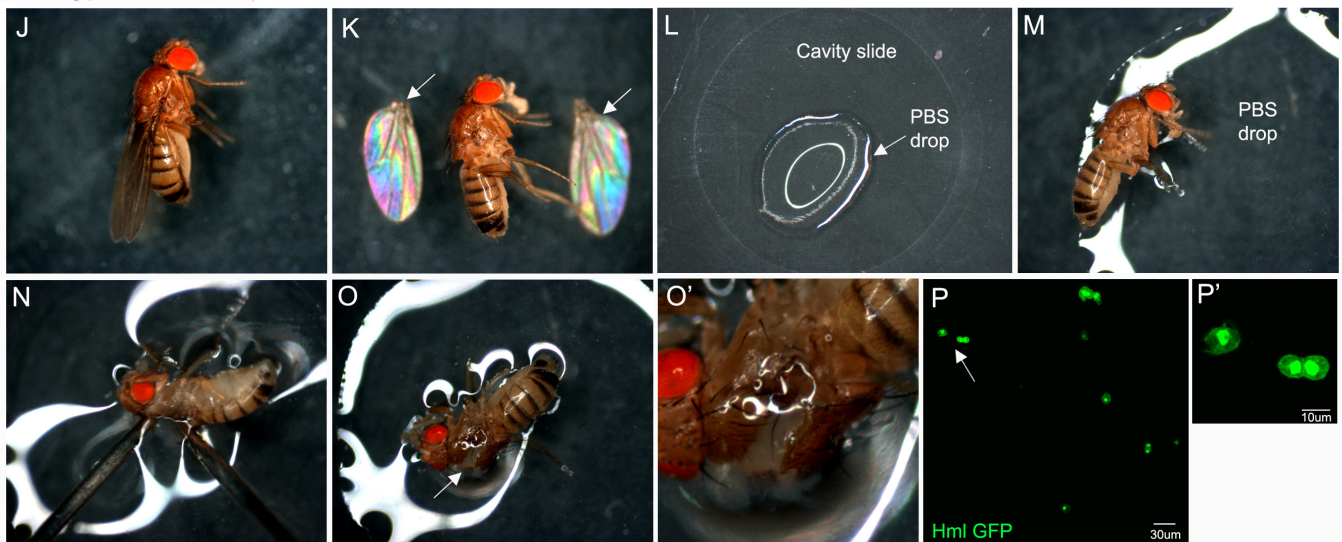


Figure 2. Adult fly dissection procedure to isolate the hematopoietic hub and adult fly bleeding procedure. (A) The anesthetized adult fly is placed on a clean glass slide at an angle of 45° (held by a dissecting needle). (B–C) The higher magnification shows the exact position of the adult fly. The fly wings are stretched apart so that the dorsal surface of the abdomen is submerged in the PBS solution. (D) A fine surgical scissor is placed parallel to the fly and (E) An incision is made with the scissor from the posterior tip of the anus and vaginal plate running ventrally to the anterior end of the fly. (F) The ventral side of abdomen, legs and the head are removed carefully. The dissected sample consists of only the dorsal side of the thorax and the abdomen (G) The wings are removed with the help of needles. (H) The tissues (gut, ovaries, malpighian tubules) present inside the abdomen are gently removed (arrow) without disturbing the dorsal diaphragm. (I) Once the dissection is complete, the sample containing the dorsal side of the thorax and exposed abdomen is processed for immunostaining. (J) Anesthetised adult fly is placed on a clean glass slide. (K) The wings (arrow) are removed with the help of scissor. (L) A drop of 20µl PBS is placed inside a cavity slide. (M) The fly from K is now placed in the drop of PBS. (N) The thorax is opened with the two fine needles. (O) Hemolymph oozes out from the exposed region of the thorax and gets collected in the drop of PBS. (O') Higher magnification of (O) shows the exposed thoracic region. (P–P') A snapshot of the bleed containing Hml GFP expressing hemocytes.

1. The dissected samples are fixed in freshly prepared 4% para-formaldehyde for 45 min in 1X PBS on a shaker.
2. Post fixation the samples are washed thrice in 1X PBS for 10 min each followed by 30 min incubation in 0.3%PBT + 0.3% Sodium Deoxycholate on a shaker
3. The permeabilized tissues are incubated in freshly prepared blocking solution (10% bovine serum albumin [BSA] in 1X PBS) overnight at 4°C.

(0.3%PBT = 0.3% TritonX 100 in 1X PBS.) [Both Triton X100 and Sodium Deoxycholate are used for permeabilization of the membrane].

4. Post blocking, samples are incubated in primary antibody (mouse anti-GFP, 1:50) diluted in 1X PBS for 45hr at 4°C (primary antibody is supplemented with 1µl of 0.02% Sodium azide). [Addition of *sodium azide reduces the chance of infection during this long duration of antibody incubation*].
5. Samples are then washed thrice in 1X PBS for 10 min each and subsequently incubated in blocking solution on a shaker.
6. The blocking solution is replaced by Secondary antibody (anti-mouse FITC, 1:400, diluted in 1XPBS) and the tissues are incubated for 45hr at 4°C. (Henceforth, all incubations are carried out in a dark chamber).
7. Three washes in 1X PBS of 10 min each are done post secondary antibody incubation.
8. 1X PBS is aspirated out and the tissues are briefly treated with 0.3%PBT + 0.3% Sodium deoxycholate for 15 min [The brief wash ensures efficient permeabilization]. This step is followed by a wash in 1X PBS for 10 min to remove the detergents.
9. The tissues are then re-fixed with 4% para-formaldehyde for 20 min on a shaker.
10. Post-fixation samples are washed thrice with PBS for 10 min each.
11. For depurination step, PBS is replaced by 2N HCl (freshly prepared in PBS) and the sample is first incubated for 10 min on a shaker followed by 30min incubation without shaking. [HCl treatment denatures the DNA and thus allows the anti-BrdU antibody access to the BrdU within the DNA. As HCl treatment is harsh for the cells, therefore, they were subjected to a minimum shaking time.].
12. Traces of HCl is removed by one quick and two 10min washes in 1X PBS.
13. Samples are then blocked in 10% BSA for an hour followed by incubation in rat anti-BrdU primary antibody (1:100, prepared in 1X PBS) for 45hr at 4°C.
14. Three washes in PBS are done before adding the secondary antibody (anti-rat Cy3, 1:400) for 45hr at 4°C.
15. Post incubation two gentle PBS washes of 10 min each are done.
16. To visualize the nucleus of cells, the tissues are incubated in DAPI solution (prepared in 1X PBS) at 4°C for overnight followed by washing twice in PBS (10 min each).
17. Finally, samples were mounted in mounting media (Vectashield).

Colchicine feeding of adult fly and PH3 staining. Colchicine is known to interfere with cell division by inhibiting microtubule polymerization and therefore an exposure to it is sufficient enough to arrest cells in mitosis (Taylor, 1965; Viktorinová & Dahmann, 2013).

To assay the rare event of adult hemocyte proliferation by Phospho-histone 3 expression, colchicine is administered through food. A suitable exposure to colchicine induces accumulation of metaphase-blocked mitoses. Phospho-histone H3 (PH3), an immunomarker specific for cells undergoing mitoses was employed to track the proliferative hemocyte.

Colchicine administration results mitotic arrest that facilitated the chances of marking the proliferative hemocytes with PH3, post bacterial infection.

A synchronised collection of freshly eclosed flies are collected.

1. 5th day old adult flies (+;hmlGal4-UASGFP;+) are transferred from normal food to cornmeal yeast fly food (2ml volume) which is supplemented with freshly prepared Colchicine (in water, final concentration 1mg/ml in food).
2. The flies are reared for 6hrs in this Colchicine containing food. [Colchicine is an alkaloid which blocks the microtubule polymerisation in cells and thus arrests the dividing cells in different stages of mitosis]
3. Flies are then infected with *E.coli* by following the procedure as mentioned above section ‘Bacterial infection of adult fly’.
4. Infected flies are re-transferred in Colchicine supplemented food and reared for 18hrs before dissection. [Post infection Colchicine feeding is important to maintain the level of Colchicine which increases the chance of mitotic arrest of these rarely dividing hemocytes in response to bacterial infection.]
5. The flies are dissected and immuno-stained by following the above-mentioned section ‘Immunostaining of adult hematopoietic hub’ (follow point number 1-7) where primary and secondary antibody used anti-PH3 antibody, 1:100 and anti-rabbit Cy3, 1:400 respectively (to mark *phospho-histone 3 expression*). Co-localization of *hml GFP* and anti-PH3 antibody expression ensured that the cell is certainly a proliferating adult hemocyte.

