Video Article

Methods to Examine the Lymph Gland and Hemocytes in *Drosophila* Larvae

Theresa A. Reimels[1,2], Cathie M. Pfleger[1,2]

[1]The Graduate School of Biomedical Sciences, The Icahn School of Medicine at Mount Sinai

[2]Department of Oncological Sciences, The Icahn School of Medicine at Mount Sinai

Correspondence to: Cathie M. Pfleger at cathie.pfleger@mssm.edu

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Abstract

Many parallels exist between the *Drosophila* and mammalian hematopoietic systems, even though *Drosophila* lack the lymphoid lineage that characterize mammalian adaptive immunity. *Drosophila* and mammalian hematopoiesis occur in spatially and temporally distinct phases to produce several blood cell lineages. Both systems maintain reservoirs of blood cell progenitors with which to expand or replace mature lineages. The hematopoietic system allows *Drosophila* and mammals to respond to and to adapt to immune challenges. Importantly, the transcriptional regulators and signaling pathways that control the generation, maintenance, and function of the hematopoietic system are conserved from flies to mammals. These similarities allow *Drosophila* to be used to genetically model hematopoietic development and disease.

Here we detail assays to examine the hematopoietic system of *Drosophila* larvae. In particular, we outline methods to measure blood cell numbers and concentration, visualize a specific mature lineage *in vivo*, and perform immunohistochemistry on blood cells in circulation and in the hematopoietic organ. These assays can reveal changes in gene expression and cellular processes including signaling, survival, proliferation, and differentiation and can be used to investigate a variety of questions concerning hematopoiesis. Combined with the genetic tools available in *Drosophila*, these assays can be used to evaluate the hematopoietic system upon defined genetic alterations. While not specifically outlined here, these assays can also be used to examine the effect of environmental alterations, such as infection or diet, on the hematopoietic system.

Video Link

The video component of this article can be found at https://www.jove.com/video/54544/

Introduction

The complex mechanisms regulating the transcription factors and signaling pathways that coordinate the development of the hematopoietic system and that malfunction in hematological diseases remain poorly understood. These transcription factors and signaling pathways, as well as their regulation, are highly conserved between *Drosophila* and mammalian hematopoiesis[1-5]. Thus the *Drosophila* hematopoietic system represents an excellent genetic model to define the molecular mechanisms controlling hematopoiesis and underlying hematological diseases.

Similar to mammals, *Drosophila* generate blood cells, called hemocytes, in spatially and temporally distinct phases of hematopoiesis. Traditionally, *Drosophila* hematopoiesis was thought to be restricted to phases in the embryonic mesoderm and in the larval lymph gland. Recent studies provide evidence that hematopoiesis also occurs in larval sessile clusters and in the adult abdomen[6-8]. All hematopoietic phases produce two types of mature hemocytes: plasmatocytes and crystal cells. Plasmatocytes are macrophage-like cells involved in phagocytosis, innate immunity, and wound healing. Crystal cells contain pro-phenoloxidases required for melanization, a reaction used in insect immune responses and wound healing. Larval hematopoiesis can generate a third mature hemocyte type, called a lamellocyte, in response to certain immune challenges such as parasitoid wasp infection[9,10]. Lamellocytes are large, adherent cells that function, in conjunction with plasmatocytes and crystal cells, to encapsulate and neutralize wasp eggs laid in *Drosophila* larvae. In the absence of parasitization, lamellocytes are not found in wild-type larvae. Melanotic masses resemble melanized, encapsulated wasp eggs; many mutant *Drosophila* strains develop melanotic masses in the absence of parasitization. The presence of lamellocytes and/or melanotic masses can be indicative of hematopoietic abnormalities. In fact, the melanotic mass phenotype has been used to identify genes and pathways involved in hematopoiesis[11-14].

The larval hematopoietic system is the most extensively studied to date. It is comprised of hemocytes circulating in the hemolymph, sessile hemocyte clusters patterned under the cuticle, and hemocytes residing in the lymph gland. The lymph gland is a series of bilateral lobes attached to the dorsal vessel. Each primary lobe of the lymph gland is divided into three main zones. The outermost zone is known as the cortical zone and contains maturing hemocytes. The innermost zone is called the medullary zone and is comprised of quiescent hemocyte precursors. The third zone, the posterior signaling center, is a small group of cells at the base of the lymph gland that act as a stem cell-like niche. Early work established critical functions for Notch[15-18], Hedgehog[19,20], JAK-STAT[18], and Wingless[21] activity to regulate larval lymph gland development. More recent studies have demonstrated that BMP[22], FGF-Ras[23], and Hippo[24,25] signaling also function within the larval lymph gland.

