Detecting proliferation of adult hemocytes in *Drosophila* by BrdU incorporation [version 1; referees: 2 approved, 1 not approved]

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**Abstract**

*Drosophila* and mammalian hematopoiesis share several similarities that range from phases to the battery of transcription factors and signaling molecules that execute this process. These resounding similarities 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 conditions. The larval system is the most extensively studied to 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 method. 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 tumorigenic conditions as well as scenarios of environmental and metabolic stresses during adulthood.

**Keywords**

Adult Drosophila, Hematopoiesis, Proliferation, Macrophage, Infection

This article is included in the Wellcome Trust/DBT India Alliance gateway.
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Author roles: Ghosh S: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Mandal S: Formal Analysis, Writing – Original Draft Preparation, Writing – Review & Editing; Mandal L: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work is supported by the Wellcome Trust DBT/India Alliance Senior Fellowship [IA/S/17/1/503100] to LM. This work was also funded by Indian Institute of Science Education and Research Mohali, to S.M. and Council of Scientific and Industrial Research India (CSIR) fellowship to S.G.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Ghosh S, Mandal S and Mandal L. Detecting proliferation of adult hemocytes in Drosophila by BrdU incorporation [version 1; referees: 2 approved, 1 not approved] Wellcome Open Research 2018, 3:47 (doi: 10.12688/wellcomeopenres.14560.1)

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; Lebeský 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; 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). 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., 2017; Makhijani et al., 2011; 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., 2017; Makhijani et al., 2011). 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). The mature lymph gland then ruptures and releases a huge number of hemocytes at the time of pupation (Grigorian et al., 2011; Jung et al., 2005; Lebeský 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 (Hontí 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 adult stage and it seems to be relevant for combating any infection. Moreover, the study also demonstrated that the adult macrophages although majorly blocked in senescence can still proliferate in response to 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 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)
- 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)
- Vectashield (Vector Laboratories, Cat. No. H-100)
- HmlGal4.UAS GFP flies (BDSC Cat# 30140, RRID: BDSC_30140)
- Escherichia coli expressing RFP (pFPV25.1 RFP plasmid)
- 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)
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- HmlGal4.UAS GFP flies (BDSC Cat# 30140, RRID: BDSC_30140)
- Escherichia coli 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
- Fine surgical Scissors
- 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, Photoshoph 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 are collected. The feeding protocol is adapted and further modified from (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 200μl of 6mg/ml BrdU. (BrdU solution freshly prepared in PBS).
2. The flies are then reared for two days in this BrdU containing food. [The feeding of BrdU will generate a pool of modified nucleotide base in the system which will subsequently get incorporated in tissues undergoing any cell division].
3. Flies are then infected with *E.coli* by following the procedure as mentioned below.

**Bacterial infection of adult fly.**

1. The sharp end of the capillary is dipped in a colony of *Escherichia coli* expressing RFP (pFPV25.1 RFP plasmid) (Figure 1A–B).
2. Using this fine glass capillary, lateral side of the thorax of a pre-anaesthetized adult fly is pierced (Figure 1A–C’).
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 hml-GFP cells), and anti-BrdU antibody.

**Adult fly dissection for Hematopoietic Hub isolation.** BrdU fed adult flies are anaesthetized 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).
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 obstructs the dorsal abdomen of the fly to dip in 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

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**Figure 1. Infection procedure in adult Drosophila.** (A) The anesthetized adult fly is infected by a fine glass capillary which has a pointed tip (arrow). (B) The image shows a colony of RFP expressing *E.coli* picked up at the tip of the capillary (arrow). (C–C’) The lateral side of the thoracic region is pierced with the fine capillary. Higher magnification shows the position at which the capillary is inserted in the thorax (arrow). (D–D’) 24 hour post infection; the site of insertion in the thorax can be detectable by the black melanised spot.
Figure 2. Adult fly dissection procedure to isolate the hematopoietic hub. (A) The anesthetised 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 at the angle of 45°. 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 are processed for immunostaining.

8. The samples are now ready for immunostaining (Figure 2).

Immunostaining of adult hematopoietic hub. Please note all incubation and washings are done at room temperature unless otherwise mentioned.

1. The dissected samples are fixed in freshly prepared 4% para-formaldehyde for 45 min in 1X PBS on a shaker.

2. The dissected samples are washed thrice in 1X PBS for 10 min each followed by 30 mins incubation in 0.3%PBT + 0.3% Sodium Deoxycholate on a shaker (0.3%PBT = 0.3% TritonX 100 in 1X PBS.) [Both Triton X100 and Sodium Deoxycholate are used for permeabilization of the membrane].

3. The permeabilized tissues are incubated in freshly prepared blocking solution (10% bovine serum albumin [BSA] in 1X PBS) overnight at 4°C.

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.

7. Three washes in 1X PBS of 10 mins 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, a minimum shaking time is provided.]

12. Traces of HCL is removed by one quick and two 10mins 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. (Henceforth, all incubations are carried out in a dark chamber).

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) following the procedure below.

**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 45º 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 cover-slip is gently placed on them. In order to prevent drying, 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 (+;hs Gal4- UAS GFP;) female flies are anesthetized and the wings are removed using fine scissors.