Mounting of adult fly samples. The mounting steps of the adult abdominal samples are critical for successful observation of adult Hematopoietic Hub. Before mounting, trimming of the cuticle on either side of the abdomen is required. This cuticle along the edges otherwise attributes unwanted thickness to the tissue.

1. A drop of 1X PBS is taken on a clean glass slide. As described previously in the “dissection” section, the

sample is placed at an angle of 45° and thorax and the curved cuticle is removed by applying a very sharp cut along the extreme edge of the abdomen where the curvature initiates.

2. After trimming both sides of the abdomen, the thorax is removed very carefully. [*The 1st hematopoietic hub along with the conical chamber of the heart is tightly associated with a thoracic-abdominal junction, therefore this step is extremely crucial*].
3. Samples are next transferred on a clean slide containing a drop of Vectashield and incubated for 15–20 min. [*Incubation of samples inside dense Vectashield is an essential step to reduce the opacity observed due to aqueous layer associated with a thick layer of the fat body which makes deep tissue imaging under the microscope a bit challenging*].
4. Finally, the tissues are arranged in a row and a coverslip is gently placed on them. In order to prevent drying, edges of the cover slip is sealed with a transparent nail-polish. The mounted samples are ready for immediate imaging or can be stored in 4°C.

Fly Bleeding.

1. To assay the cell proliferation in adult circulatory hemocytes, a batch of synchronously eclosed adult flies are reared in BrdU containing food before and after the bacterial infection, following the above-mentioned procedure (Section: ‘*BrdU feeding of adult fly*’).
2. The synchronized culture of (+;*hml Gal4- UAS GFP*;+) female flies are anesthetized and the wings are removed using fine scissors (Figure 2J–K).
3. The fly is then placed in a drop of 1X PBS (20µl) on a clean, pre chilled glass cavity slide (Figure 2L–M). [*Chilled glass slide minimizes the evaporation of the small amount of PBS*]
4. With the aid of two needles, a fine incision is made on the lateral side of the thorax without disturbing the adult abdomen proper. The exposed region of the thorax is carefully positioned in the drop of PBS for 20 seconds in order to collect the hemolymph along with blood cells (Figure 2N–P’). (*A total of 8 flies are taken to bleed in a drop of 20µl PBS*).
5. The hemocytes are allowed to adhere to the glass surface for 20 min inside a moist chamber.
6. Fixation of the hemocytes is done by adding freshly prepared 4% para-formaldehyde for 30min inside a moist chamber.
7. After three washes in 1X PBS for 5min each, 0.3%PBT + 0.3% Sodium Deoxycholate is added on the hemocytes for 5 min. [*For permeabilization of the membranes*]
8. Sodium Deoxycholate is removed by two washes in 1X PBS for 10min each.
9. The cells are then incubated in the blocking solution (10% bovine serum albumin (BSA) prepared in 1X PBS) for 30min.
10. The blocking solution is replaced by the primary antibody (mouse anti-GFP, 1:50, diluted in 1X PBS). Incubation is done for 18hr at 4°C. Cells are then washed thrice in 1X PBS for 5min each.
11. Secondary antibody (anti-mouse FITC, 1:400) incubation is carried out in dark chamber, for 4hr at RT followed by three 10 min washes in 1X PBS.
12. For depurination step, 2N HCl (freshly prepared in 1XPBS) is added for 20 min without shaking.
13. Two washes in 1XPBS for 10min each is next done to remove traces of HCl.
14. The samples are next incubated in rat anti-BrdU primary antibody (1:100), prepared in 1X PBS for 4hr.
15. Post incubation, two washes in 1XPBS for 10min is carried out before blocking it with 10% BSA block for 30min.
16. Secondary antibody anti-rat Cy3 (1:400) is added and incubated for 4hr. This was followed by two-three washes in PBS for 10min each.
17. The nucleus of cells is next labeled with DAPI. (A incubation in DAPI solution for 10min followed by two washes of 10 min each in 1X PBS was done).
18. Samples are mounted in mounting media Vectashield for immediate imaging.

Imaging.

1. The circulating hemocytes or hematopoietic hub are imaged using a combination of laser lines 405nm, 488nm, and 561nm in a confocal microscope.
2. Images are further processed in ImageJ and Photoshop software. In ImageJ, the raw confocal data file (.lsm/.LIF) are opened and visualized the combined image (Image>Color>Make composite). Then each channel are analysed by following Image>Color>Channel tool option. Brightness/contrast are adjusted in equal level both in control and experiment following Image>Adjust>Brightness/Contrast. Finally, the images are converted to RGB (Image>Color>Stake to RGB) and saved in TIFF format (File>Save as>Tiff). Photoshop software is used to arrange and prepare the high-resolution figure panels.
3. 3D surface rendered models are generated using Imaris 64X software (7.6 version).

The steps below were followed:

- a. In Imaris, the raw confocal data of multiple Z stacks are opened and visualised in 'Surpass' mode.
- b. Open 'Display Adjustment' window to analyse each color channel (GFP, Cy3, DAPI).
- c. Next, click on to the 'Surface' option from the 'Volume properties' in the menu bar and follow the subsequent three steps (of the algorithm) to create the 3D surface model.
- d. Next step 'Source Channel': select the Channel (GFP/Cy3/DAPI) and click 'smooth' and 'absolute intensity'.
- e. After that is 'Threshold': in this step effort has to be made to ensure that the selected object is perfectly covered by the newly created 3D surface. This depends on the volume observed from the respective fluorescence intensity of the object present in the image file.
- f. Next is 'Classify surface': in this step click and finalise the 3D surface. After finalising the 3D surface selection click on to 'Color' panel and subsequent adjustment of the colour tone, transparency, intensity in the multicolour 3D model can be done according to the interest.
- g. Finally, for saving the 3D model as single image select the 'Snapshot' and for 3D rotation select 'Animation' tool.

Results

Response against infected *E. coli* in adult hemocytes

A. Phagocytic Response. Upon 30 minutes post infection with *E. coli*, the resident hemocytes of the hematopoietic hub are seen actively engaged in phagocytosis of the invading bacteria (Figure 3A–B"). Instances can also be documented where multiple bacteria are phagocytosed by single hemocyte illustrating their rapid response to clear the huge infection load (Figure 3B–B").

Similar behavior is seen from the circulating hemocytes (Figure 3C–E"). Here also, single hemocytes can be seen throwing multiple filopodial extensions to engulf several *E. coli* cells from the infected hemolymph.

B. Proliferative response. Previous attempts to evaluate the cell division potentiality of adult hemocytes, primarily involved infecting the flies and then subjected them to proliferation assay with BrdU. These assays followed normal protocol, which is generally employed to assay proliferation in eye disc or lymph glands (Escudero & Freeman, 2007; Grigorian *et al.*, 2011; Jung *et al.*, 2005). In majority of these methods, dissected tissues are incubated in BrdU solution for 30–60 min so that any cell division happening at that time of incubation will incorporate the modified nucleotide source thereby getting labeled. Employing this strategy no division was detected in plasmatocytes, although, BrdU incorporation was evident in endoreplicating fat cells of both the control and infected adult flies.

Employing the current method, no BrdU incorporation within the hub resident hemocytes is seen in an uninfected individual as well as in mock infection. Although the fat body cells (arrow in Figure 4A,B) which are endoreplicating in nature positively incorporated BrdU (Figure 4A–B").

In contrast, the infected flies showed several BrdU labeled plasmatocytes inside the hematopoietic hub along with nearby fat cells (arrowhead in Figure 4C–C"). The BrdU incorporation was specific to plasmatocyte nucleus was further confirmed by constructing 3D surface modeling at a single cell resolution (Figure 4 C1–F6 and Supplemental Movie S1 and Supplemental Movie S2).

However, employing the similar infection regime and BrdU assay the circulating hemocytes in adult fruit fly failed to incorporate BrdU (Figure 5). While the fat body cells served as positive control for successful BrdU labeling (Figure 5A–A"), hemocytes from both uninfected (Figure 5 B–D") and infected (Figure 5E–G") individuals lacked any incorporation.