Four larval hematopoietic assays outlined here describe 1) measuring circulating hemocyte concentration, defined as number of cells per unit volume, 2) isolating and fixing circulating hemocytes for immunohistochemistry, 3) visualizing crystal cells *in vivo*, and 4) dissecting, fixing, and mounting lymph glands for immunohistochemistry. These assays can be used as hematopoietic readouts to assess the functions and regulations of signaling pathways in the larval hematopoietic system. While these methods have been used previously in the field, visual documentation of these assays has begun only recently[8,26-30]. Several publications cited here are helpful resources describing similar methods and hematopoietic markers[26,31-33]. Additionally, Trol and Viking are useful markers of the lymph gland basement membrane.

Protocol

1. Circulating Hemocyte Concentration

1. To obtain larvae of roughly the same developmental stage for this assay, restrict egg collection by allowing females to lay eggs for a fixed time period of 2 - 6 hr.
2. Collect larvae in dissecting dish wells filled with 1x phosphate buffered saline (PBS, **Table 1**).
3. For each larva, place 10 µl 1x PBS in a microcentrifuge tube on ice and 10 µl 1x PBS on a clean dissecting pad. Place the dissecting pad on an illuminated stereomicroscope base.
4. Dry an individual larva by placing it on a tissue wipe before transferring it to a PBS drop on the dissection pad.
5. Using a pair of forceps, gently tear open and carefully invert the cuticle to release the hemolymph.
   NOTE: Take care not to scrape or jab the cuticle as this could potentially release sessile hemocytes[29].
6. Using a pipet, collect the hemolymph from the dissecting pad. Avoid collecting larval debris such as the fat body.
7. Add the hemolymph to a microcentrifuge tube and mix by pipetting up and down.
   NOTE: Several samples can be collected at once and kept on ice for up to an hour.
8. Optional: Mix Trypan blue with the hemolymph sample in equal parts to dye and exclude dead cells. Count cells within 3 min of adding Trypan blue because it is toxic to cells.
9. Mix the sample by pipetting up and down before loading 10 µl into a hemocytometer chamber.
10. If using an automated cell counter, set appropriate parameters for cell size and circularity. For example, set a minimum cell size of 2 µm, a maximum cell size of 22 µm, and a circularity of 75 - 80% roundness to detect normal, round hemocytes[34]. Experiment with different parameters to detect larger and spindle-shaped hemocytes, such as lamellocytes. Alternatively, use a hemocytometer to manually count hemocytes.