3. The fly is then placed in a drop of 1X PBS (20μl) on a clean, pre chilled glass cavity slide. [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. (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 are 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 then 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 5mins each.
11. Secondary antibody (anti-mouse FITC, 1:400) incubation is carried out 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 as followed by two-three washes in PBS for 10min each.

17. The nucleus of cells are next labelled with DAPI. (An 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.

3. 3D surface rendered models are generated using Imaris 64X software.

**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–C′′). Instances can also be documented where multiple bacteria are phagocytosed by single hemocyte illustrating their rapid response to clear the huge infection load (Figure 3C–C′′).

Similar behavior is seen from the circulating hemocytes (Figure 3D–F′′). Here also, single hemocytes can be seen throwing numerous 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 are 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 endo-replicating 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. Although the fat body cells (arrow in Figure 4A) which are endo-replicating in nature positively incorporated BrdU (Figure 4A–A′′). In contrast, the infected flies showed several BrdU labeled plasmatocytes inside the hematopoietic hub along with nearby fat cells (arrowhead in Figure 4B–B′′). The BrdU incorporation was specific to plasmatocyte nucleus was further testified by constructing 3D surface modeling at a single cell resolution (Figure 4 B1–E6 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. It is thus very clear that the hub resident hemocytes still retain the proliferative response, whereas the circulating hemocytes must have lost it.
Figure 3. Phagocytic response against infected *E. coli* in hemocytes of hematopoietic hub and circulation. (A–C"") 30 minutes post infection the hub hemocytes (green) shows a strong phagocytic response against the bacteria (red). (B–B"") A portion of hemocyte cluster in A shows majority of the hemocytes have engulfed *E. coli*. (C–C"") Higher magnification of B shows multiple *E. coli* are engulfed by a single hemocyte present in the hematopoietic hub. (D–F"") Multiple examples of circulating hemocytes show phagocytosis of *E. coli* (red) from the infected hemolymph. (D–D"") The hemocyte throws filopodial extensions to form phagocytic cup around the *E. coli* prior to phagocytosis. Scale bar: 20µm (A–C""), 10µm (D–F"").
**Figure 4. In response to bacterial infection hub hemocytes undergo proliferation.** (A–A’’) In wild-type, adult flies hemocytes do not incorporate BrdU, while endoreplicating fat body (fb, arrow) cells incorporate BrdU and serves as control. (B–B’’) Infected individuals show BrdU incorporation in hub resident hemocytes indicating their proliferative state in response to infection. Higher magnification of B shows hml GFP+ BrdU+ hub hemocyte (arrowhead) juxtaposed to a BrdU positive fat body cells (B’–B’’). (B1–B6) BrdU incorporation in the hemocyte visualized in a single-cell resolution (B1–B3) and 3D surface rendering of (B1) reveals the BrdU signal is present inside the hemocyte. (C–E6) Transparent 3D surface model of individual hub hemocytes shows incorporation of BrdU in nucleus respectively. Scale bar: 20µm (A–B’’), 10µm (B1–E6).
Figure 5. Circulating hemocytes do not show any proliferation. (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 retain some E.coli cells (tiny red dots present outside nucleus). Scale bar: 20µm
Discussion

*Drosophila* larval stage is a preparatory phase that enables the late larvae to achieve certain required volume for metamorphosis. Majorly all cell types including the macrophages/plasmocytes proliferate and increase their number in larval developmental stages (Makhijani et al., 2011). Prior to the identification of hematopoietic hub (Ghosh et al., 2015), it was believed that there is no active site of hematopoesis 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 such a scenario, they lose a major number of macrophages engaged in combating the infection. In vertebrates, such threat is tackled by different type of blood cells that get routinely regenerated. Studies on adult mosquito, on the other hand reveals that indeed hemocytes can proliferate upon bacterial infection to increase their number (King & Hillyer, 2012; Sigle & Hillyer, 2016). This raised the possibility that may be 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 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. 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 tumorigenic conditions, viral infections (Tassetto et al., 2017) as well as scenarios of environmental and metabolic stresses during adulthood.

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

This work is supported by the Wellcome Trust DBT/India Alliance Senior Fellowship [IA/S/17/1/503100] to LM.

This work was also funded by Indian Institute of Science Education and Research Mohali, to S.M. and Council of Scientific and Industrial Research India (CSIR) fellowship to S.G.

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

Acknowledgements

We thank D. Chakravortty for providing the plasmid and all the lab members for discussions and their inputs. We thank IISER Mohali’s Confocal Facility and Bloomington Drosophila Stock Center for the flies. We would also like to thank S. K. Sharma and P. Ramesh assisting in photography.

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


Open Peer Review

Current Referee Status: ✓ ✓ ✗

Version 1

Referee Report 11 June 2018

doi: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 then 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-3B’-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.

István Ando 1, Viktor Honti 2
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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 synthetized strand of the DNA, however BrdU incorporation does not necessarily reflect division1-7.

It was suggested previously by the same authors8that 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


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.
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 synthetized 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 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.

Referee Report 18 May 2018

doi:10.21956/wellcomeopenres.15848.r33030

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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 study\(^1\) 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

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 DH5α. 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.