The hemocyte proliferation in the hematopoietic hub of the adult was further validated by another independent assay of cell proliferation, the Phospho-histone 3 (PH3) labeling which marks the mitotically active cells only (Aune *et al.*, 2011; Ribalta *et al.*, 2004; Sawicka & Seiser, 2012). The BrdU incorporation assay revealed that the hemocytes undergoing replication in the adult are very less number. In addition to this, the event of replication seems to be random (Ghosh *et al.*, 2015). Therefore, to trace a mitotically active hemocyte in adult fly post-immune challenge is technically difficult. Considering the less duration of M phase compared to S phase in the cell cycle, a strategy was adopted to mark this rare mitotic phase of adult hemocytes. The adult flies were reared in food supplemented with colchicine (1mg/ml) to arrest the rarely dividing cells in mitosis thereby increases the probability of marking the mitotically active hemocytes upon bacterial infection (by PH3 labeling).

Employing the above method, no PH3 expression is seen within the hub resident hemocytes of an uninfected individual. Interestingly, mitotically active plasmatocytes (PH3⁺) can be seen in the hematopoietic hub of the infected individuals (arrowhead in Figure 6A–D")

It is, thus, very clear that the hemocytes present in the hub still retain the capacity of proliferation, whereas employing the same method; we were unable to detect any proliferation in the circulating hemocytes. These observations suggest that the circulating hemocytes in adult fly might have lost the proliferation capacity unlike their larval counterpart (Anderl *et al.*, 2016; Makhijani *et al.*, 2011).

Discussion

Hemocytes play multiple crucial roles in the *Drosophila* immune response be it embryonic or larval stages. The important mechanisms of cellular immune response provided by hemocytes are phagocytosis, encapsulation, melanization, and coagulation (Lemaitre & Hoffmann, 2007). Moreover, during systemic

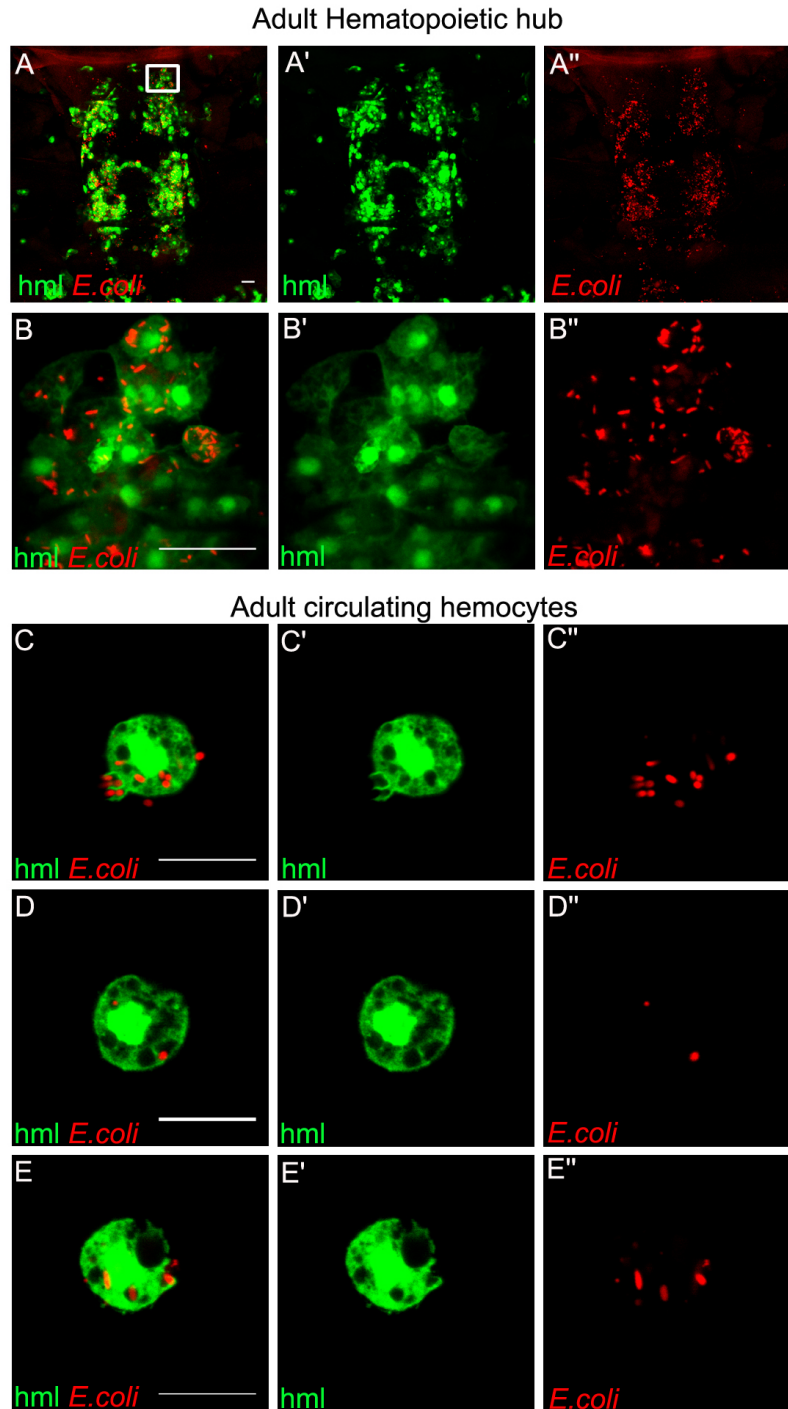


Figure 3. Phagocytic response against infected *E. coli* in hemocytes of hematopoietic hub and circulation. (A–B'') 30 minutes post infection the hub hemocytes (green) shows a strong phagocytic response against the bacteria (red). (B–B'') Higher magnification of A shows majority of the hemocytes has engulfed *E. coli*. Image shows multiple *E. coli* is engulfed by a single hemocyte present in the hematopoietic hub. (C–E'') Multiple examples of circulating hemocytes engaged in phagocytosis of *E. coli* (red). (C–C'') The hemocyte throws filopodial extensions to form phagocytic cup around the *E. coli* prior to phagocytosis. Scale bar: 20µm (A–B''), 10µm (C–E'').

infection the hemocytes produce antimicrobial peptide (AMP) and also communicate with other tissues like fat body via the production of cytokines (Upd3, Spz) and TGF-β signaling (Agaïsse *et al.*, 2003; Dimarcq *et al.*, 1997; Kounatidis & Ligoxygakis, 2012; Shia *et al.*, 2009). *Drosophila* larval stage is a preparatory

phase that enables the late larvae to achieve certain required volume for metamorphosis (Ono, 2014). The robust proliferation of all the major cell types including the macrophages/plasmacytes enables the larvae to reach the required volume. Prior to the identification of hematopoietic hub (Ghosh *et al.*, 2015),