2. Circulating Hemocyte Immunohistochemistry

1. To obtain larvae of roughly the same developmental stage for this assay, restrict egg collection by allowing females to lay eggs for a fixed time period of 2 - 6 hr.
2. Place one coverslip in each well of a 6- or 12-well plate.
   NOTE: Square coverslips (22 mm) fit in 6-well plates and round coverslips (18 mm diameter) fit in 12-well plates.
3. Collect larvae in dissecting dish wells filled with 1x PBS.
4. Place 5 µl 1x PBS in the center of each coverslip.
5. Place the plate on an illuminated stereomicroscope base.
6. Dry an individual larva on a tissue wipe and then place in the PBS on the coverslip.
7. Using a pair of forceps, gently tear and carefully invert the cuticle. Before removing the larval carcass, use it to spread the hemolymph evenly.
   NOTE: Take care not to scrape or jab the cuticle as this could potentially release sessile hemocytes[29].
8. Repeat for all larvae, collecting the hemolymph of one larva per coverslip. Allow hemocytes to adhere to the coverslips for 5 - 8 min at room temperature RT. **Do not exceed 30 min before fixation.**
9. Fix hemocytes by adding 5 µl of 7.5% formaldehyde (**Table 1**) to each coverslip for 15 min at room temperature.
10. Wash coverslips three times with 1x PBS. Tip the plate and aspirate PBS after each wash. To prevent run-off of the permeabilization solution in the next step, use the aspirator to remove excess PBS from perimeter of the coverslips.
11. Add 100 µl permeabilization solution (**Table 1**) to each coverslip for 20 min at RT.
12. Tip and gently tap the plate to aspirate the permeabilization solution.
13. Dilute primary antibody with antibody solution (**Table 1**) according to provider's specifications. Add at least 500 µl primary antibody solution to coverslips in 12-well plates. Add at least 1.5 ml in 6-well plates. Incubate at 4 °C overnight.
14. Remove the primary antibody solution and wash coverslips with 1x PBS for 10 min on an orbital shaker, three times.
15. Dilute secondary antibody with antibody solution according to provider's specifications. Add at least 500 µl secondary antibody solution to coverslips in 12-well plates. Add at least 1.5 ml for 6-well plates. Incubate for at least 2 hr at RT.
16. Remove the secondary antibody solution and wash coverslips with 1x PBS for 10 min on an orbital shaker as in Step 2.10, three times.
17. During the wash steps, clean glass microscope slides with 70% ethanol (**Table 1**) using tissue wipes. For each coverslip, place 5 µl mounting buffer (**Table 1**) on the glass slide.
    NOTE: Two coverslips fit on one slide.
18. Aspirate the final PBS wash.
19. Use forceps to carefully remove the coverslips from the plates and place, inverted, on top of the mounting buffer.
    Note: Microscope slides can be stored at 4 °C prior to imaging. Maximum storage time depends on the stability of the antibodies used. 6- and 12-well plates can be rinsed and reused.

3. *In Vivo* Crystal Cell Melanization

1. To obtain larvae of roughly the same developmental stage for this assay, restrict egg collection by allowing females to lay eggs for a fixed time period of 2-6 hours.

2. Before beginning, set a heating source at 60 °C.
   NOTE: A thermal cycler program of 60 °C for 10 min (followed by a 25 °C hold) works best, but a water bath or other heating source is sufficient as long as the heat is distributed consistently and evenly over each larva.
3. Collect larvae in dissecting dish wells filled with 1x PBS (**Table 1**).
4. One at a time, dry a larva on a tissue wipe and place at the bottom of a PCR tube. Place each larva in a separate PCR tube. (See **Figure 3A**.)
5. For consistent results, ensure that larvae stay at the bottom of the PCR tubes by chilling larvae in the tubes at 4 °C for 10 - 15 min and/or gently tapping the tubes prior to heating.
6. Place the PCR tubes in the thermal cycler (or water bath). Heat at 60 °C for 10 min.
7. Carefully remove larvae from the PCR tubes into dissecting dish wells filled with fresh 1x PBS.
8. Dry larvae on a tissue wipe and arrange on a flat surface for imaging under a stereomicroscope.
9. Score images of larvae blindly by multiple individuals.

4. Larval Lymph Gland Immunohistochemistry

NOTE: The lymph gland is located approximately one-third length from the anterior end of a larva slightly below the brain on the dorsal side. (See arrow in **Figure 3B**.) The lymph gland flanks the dorsal vessel and is most easily dissected attached to the mouth hooks or to the brain. Wild-type, third instar lymph glands are very small structures; the primary lobes are approximately 100 - 200 µm in length. (See **Figure 4A**.)