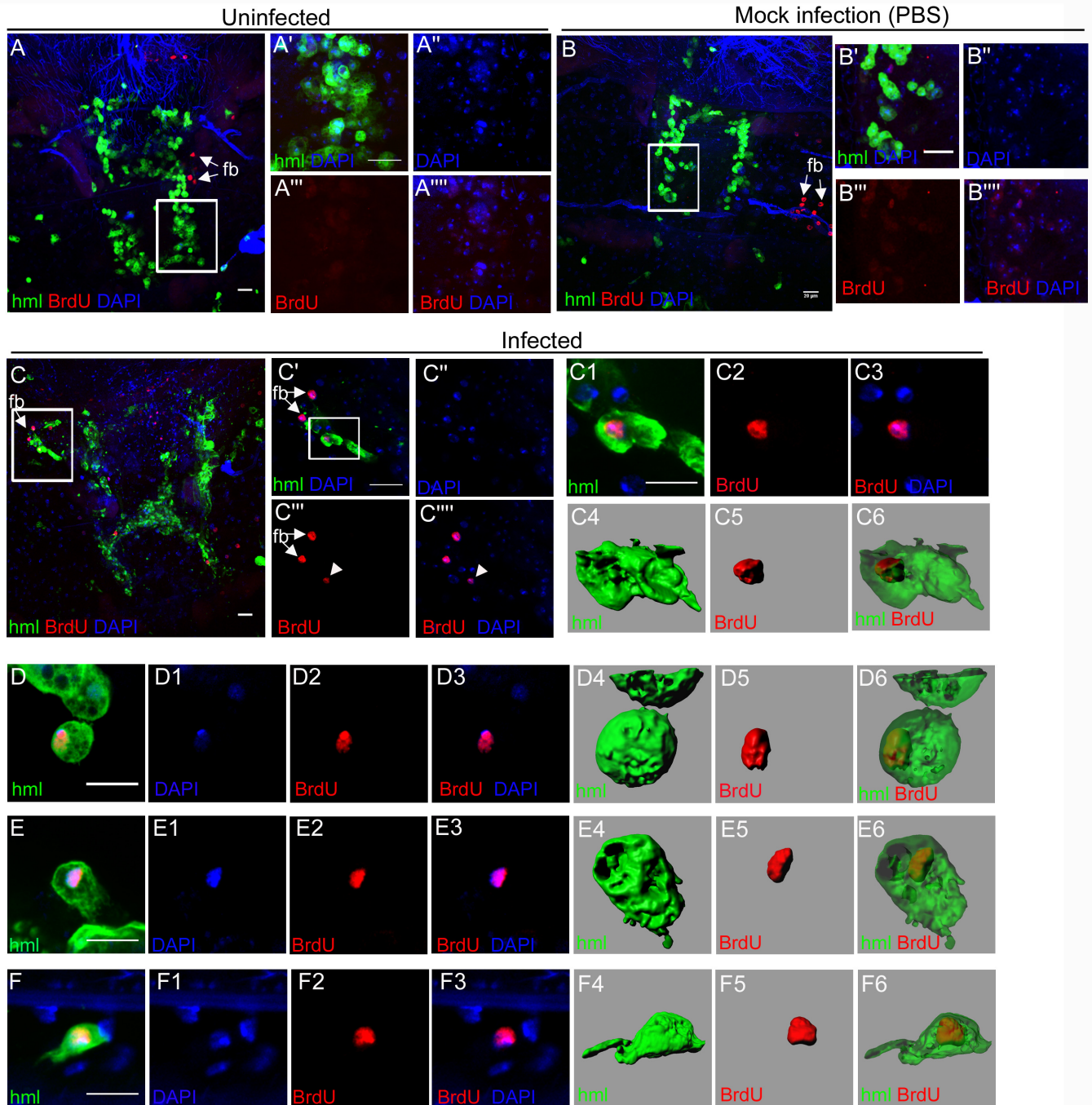


Figure 4. In response to bacterial infection hub hemocytes undergo proliferation. (A–A''') In wild-type, adult fly hemocytes do not incorporate BrdU, while endo-replicating fat body (fb, arrow) cells incorporate BrdU and serves as an uninfected control. (B–B''') Mock infection control set also does not show BrdU incorporation in the hemocytes, fat body cells showing BrdU signal serves as control tissue (arrow). (C–C''') Infected individuals show BrdU incorporation in hub resident hemocytes indicating their proliferative state in response to infection. Higher magnification of C shows hml GFP⁺ BrdU⁺ hub hemocyte (arrowhead) juxtaposed to BrdU positive fat body cells (C'–C'''). (C1–C6) BrdU incorporation in the hemocyte visualized in a single-cell resolution (C1–C3) and 3D surface rendering (of C1) reveals the BrdU signal is present inside the hemocyte. (D–F6) Transparent 3D surface model of individual hub hemocytes shows incorporation of BrdU in nucleus respectively. Scale bar: 20µm (A–C'''), 10µm (C1–F6).

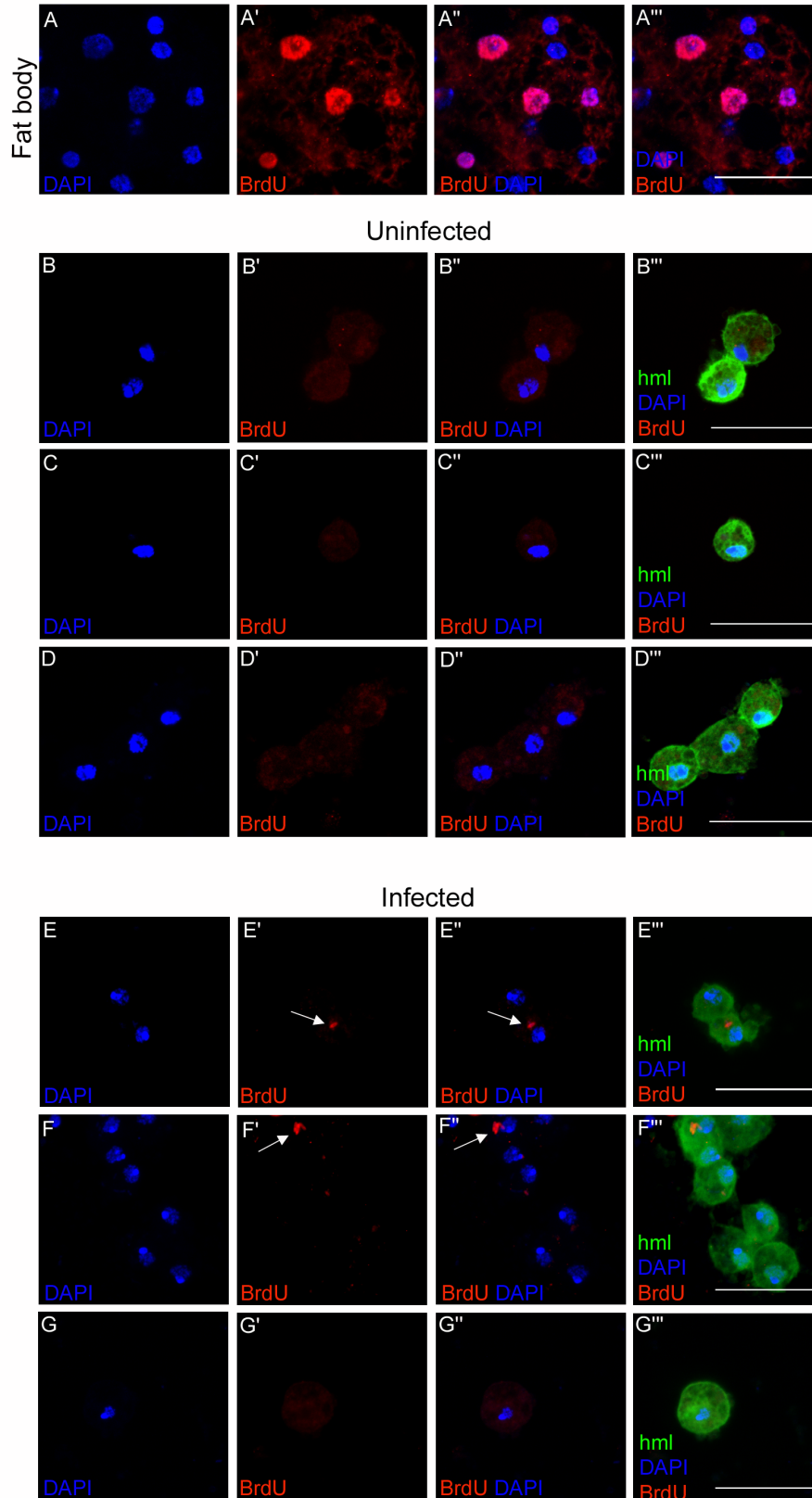


Figure 5. Circulating hemocytes do not show any proliferation. (A– A''') The adult endoreplicating fat body cells act as a positive control as they show BrdU (red) incorporation. (B–D''') The uninfected circulating hemocytes of adult flies are in their non-dividing state thus does not show any BrdU incorporation. (E–G''') Circulating hemocytes from *E. coli* infected Adult fly do not show any BrdU positive cells. Some hemocytes in this figure still retains some *E.coli* cells (tiny red dots present outside nucleus). Scale bar: 20µm.

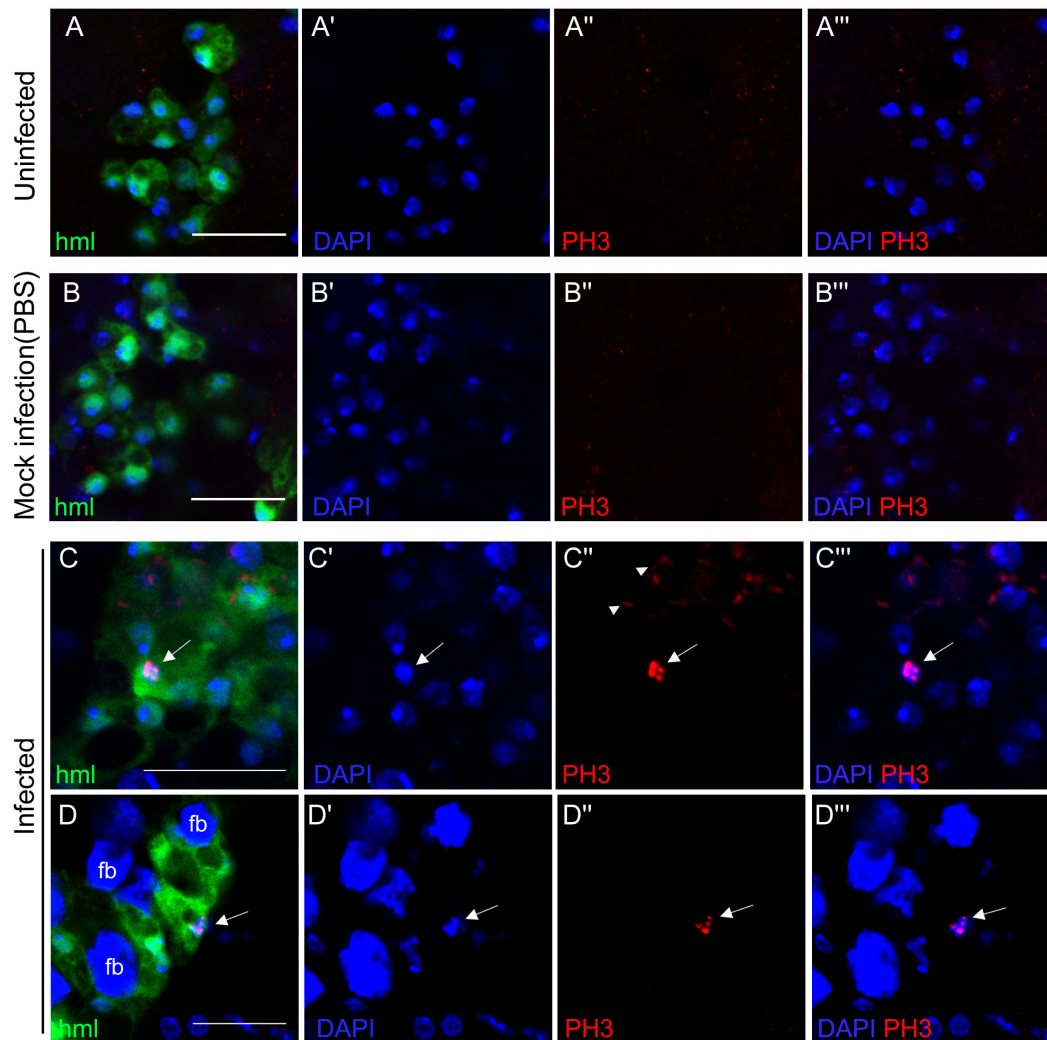


Figure 6. PH3 expression marks the mitotically active adult hemocytes in response to infection. (A–A'') Control uninfected flies do not show PH3 expression in the Hml positive hub resident hemocytes. (B–B'') Mock infection with sterile PBS does not evoke a proliferative response from the hemocytes (PH3⁺). (C–C'') and (D–D'') Two examples from infected adult flies whose hematopoietic hub shows mitotically active (PH3⁺) hemocytes (arrow). Arrowhead marks the hemocytes that have engulfed *E.coli*. Scale bar: 20µm.

it was believed that there is no active site of hematopoiesis in the adult fruit fly (Evans & Banerjee, 2003; Evans *et al.*, 2003; Honti *et al.*, 2014; Wang *et al.*, 2013). Thus, it was thought that hemocytes from embryonic and larval lineages constitute the adult blood cell repertoire (Holz *et al.*, 2003). It was further inferred that these hemocytes from earlier stages of development lose their proliferative capacity and enter into senescence in adulthood (Honti *et al.*, 2014).

However, if adult fly is unable to produce new blood cells how do they survive basic hazards of life like a bacterial infection that requires a quick and spontaneous immune response? In vertebrates, such threat is tackled by different type of blood cells that get routinely regenerated. In case of bacterial infection the response of the hematopoietic hub is akin to the sessile patches

of the larvae. Within first few hours of infection in larvae, the number of hemocytes in the sessile patches are significantly reduced compared to control (Márkus *et al.*, 2009). A similar response is seen in the case of the hub resident hemocytes (Ghosh *et al.*, 2015). Studies on the adult mosquito, on the other hand, reveal that indeed hemocytes can proliferate upon bacterial infection to increase their number (King & Hillyer, 2012; Sigle & Hillyer, 2016). This raised the possibility that maybe this is true for *Drosophila* adult too. Due to the limitation of the previous protocols, maybe we are missing the phenomenon. Thus, we attempted to come up with an alternate protocol of proliferation assay sensitive enough to document such an event.

The current protocol is efficient and successful in unraveling the proliferation capacity of hemocytes in adult fly which was

previously unappreciated. Although the senescence is prevalent in hemocytes of adult, with this efficient method we have been able to identify the rare proliferation events that can be encountered upon bacterial infection. This, in turn, has led to a new understanding that the hemocyte within the hub has not completely lost their proliferative capacity.

Remarkably, with the same BrdU feeding and infection regime, the circulating hemocytes do not demonstrate any proliferative activity. This contrasting observation evokes few interesting possibilities of hemocyte behaviour in response to infection. First possibility is that the hub resident hemocytes post infection can only undergo proliferation. Secondly, after detecting an infection, hemocytes from circulation migrate to the hub and proliferate there. In that case, the hub environment is permissive and mandatory for proliferation. However, there is also a slim chance that soon after infection, a rare fraction of hub resident hemocytes that undergo proliferation in the hub subsequently enters into circulation. The reason why we are unable to detect them with the same protocol might be that the *ex vivo* bleeding technique does not assure 100% analyses of the entire pool of circulating hemocytes. However, the current protocol is sensitive enough to detect the rare events of proliferation happening in the hub.

Thus, the protocol has helped us to differentiate the hub resident hemocytes from the ones in circulation based on their proliferative capacity or the rigidity of senescence.

We envisage that this protocol can also be used to characterize the proliferative responses of hemocytes in various cancerous fly model (leukemia or tumors) (Dearolf, 1998; Levinson & Cagan, 2016; Petkau *et al.*, 2017; Tipping & Perrimon, 2014), viral infections (Tassetto *et al.*, 2017) as well as

scenarios of environmental and metabolic stresses during adulthood (Dionne, 2014).

Data availability

The data underlying this study is available from OSF. Dataset 1: Wellcome Open Research Manuscript 14560: Detecting proliferation of adult hemocytes in *Drosophila* by BrdU incorporation

DOI <http://doi.org/10.17605/OSF.IO/8V9XE> (Mandal, 2018)

Data is available under CC0 1.0 Universal

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in in study design, data collection, preparation and publishing of the protocol

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Supplementary material

Movie S1: Two examples illustrating that the BrdU incorporation is indeed specific to the hemocyte nucleus.

[Click here to access the data.](#)

Movie S2: Two more evidences illustrating that the BrdU signal seen within the hemocytes are not a part of any cell that might have been engulfed by the macrophage rather the hemocyte nucleus is positive for it.

[Click here to access the data.](#)

References

Agaisse H, Petersen UM, Boutros M, *et al.*: Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. *Dev Cell.* 2003; 5(3): 441–450.
[PubMed Abstract](#) | [Publisher Full Text](#)

Anderl I, Vesala L, Ihalainen TO, *et al.*: Transdifferentiation and Proliferation

in Two Distinct Hemocyte Lineages in *Drosophila melanogaster* Larvae after Wasp Infection. *PLoS Pathog.* 2016; 12(7): e1005746.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Aune G, Stunes AK, Tingulstad S, *et al.*: The proliferation markers KI-67/MIB-1,