1. To obtain larvae of roughly the same developmental stage for this assay, restrict egg collection by allowing females to lay eggs for a fixed time period of 2 - 6 hr.
2. Lymph gland dissection
   1. For each experimental condition, add 1 ml 1x PBS (**Table 1**) to one well of a 24-well plate.
   2. Add 1 drop of 0.1% PBST (**Table 1**) to each well using a disposable transfer pipet.
      NOTE: Detergent, such as Tween 20, is added to lower the surface tension, allowing the tissue to sink to the bottom of the well.
   3. Place the plate flat on ice.
   4. Collect larvae in dissecting dish wells filled with 1x PBS.
   5. Place a clean dissecting pad on an illuminated stereomicroscope base. Use a disposable transfer pipet to place small drops of 0.01% PBST (**Table 1**) on the pad. Transfer one larva to a PBST drop for dissection.
   6. Hold the larva with one pair of forceps approximately one-quarter length from the posterior end, dorsal side up.
   7. Use another pair of forceps to grab the cuticle immediately anterior to the forceps that are holding the larva. Gently pull the cuticle toward the anterior end until the mouth hooks are exposed.
      NOTE: The aim is to peel the cuticle without disturbing any internal structures.
   8. Release the cuticle and use both forceps to cut the larva in two. Remove the posterior end from the PBST drop.
      NOTE: If the cuticle does not peel all the way to the mouth hooks, cut the larva in two and remove the posterior end. Use one pair of forceps to hold the edge of the cuticle, and use the other pair of forceps to push the mouth hooks through the opening. This will invert the cuticle and expose the mouth hooks. This can be used as an alternate dissection method as well.
   9. Use one pair of forceps to pin down the cuticle (either the ventral cuticle, the dorsal cuticle flap, or both) for stability.
   10. Use another pair of forceps to grab the exposed mouth hooks and gently pull them out.
       NOTE: This will separate the cuticle from the internal structures. Ideally, the eye/antennal imaginal discs, brain, ring gland, and lymph gland flanking the dorsal vessel will remain attached to the mouth hooks.
   11. While still holding the mouth hooks, carefully remove unwanted structures such as the salivary glands, fat body, and intestine.
       NOTE: If the brain separates from the mouth hooks, the lymph gland might still be attached to and collected with the brain. In this case, hold the ventral nerve cord instead of the mouth hooks.
   12. Using the mouth hooks or ventral nerve cord as a handle, transfer the dissected complex containing the lymph gland to the well on ice. **Do not exceed 30 min before fixation.**

3. Fixation and immunohistochemistry
   1. Place the 24-well plate on the stereomicroscope base.
   2. Use a p200 pipette to carefully remove the PBST from the well.
      NOTE: Empty, neighboring wells are useful to temporarily deposit waste.
   3. Gently add 200 µl 3.7% formaldehyde (**Table 1**) down the side of the well and swirl the plate to ensure that the dissected tissues are completely submerged.
   4. Return the plate to ice for 30 min. If the lymph glands express fluorescent protein(s), keep the plate covered to prevent photobleaching.
   5. Use a p200 pipette to carefully remove the fixative.
   6. Wash by adding 200 µl 1x PBS to the well and placing the plate on an orbital shaker for 5 min at room temperature. Use a p200 pipette to carefully remove the PBS.
      NOTE: Fixed lymph glands can be left on ice in PBS until lymph glands for all experimental conditions are dissected and fixed.
   7. Repeat the wash steps two more times.
   8. Add 200 µl permeabilization solution (**Table 1**) to the well. Set the plate on an orbital shaker for 45 min at RT.
      NOTE: 45 min is the "gold standard" for lymph gland permeabilization but the authors have success after only 20 min.
   9. Remove the solution with a p200 pipette.
   10. Dilute primary antibody with antibody solution (**Table 1**) according to provider's specifications. Add 300 µl primary antibody solution to the well and ensure that the dissected tissues are completely submerged. Incubate at 4 °C overnight.
   11. Use a p200 pipette to remove the primary antibody.
   12. Wash by adding 200 µl 1x PBS to the well and placing the plate on an orbital shaker for 10 min at room temperature. Use a p200 pipette to carefully remove the PBS.
   13. Repeat the wash steps two more times.

14. Dilute secondary antibody with antibody solution according to provider's specifications. Add 300 µl secondary antibody solution to the well and ensure that the dissected tissues are completely submerged. Incubate on an orbital shaker for at least 2 hr at RT.
15. Use a p200 pipette to remove the secondary antibody and wash as described in Steps 4.3.12 & 4.3.13. Do not remove the PBS after the final wash.