- phosphohistone H3, and survivin may contribute in the identification of aggressive ovarian carcinomas. *Int J Clin Exp Pathol*. 2011; 4(5): 444–453.
[PubMed Abstract](#) | [Free Full Text](#)
- Brückner K, Kockel L, Duchek P, *et al.*: The PDGF/VEGF receptor controls blood cell survival in *Drosophila*. *Dev Cell*. 2004; 7(1): 73–84.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Bunt S, Hooley C, Hu N, *et al.*: Hemocyte-secreted type IV collagen enhances BMP signaling to guide renal tubule morphogenesis in *Drosophila*. *Dev Cell*. 2010; 19(2): 296–306.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Crozatier M, Meister M: *Drosophila* haematopoiesis. *Cell Microbiol*. 2007; 9(5): 1117–1126.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Dearolf CR: Fruit fly “leukemia”. *Biochim Biophys Acta*. 1998; 1377(1): M13–23.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Dimarcoq JL, Imler JL, Lanot R, *et al.*: Treatment of I(2)mbn *Drosophila* tumorous blood cells with the steroid hormone ecdysone amplifies the inducibility of antimicrobial peptide gene expression. *Insect Biochem Mol Biol*. 1997; 27(5): 877–886.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Dionne M: Immune-metabolic interaction in *Drosophila*. *Fly (Austin)*. 2014; 8(2): 75–79.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Escudero LM, Freeman M: Mechanism of G1 arrest in the *Drosophila* eye imaginal disc. *BMC Dev Biol*. 2007; 7: 13.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Evans CJ, Banerjee U: Transcriptional regulation of hematopoiesis in *Drosophila*. *Blood Cells Mol Dis*. 2003; 30(2): 223–228.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Evans CJ, Hartenstein V, Banerjee U: Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. *Dev Cell*. 2003; 5(5): 673–690.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Evans IR, Hu N, Skaer H, *et al.*: Interdependence of macrophage migration and ventral nerve cord development in *Drosophila* embryos. *Development*. 2010; 137(10): 1625–1633.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Franc NC, Dimarcoq JL, Lagueux M, *et al.*: Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells. *Immunity*. 1996; 4(5): 431–443.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Ghosh S, Singh A, Mandal S, *et al.*: Active hematopoietic hubs in *Drosophila* adults generate hemocytes and contribute to immune response. *Dev Cell*. 2015; 33(4): 478–488.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Grigorian M, Mandal L, Hartenstein V: Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the *Drosophila* lymph gland. *Dev Genes Evol*. 2011; 221(3): 121–131.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Holz A, Bossinger B, Strasser T, *et al.*: The two origins of hemocytes in *Drosophila*. *Development*. 2003; 130(20): 4955–4962.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Honti V, Csordás G, Kurucz É, *et al.*: The cell-mediated immunity of *Drosophila melanogaster*: hemocyte lineages, immune compartments, microanatomy and regulation. *Dev Comp Immunol*. 2014; 42(1): 47–56.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Jung SH, Evans CJ, Uemura C, *et al.*: The *Drosophila* lymph gland as a developmental model of hematopoiesis. *Development*. 2005; 132(11): 2521–2533.
[PubMed Abstract](#) | [Publisher Full Text](#)
- King JG, Hillyer JF: Infection-induced interaction between the mosquito circulatory and immune systems. *PLoS Pathog*. 2012; 8(11): e1003058.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kounatidis I, Ligoxygakis P: *Drosophila* as a model system to unravel the layers of innate immunity to infection. *Open Biol*. 2012; 2(5): 120075.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lebestky T, Chang T, Hartenstein V, *et al.*: Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. *Science*. 2000; 288(5463): 146–149.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Lemaitre B, Hoffmann J: The host defense of *Drosophila melanogaster*. *Annu Rev Immunol*. 2007; 25: 697–743.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Leitão AB, Sucena É: *Drosophila* sessile hemocyte clusters are true hematopoietic tissues that regulate larval blood cell differentiation. *eLife*. 2015; 4: e06166.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Levinson S, Cagan RL: *Drosophila* Cancer Models Identify Functional Differences between Ret Fusions. *Cell Rep*. 2016; 16(11): 3052–3061.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Makhijani K, Alexander B, Rao D, *et al.*: Regulation of *Drosophila* hematopoietic sites by Activin- β from active sensory neurons. *Nat Commun*. 2017; 8: 15990.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Makhijani K, Alexander B, Tanaka T, *et al.*: The peripheral nervous system supports blood cell homing and survival in the *Drosophila* larva. *Development*. 2011; 138(24): 5379–5391.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Mandal L: Wellcome Open Research Manuscript 14560: Detecting proliferation of adult hemocytes in *Drosophila* by BrdU incorporation. 2018.
<http://www.doi.org/10.17605/OSF.IO/8V9XE>
- Márkus R, Laurinyecz B, Kurucz E, *et al.*: Sessile hemocytes as a hematopoietic compartment in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*. 2009; 106(12): 4805–4809.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Meister M: Blood cells of *Drosophila*: cell lineages and role in host defence. *Curr Opin Immunol*. 2004; 16(1): 10–15.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Micchelli CA, Perrimon N: Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. *Nature*. 2006; 439(7075): 475–479.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Ono H: Ecdysone differentially regulates metamorphic timing relative to 20-hydroxyecdysone by antagonizing juvenile hormone in *Drosophila melanogaster*. *Dev Biol*. 2014; 391(1): 32–42.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Parsons B, Foley E: The *Drosophila* platelet-derived growth factor and vascular endothelial growth factor-receptor related (Pvr) protein ligands Pvf2 and Pvf3 control hemocyte viability and invasive migration. *J Biol Chem*. 2013; 288(28): 20173–20183.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Petkau K, Ferguson M, Guntermann S, *et al.*: Constitutive Immune Activity Promotes Tumorigenesis in *Drosophila* Intestinal Progenitor Cells. *Cell Rep*. 2017; 20(8): 1784–1793.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Ramond E, Meister M, Lemaitre B: From Embryo to Adult: Hematopoiesis along the *Drosophila* Life Cycle. *Dev Cell*. 2015; 33(4): 367–368.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Ribalta T, McCutcheon IE, Aldape KD, *et al.*: The mitosis-specific antibody anti-phosphohistone-H3 (PHH3) facilitates rapid reliable grading of meningiomas according to WHO 2000 criteria. *Am J Surg Pathol*. 2004; 28(11): 1532–1536.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Sawicka A, Seiser C: Histone H3 phosphorylation - a versatile chromatin modification for different occasions. *Biochimie*. 2012; 94(11): 2193–2201.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Shia AK, Glittenberg M, Thompson G, *et al.*: Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spätzle secreted by haemocytes. *J Cell Sci*. 2009; 122(Pt 24): 4505–4515.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Sigle LT, Hillyer JF: Mosquito hemocytes preferentially aggregate and phagocytose pathogens in the peristomal regions of the heart that experience the most hemolymph flow. *Dev Comp Immunol*. 2016; 55: 90–101.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Tassetto M, Kunitomi M, Andino R: Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in *Drosophila*. *Cell*. 2017; 169(2): 314–325.e313.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Taylor EW: The Mechanism Of Colchicine Inhibition Of Mitosis. I. Kinetics Of Inhibition And The Binding Of H3-Colchicine. *J Cell Biol*. 1965; 25(SUPPL): 145–160.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Tipping M, Perrimon N: *Drosophila* as a model for context-dependent tumorigenesis. *J Cell Physiol*. 2014; 229(1): 27–33.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Tucker PK, Evans IR, Wood W: Ena drives invasive macrophage migration in *Drosophila* embryos. *Dis Model Mech*. 2011; 4(1): 126–134.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Viktorinová I, Dahmann C: Microtubule polarity predicts direction of egg chamber rotation in *Drosophila*. *Curr Biol*. 2013; 23(15): 1472–1477.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Wang L, Kounatidis I, Ligoxygakis P: *Drosophila* as a model to study the role of blood cells in inflammation, innate immunity and cancer. *Front Cell Infect Microbiol*. 2013; 3: 113.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wood W, Faria C, Jacinto A: Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in *Drosophila melanogaster*. *J Cell Biol*. 2006; 173(3): 405–416.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wood W, Jacinto A: *Drosophila melanogaster* embryonic haemocytes: masters of multitasking. *Nat Rev Mol Cell Biol*. 2007; 8(7): 542–551.
[PubMed Abstract](#) | [Publisher Full Text](#)

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Current Referee Status:   

Version 2

Referee Report 02 October 2018

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We think the paper has been satisfactorily improved. It's a useful, unique study and will be a valuable addition to the literature. We think it's ready for indexing.

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 11 June 2018

doi:[10.21956/wellcomeopenres.15848.r33211](https://doi.org/10.21956/wellcomeopenres.15848.r33211)



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Summary:

The protocol is well written (grammar aside) and quite clear, but it doesn't add much more info than the 2015 paper by the same authors. Indeed, in that publication they had already described all the techniques detailed in this new methods paper, and, albeit they were written in a simpler format, the previously published methods were exhaustive enough to be easily replicated. Hence I don't see a compelling reason to describe in more detail the methods section of a previous paper, especially considering that the past article is 3 years old.

Minor comments:

- The Grammar needs to be extensively checked;
- Figures 1C and 1D have no purpose since 1C' and 1D' are the same but enlarged (and clearer);
- The fly bleeding experiments could use pictures to better describe the experiment;
- Figures 3B-B'-B'' are not really useful since they do not give more info than 3A and 3C;

- PH3 staining would be good to confirm proliferation;
- Although they are nicely described, the BrdU incorporation, hub stainings, and bleeding procedures were already detailed in their 2015 publication, albeit in a reduced format.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 02 Aug 2018

Lolitika Mandal, India Institute of Science Education and Research Mohali, India

Summary: The protocol is well written (grammar aside) and quite clear,

Thanks for your appreciation and for reviewing our protocol.