4. Lymph gland mounting
    1. Clean glass microscope slides using 70% ethanol (**Table 1**) and tissue wipes.
    2. For each experimental condition, place one drop of mounting buffer (**Table 1**) on the glass slide.
    NOTE: Two conditions fit on one slide. Mounting buffer volume depends on the number of lymph glands to be mounted. Use 2 µl for approximately 18 - 20 lymph glands, 1 µl for 10 - 12, and 0.5 µl for 5 or less.
    3. Place the microscope slide on an illuminated stereomicroscope base.
    4. For up to two conditions at a time, transfer all of the lymph glands from the well to the mounting buffer with forceps, using the mouth hooks or ventral nerve cord as a handle.
    5. Space the dissected tissues evenly in a circular or rectangular shape, spreading the mounting buffer in the process.
    6. For each of the dissected tissues, individually, slide one tong of the forceps under the dorsal vessel and gently pull toward the periphery of the mounting buffer.
    NOTE: This will draw the lymph gland out from the rest of the dissected tissues and flatten the lymph gland on the glass.
    7. Using one tong of the forceps and a sawing motion, cut the dorsal vessel between the lymph gland and the brain.
    8. Move the rest of the dissected tissues to the opposite side of the lymph gland, at the outermost edge of the buffer.
    NOTE: The unwanted dissected tissues will eventually form a perimeter around the lymph glands and serve as a support upon which the coverslip will rest.
    9. Repeat until all of the lymph glands are separated from the dissected tissues, reserving one of the unwanted dissected tissues to place in the center.
    10. Take a coverslip between two fingers. Check that the coverslip is free of dust and fingerprints. If necessary, use a tissue wipe and 70% ethanol to clean the coverslip.
    11. Ensuring that the edge of the coverslip is parallel to the edge of the glass slide, carefully lower the coverslip over the mounting buffer.
    NOTE: Microscope slides can be stored at 4 °C prior to imaging. Maximum storage time depends on the stability of the antibodies used. 24-well plates can be rinsed and reused.

5. Imaging

1. Image fixed hemocytes and mounted lymph glands on an appropriate standard fluorescence or confocal microscope at 20X or higher magnification according to manufacturer's operating manual. Image whole larvae on a standard stereomicroscope at 2X magnification according to manufacturer's operating manual.
2. Follow software provider's instructions for deconvolution, if desired.

Solution	Composition	Storage	Comments
1x PBS	200 mg/L potassium chloride	room temperature	
	200 mg/L potassium phosphate monobasic		
	8,000 mg/L sodium chloride		
	1,150 mg/L sodium phosphate dibasic		
	dH_2O		
Fixative	3.7% or 7.5% formaldehyde in 1x PBS	room temperature in the dark	Formaldehyde is toxic.
Permeabilization solution/antibody diluent	0.4% Triton	4 °C	The standard formula uses 0.4% Triton but the authors use 0.1% Tween 20 with success. Use to dilute primary and secondary antibodies according to providers' recommended concentrations.
	5% bovine serum albumin, normal goat serum, or normal donkey serum		
	1x PBS		
70% ethanol	70% 200 proof ethanol in dH2O	room temperature	
Mounting buffer	0.5% N-propyl gallate	4 °C in the dark	N-propyl gallate is harmful. DAPI is a mutagen.
	80% glycerol		
	Optional: 1 µg/ml DAPI (4',6-diamidino-2-phenylindole)		
	1x PBS		
0.1% PBST	0.1% Tween 20 in 1x PBS	room temperature	
0.01% PBST	1:10 dilution of 0.1% PBST	room temperature	

Table 1. Solutions used in this Protocol.

Representative Results

Circulating Hemocyte Concentration

Hemocyte numbers increase throughout larval development[35]. To illustrate that this method detects differences in hemocyte numbers and concentration, regardless of the biological cause, we measured hemocyte concentrations of delayed and non-delayed larvae. Loss of prothoracicotropic hormone (ptth) by genetic ablation of ptth-producing neurons (ptth>grim) produces a delay in larval development[36]. For each genotype, hemocyte concentrations were measured as described in Protocol 1 for at least 8 individual larva raised at 25 °C. At 120 hr after a 2 hr egg collection, the average hemocyte concentration per delayed larva (ptth>grim) is less than the average hemocyte concentration per control larva (ptth). Only after 9 days does the average hemocyte concentration per delayed larva approach that of controls (**Figure 1B**). Another example in which this method is used to detect differences in hemocyte concentrations has been published[37].

Images taken from an automated cell counter show a clean, desirable hemolymph sample and a sample containing debris, which might lead to inaccurate measurement (**Figure 1C**). Minimum and maximum cell sizes were set to 2 µm and 22 µm, respectively. Circularity was set to 75-80% roundness. These parameters are intended as guidelines and should be empirically optimized.