...but it doesn't add much more info than the 2015 paper by the same authors. Indeed, in that publication they had already described all the techniques detailed in this new methods paper, and, albeit they were written in a simpler format, the previously published methods were exhaustive enough to be easily replicated. Hence I don't see a compelling reason to describe in more detail the methods section of a previous paper, especially considering that the past article is 3 years old.

Here is where we beg to differ with the Reviewers. The current article is an improved version of one published in Developmental Cell. In fact, because of word limitation, we had to provide a restricted description of the protocol in our previous publication. We felt that a detailed step-by-step narration is essential for easy handling of hematopoietic hub and replication of the assays related to immuno-staining, infection and proliferation in adult fruit fly. Furthermore, the proliferation assay of circulating adult hemocytes presented here is a new data. In the revised version we have also included the PH3 labeling to endorse the mitotically active state of the hemocytes..

Minor comments: The Grammar needs to be extensively checked;
Taken care off.

Figures 1C and 1D have no purpose since 1C' and 1D' are the same but enlarged (and clearer);
We have done the necessary changes.

The fly bleeding experiments could use pictures to better describe the experiment;

Thank for your suggestion. We have incorporated pictures of adult fly bleeding in Figure 2, in the revised version.

Figures 3B-B'-B'' are not really useful since they do not give more info then 3A and 3C;
We have changed it accordingly in the revised version.

PH3 staining would be good to confirm proliferation;
Thank you so much for raising the point. We have performed the experiment to mark the mitotic phase of cell cycle by PH3 staining. The image and the protocol are incorporated in the revised manuscript (Figure 6 and steps in 'Colchicine feeding of adult fly and PH3 staining').

Although they are nicely described, the BrdU incorporation, hub stainings, and bleeding Procedures were already detailed in their 2015 publication, albeit in a reduced format.

We are thankful to all the reviewers for their constructive suggestions. The description in the publication is not elaborate. We felt the necessity to provide a step-by-step detail protocol of experiments to provide a proper replication of the results for future research. In addition, the revised manuscript also includes new protocol of hemocyte proliferation assay by PH3 expression using colchicine treatment.

Competing Interests: No competing interests were disclosed.

Referee Report 01 June 2018

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² Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary

The article provides sufficient information for reproduction of the experiments. The data are well documented.

Comments:

The conclusions on the cell division could be corroborated by using an independent marker (e.g. PHH3) for cell division. BrdU is incorporated into the newly synthesized strand of the DNA, however BrdU incorporation does not necessarily reflect division¹⁻⁷.

It was suggested previously by the same authors⁸ that cells from the hub enter the circulation “soon after infection there is a quick release of a large fraction of hemocytes from the hub (Figures S4C–S4F) highlights the contribution of the hub to fight out infection”. In this case we would expect BrdU labelled cells entering the circulation in the course of the five day labelling. The reason for this seemingly paradox situation should be discussed.

To draw the conclusion on the effect of bacterial induction, data obtained from a control group, injected with bacterium free buffer, should be presented.

In general, the technical part of the submission is appropriate, however the conclusions should be strengthened.

References

1. Breunig JJ, Arellano JI, Macklis JD, Rakic P: Everything that glitters isn't gold: a critical review of postnatal neural precursor analyses. *Cell Stem Cell*. 2007; **1** (6): 612-27 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Caldwell MA, He X, Svendsen CN: 5-Bromo-2'-deoxyuridine is selectively toxic to neuronal precursors in vitro. *Eur J Neurosci*. 2005; **22** (11): 2965-70 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Duque A, Rakic P: Different effects of bromodeoxyuridine and [3H]thymidine incorporation into DNA on cell proliferation, position, and fate. *J Neurosci*. 2011; **31** (42): 15205-17 [PubMed Abstract](#) | [Publisher Full Text](#)
4. Goldsworthy TL, Dunn CS, Popp JA: Dose effects of bromodeoxyuridine (BRUD) on rodent hepatocyte proliferation measurements. *Toxicologist*. 1992; **12** (265).
5. Kuan CY, Schloemer AJ, Lu A, Burns KA, Weng WL, Williams MT, Strauss KI, Vorhees CV, Flavell RA, Davis RJ, Sharp FR, Rakic P: Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. *J Neurosci*. 2004; **24** (47): 10763-72 [PubMed Abstract](#) | [Publisher Full Text](#)
6. Qu TY, Dong XJ, Sugaya I, Vaghani A, Pulido J, Sugaya K: Bromodeoxyuridine increases multipotency of human bone marrow-derived stem cells. *Restor Neurol Neurosci*. 2004; **22** (6): 459-68 [PubMed Abstract](#)
7. Yang Y, Geldmacher DS, Herrup K: DNA replication precedes neuronal cell death in Alzheimer's disease. *J Neurosci*. 2001; **21** (8): 2661-8 [PubMed Abstract](#)
8. Ghosh S, Singh A, Mandal S, Mandal L: Active hematopoietic hubs in Drosophila adults generate hemocytes and contribute to immune response. *Dev Cell*. 2015; **33** (4): 478-88 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Immunogenetics, Development, Immunology, Genetics

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 02 Aug 2018

Lolitika Mandal, India Institute of Science Education and Research Mohali, India

The article provides sufficient information for reproduction of the experiments. The data are well documented.

Thank you so much for your comments and appreciation.

Comments: The conclusions on the cell division could be corroborated by using an independent marker (e.g. PHH3) for cell division. BrdU is incorporated into the newly synthesized strand of the DNA, however BrdU incorporation does not necessarily reflect division .

Thank you so much for raising the point. We understand the genuine concern of reviewer. We have performed the experiment to mark the mitotic phase of cell cycle by PH3 staining. As we have noticed in BrdU incorporation assay (both in this manuscript and in our previous article on adult hematopoietic hub), the number of adult hemocytes undergoing replication is very less and the response is random. Considering the fact that the duration of M phase is shorter when compared to S phase in cell cycle, we adopted a strategy to mark the adult hemocytes that are in the M phase. We reared adult fly in food supplemented with colchicine (colchicine blocks the microtubule polymerisation in cells and thus arrest the dividing cells in different stages of mitosis) to arrest the rarely dividing cells in mitosis. This step increases the probability of tracing the mitotically active hemocytes upon bacterial infection (by PH3 labelling). Using this protocol(1mg/ml concentration of colchicine), we have successfully demonstrated the Hml GFP positive PH3 positive adult hemocytes undergo proliferation in response to bacterial infection. The images and the protocol are incorporated in the revised manuscript (Figure 6 and steps in 'Colchicine feeding of adult fly and PH3 staining').

It was suggested previously by the same authors that cells from the hub enter the circulation "soon after infection there is a quick release of a large fraction of hemocytes from the hub (Figures S4C–S4F) highlights the contribution of the hub to fight out infection". In this case we would expect BrdU labelled cells entering the circulation in the course of the five day labelling. The reason for this seemingly paradox situation should be discussed.

Thank you for raising this point. We have discussed the different possible outcome of the result. It might very well be possible that after detecting an infection, hemocytes migrate to the hub and proliferate. In that case, the hub environment permits proliferation. There is also a slim chance that soon after infection, a rare fraction of hub resident hemocytes that undergo proliferation in the hub subsequently enters into circulation. The reason why we are unable to detect them with the same protocol might be that the ex vivo bleeding technique does not assure 100% analyses of the entire pool of circulating hemocytes. However, we want to draw your attention that the sensitivity of the current protocol allows the rare events of proliferation to be documented in case of the hematopoietic hub.

To draw the conclusion on the effect of bacterial induction, data obtained from a control group, injected with bacterium free buffer, should be presented.

We have performed control mock infection experiment earlier and now have incorporated in the revised manuscript. We have mentioned the step of control experiment at point 4. "As a control experiment a mock infection was performed. Similarly aged thorax of an adult flies are pierced with fine glass capillary dipped in a sterile 1X PBS (similar to point 2) and the flies are subjected to all the steps similar to the experimental flies).

In general, the technical part of the submission is appropriate, however the conclusions should be strengthened.

We have worked on to the conclusion part in the revised version.

Deeply appreciate your inputs and comments in enriching our work.

Competing Interests: No competing interests were disclosed.

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SUMMARY

Drosophila is routinely used for the study of blood cell development, migration, and function. Until recently, there was a general assumption that hemocytes were non-proliferative in the adult. However, a recent study¹ showed the existence of proliferative hemocytes within the adult hematopoietic hub. This manuscript presents a straightforward procedure to isolate and visualize the hub from adult *Drosophila*. The protocol is well-written and easy to follow. The method should be easy for interested group to replicate, and is a valuable addition to tools for studying blood cells in *Drosophila*.