Circulating Hemocyte Immunohistochemistry

Circulating hemocytes expressing green fluorescent protein (GFP) were collected, fixed, and incubated with a mixture of plasmatocyte-specific antibodies (P1a and P1b, István Andó)[31] as described in Protocol 2 (**Figure 2**). The image was taken on a standard fluorescence microscope and is shown unaltered and after constrained iterative deconvolution. In this instance, deconvolution did not drastically improve the image quality.

In Vivo Crystal Cell Melanization

Larvae were placed at the bottom of PCR tubes prior to heat-induced crystal cell melanization as described in Protocol 3 (**Figure 3A**). Red arrows indicate tubes in which larvae are too far from the bottom and might be heated unevenly. Uneven distribution of heat across individual larva can increase variability in the melanization of crystal cells within the larva.

A wild-type larva imaged on a standard stereomicroscope shows the typical pattern of melanized crystal cells in sessile clusters after heat exposure (**Figure 3B**). Melanized crystal cells in the lymph gland are sometimes seen.

Larval Lymph Gland Immunohistochemistry

Third instar larval lymph glands were dissected, fixed, and mounted as described in Protocol 4. A differential interference contrast (DIC) image taken on a standard fluorescence microscope shows the primary and secondary lymph gland lobes flanking the dorsal vessel (**Figure 4A**).

A lymph gland in which the medullary zone and the posterior signaling center were genetically marked with enhanced blue fluorescent protein (EBFP2) and GFP, respectively, was stained with an antibody against the Notch intracellular domain (C17.9C6, Developmental Studies Hybridoma Bank) as described in Protocol 4. Z-stack images were taken on a standard fluorescence microscope. A single maximum intensity projection image appears unaltered and after constrained iterative deconvolution (**Figure 4B**). In this instance, deconvolution drastically improved the quality and detail of the image.

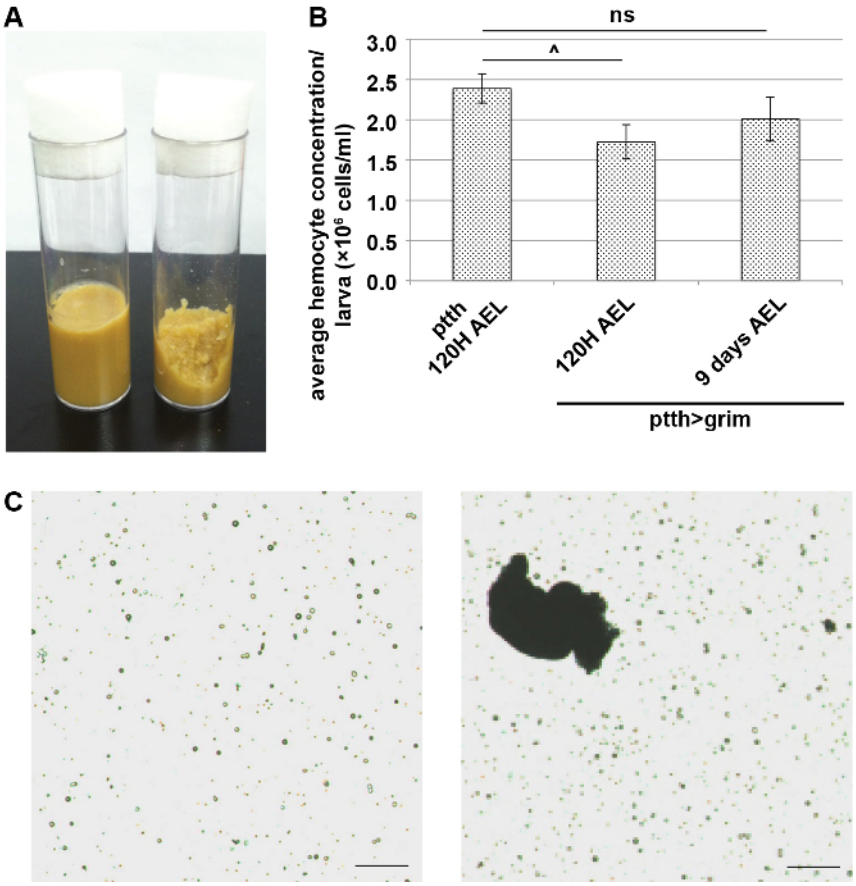

Figure 1. Circulating Hemocyte Concentration. A) A portion of standard *Drosophila* medium was removed (right) to promote egg laying during a 2 hr egg collection period. **B)** The average hemocyte concentration per developmentally delayed larva (ptth>grim) was lower than the average hemocyte concentration per control larvae (ptth) 120 hr after egg laying (AEL). The average hemocyte concentration per delayed larva approached control level 9 days AEL. Error bars represent ± s.e.m. **C)** Hemolymph samples without (left) and with (right) debris. Scale bars represent 0.1 mm. Please click here to view a larger version of this figure.

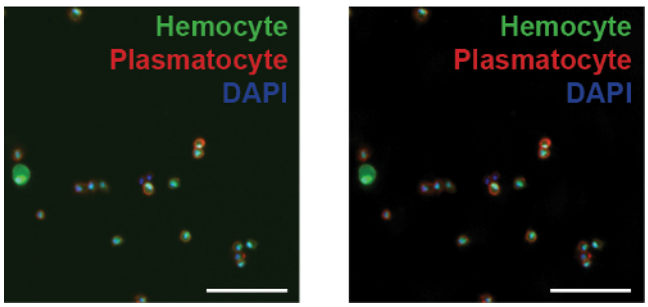

Figure 2. Circulating Hemocyte Immunohistochemistry. Hemocytes genetically expressing GFP were collected and fixed to a coverslip. A plasmatocyte-specific antibody was used to stain plasmatocytes (red). DAPI staining is shown in blue. Unaltered image taken with a fluorescence microscope (left). The same image after deconvolution (right). Scale bars represent 50 µm. Please click here to view a larger version of this figure.

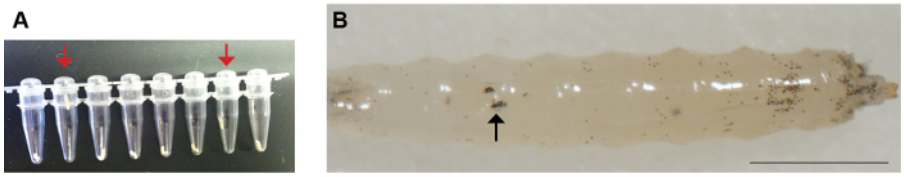

Figure 3. *In Vivo* Crystal Cell Melanization. A) Larvae were placed individually in PCR tubes prior to heating. Red arrows indicate larvae that are not at the bottom of the tubes. **B)** A typical crystal cell melanization pattern after heating, which sometimes reveals the lymph gland (arrow; dorsal side shown, anterior is left). Scale bar represents 1 mm. Please click here to view a larger version of this figure.