Most of my comments are relatively minor.

1. The title only discusses proliferation. However, the data and protocol show how this method can be used to visualize phagocytosis and proliferation. I recommend that the authors amend the title accordingly.
2. The hematopoietic hub is poorly defined in the introduction. The authors should consider a brief summary of what we know about the hub.
3. There is a tendency to verbose statements “resounding similarities” “huge number”, “any infection”, “majorly blocked” “huge infection load” “throwing numerous filopodial extensions” “Majorly all cell types” “a major number of macrophages”. I encourage the authors to consider a more measured approach.
4. In the introduction, it would be helpful to acknowledge that hemocytes also have effector roles (e.g. antimicrobial peptide production) and signaling roles (e.g. release of upd cytokines).
5. It is not clear what is meant by “phases” (abstract), and “tumorigenic conditions” (abstract and discussion).
6. In the section dealing with feeding BrdU to adults, the following sentence is difficult to follow: “The feeding of BrdU will generate a pool of modified nucleotide base in the system”. I recommend the authors re-write this sentence to clarify that BrdU is incorporated in DNA during S phase, and can be detected using commercially available anti-BrdU antibodies.
7. The section dealing with Bacterial infection of the adult fly is missing an important control. Did the authors do a mock infection lacking *E. coli*? If so, they should include a sentence clarifying how to do this. Step 4 should clarify that the anti-BrdU antibody marks replicating cells, including hemocytes.
8. The authors should provide details on the *E. coli* strain used in this study, and on the vendor and catalog numbers required for fine surgical forceps and scissors.
9. I’m concerned by the claim in the results section that circulating hemocytes “must have lost” proliferating capacity. Aren’t alternative explanations possible? For example, isn’t it possible that circulating hemocytes detect infection, migrate to the hub, and start to divide? In this case, a circulating hemocyte still has proliferative capacity. The authors may well be correct with their assumption, but I recommend replacing “must” with slightly less definitive terminology.
10. The imaging section is short. More details are needed about how images are processed in ImageJ and Imaris.
11. Minor comment: “was further testified” should read “was confirmed”
12. What is the evidence that adult flies “lose a major number of macrophages during infection”? In the discussion, it would be helpful to briefly cover what we know about the roles of hemocytes in the immune response of *Drosophila*. There are several studies that looked at this question.

References

1. Ghosh S, Singh A, Mandal S, Mandal L: Active hematopoietic hubs in *Drosophila* adults generate hemocytes and contribute to immune response. *Dev Cell*. 2015; **33** (4): 478-88 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 02 Aug 2018

Lolitika Mandal, India Institute of Science Education and Research Mohali, India

SUMMARY : Drosophila is routinely used for the study of blood cell development, migration, and function. Until recently, there was a general assumption that hemocytes were non-proliferative in the adult. However, a recent study showed the existence of proliferative hemocytes within the adult hematopoietic hub. This manuscript presents a straightforward procedure to isolate and visualize the hub from adult Drosophila. The protocol is well-written and easy to follow. The method should be easy for interested group to replicate, and is a valuable addition to tools for studying blood cells in Drosophila.

Thank you so much for summarising the manuscript and for your appreciation.

Most of my comments are relatively minor. The title only discusses proliferation. However, the data and protocol show how this method can be used to visualize phagocytosis and proliferation. I recommend that the authors amend the title accordingly.

Thank you for the suggestion. We have revised the title accordingly. The revised title is: 'Detecting proliferation of adult hemocytes in Drosophila by BrdU incorporation and PH3 expression in response to bacterial infection'

The hematopoietic hub is poorly defined in the introduction. The authors should consider a brief summary of what we know about the hub.

Thank you for your input. We have now described the hematopoietic hub adequately in the introduction of the revised version.

There is a tendency to verbose statements "resounding similarities" "huge number", "any infection" "majorly blocked" "huge infection load" "throwing numerous filopodial extensions" "Majorly all cell types" "a major number of macrophages". I encourage the authors to consider a more measured approach.

We have changed the phrases accordingly in the respective sentences.

In the introduction, it would be helpful to acknowledge that hemocytes also have effector roles (e.g. antimicrobial peptide production) and signaling roles (e.g. release of upd cytokines). Thank you for your input.

We have incorporated more about the effector functions of hemocytes in the introduction as well as the discussion section.

It is not clear what is meant by “phases” (abstract), and “tumorigenic conditions” (abstract and discussion).

Sorry for the confusion created.

We have mentioned the phases (primitive and definitive hematopoiesis) and tumorigenic conditions (leukaemia and solid tumor) that might induce blood cell proliferation which could be detectable using the current protocol. This is now incorporated both in abstract and discussion of the revised version.

In the section dealing with feeding BrdU to adults, the following sentence is difficult to follow: “The feeding of BrdU will generate a pool of modified nucleotide base in the system”. I recommend the authors re-write this sentence to clarify that BrdU is incorporated in DNA during S phase, and can be detected using commercially available anti-BrdU antibodies.

Thank you for mentioning the point. Now we have clarified the role of BrdU in detection of cell proliferation. Following description has been included in the method section:

“Incorporation of Bromodeoxyuridine (BrdU), a thymidine analog is an established assay for determining cell proliferation in different organisms. Replicating cells during the S phase of cell cycle readily incorporates BrdU instead of thymidine. (Rearing flies in BrdU food generates a pool of BrdU in the system that increases the chance of incorporation in cells specially the ones that undergo rare cell division). The BrdU incorporation can be detected by commercially available anti-BrdU antibody”

The section dealing with Bacterial infection of the adult fly is missing an important control. Did the authors do a mock infection lacking E. coli? If so, they should include a sentence clarifying how to do this. Step 4 should clarify that the anti-BrdU antibody marks replicating cells, including hemocytes.

A mock infection was always done with the experimental batches.

We are sorry for not mentioning the details regarding it in the previous version. We have incorporated it into the revised version of the manuscript (‘Bacterial infection in adult fly’ at point 4. “As a control experiment a mock infection was performed. Similarly aged thorax of an adult flies are pierced with fine glass capillary dipped in a sterile 1X PBS (similar to point 2) and the flies are subjected to all the steps similar to the experimental flies).

The authors should provide details on the E. coli strain used in this study, and on the vendor and catalog numbers required for fine surgical forceps and scissors.

The E.coli strain used in our study is DH5a. Although the procedures discussed in the manuscript can be efficiently replicated using scissors and forceps obtained from Fine Science Tools (FST). We have used the fine forceps and scissors obtained from a local surgical store.

I'm concerned by the claim in the results section that circulating hemocytes "must have lost" proliferating capacity. Aren't alternative explanations possible? For example, isn't it possible that circulating hemocytes detect infection, migrate to the hub, and start to divide? In this case, a circulating hemocyte still has proliferative capacity. The authors may well be correct with their assumption, but I recommend replacing "must" with slightly less definitive terminology.

We understand your concern and do agree with your interpretation to a extent. It might very well be possible that after detecting an infection, hemocytes migrate to the hub and proliferate. In that case, the hub environment permits proliferation. There is also a slim chance that soon after infection, a rare fraction of hub resident hemocytes that undergo proliferation in the hub subsequently enters into circulation. The reason why we are unable to detect them with the same protocol might be that the ex vivo bleeding technique does not assure 100% analyses of the entire pool of circulating hemocytes. However, we want to draw your attention that the sensitivity of the current protocol allows the rare events of proliferation to be documented in case of the hub.

The imaging section is short. More details are needed about how images are processed in ImageJ and Imaris.

We have incorporated in details the step-by-step procedure both for ImageJ and Imaris software mentioned in 'Imaging section'.

Minor comment: "was further testified" should read "was confirmed"

We have reframed the sentence with the advised phrase in the result section.

What is the evidence that adult flies "lose a major number of macrophages during infection"?

Our analyses of the hematopoietic hub revealed that soon after infection there is a rapid release of macrophages from the hub. In such a scenario, the response of the hematopoietic hub is akin to the sessile patches of the larvae. Within first few hours of infection the number of hemocytes in the sessile patches are significantly reduced compared to control (Markus et al., 2009). Based on the fact that there is loss of the resident hemocytes in both cases, we made that inference. We have explained this in the revised version.

In the discussion, it would be helpful to briefly cover what we know about the roles of hemocytes in the immune response of Drosophila. There are several studies that looked at this question.

We have incorporated different roles of hemocyte in immune response in the revised manuscript. Thanks for your inputs.

Competing Interests: No competing interests were disclosed.