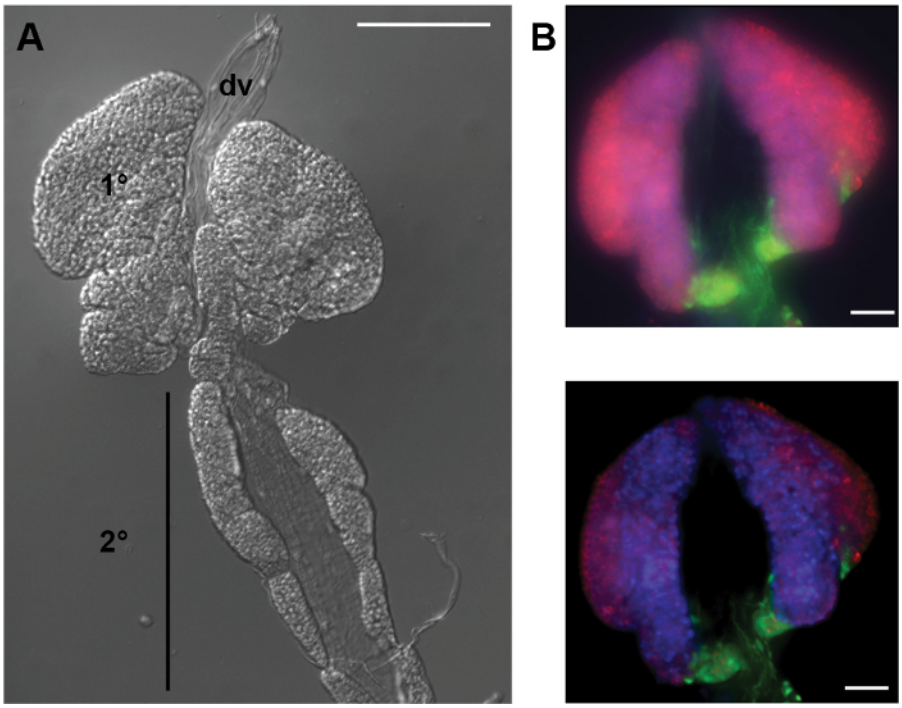

Figure 4. Larval Lymph Gland Immunohistochemistry. A) A DIC image of a third instar larval lymph gland showing the dorsal vessel (dv), primary lobes (1°), and secondary lobes (2°). Scale bar represents 100 µm. **B)** A representative third instar larval lymph gland from a larva genetically expressing EBFP2 in the medullary zone and GFP in the posterior signaling center. The lymph gland was stained with an antibody against the Notch intracellular domain (red). Unaltered image taken with a fluorescence microscope (top). The same image after deconvolution (bottom). Scale bars represent 20 µm. Please click here to view a larger version of this figure.

Discussion

Upon genetic or environmental alteration, the four methods described here can be used individually or in conjunction to analyze distinct processes during hematopoiesis such as signaling, survival, proliferation, and differentiation. *Drosophila* hematopoiesis is a dynamic process; the number of hemocytes per animal increases[35] and the structure and gene expression of the lymph gland changes[32] during development. Prior to performing these assays, therefore, it is critical to restrict egg collections by allowing females to lay eggs for a fixed amount of time and to confirm the desired larval developmental stage. For shorter egg collections (less than 6 hr) or for instances in which females are unhealthy or scarce, egg laying can be encouraged by creating crevices in the food. This can be accomplished by removing a portion of food with an ethanol-cleaned spatula. If excess liquid accumulates in the food, use tissue wipes to soak up the liquid. (See **Figure 1A.**)

Alone, the methods described here have limitations. For instance, measuring circulating hemocyte concentration captures changes in hemocyte survival or proliferation but provides no information about hemocyte lineage distribution or any lineage disruption that might be consequent to genetic or environmental alteration. Conversely, immunohistochemistry of circulating hemocytes reveals changes in specific hemocyte lineages in the hemolymph but only in relative, not absolute, numbers. Crystal cell melanization *in vivo* is difficult to quantitate as crystal cell distribution is variable and sessile hemocytes are dynamic[8,10]. Rather, multiple individuals should score crystal cell melanization blindly. Finally, observations made in one hemocyte compartment might not necessarily hold true in other compartments.

When used together, these assays can be applied to distinguish genetic alterations that regulate proliferation or survival from those that regulate gene expression or differentiation. For instance, a genetic alteration can increase proliferation of hemocytes such that hemocyte concentration increases but the relative proportions of hemocyte lineages remains the same. Alternately, a genetic alteration can promote changes in hemocyte differentiation into specific lineages with no effect on overall hemocyte concentration. The crystal cell population can be interrogated quickly and easily using the *in vivo* melanization method, facilitating genetic screens and genetic interaction studies. This method can be used in corroboration with genetic studies that utilize *prophenoloxidase* 1 (*PPO1*, also called *black cells, Bc*) mutant alleles or immunohistochemical assays that utilize crystal cell-specific antibodies. The larval lymph gland can be used to address similar questions regarding hematopoietic

cellular processes. Numerous signaling pathways are involved in establishing and maintaining the lymph gland and its major zones. Each zone has distinct gene expression and function in larval hematopoiesis. Additionally, utilizing the lymph gland can reveal autonomous and non-autonomous functions within the lymph gland zones or lymph gland hemocytes.

Relevant methods for studying *Drosophila* hematopoiesis have been compiled in comprehensive text-based resources only recently[26,27]. Though indispensable to the field, these resources are limited in scope. Methods for measuring hemocyte concentration, for example, were not included. Written methods, especially those describing dissection techniques, can be challenging to master quickly. Additional contributions have been made, providing visual resources to assist with methods, but were still limited in number and in scope[28,29]. The methods described here, while also not comprehensive, add to the resources available to aid in the study of *Drosophila* hematopoiesis.

We offer modifications and alternatives to existing protocols. For example, there are several advantages to measuring hemocyte concentration with an automated cell counter rather than a manual hemocytometer, including increased speed, ease, and objectivity. In our experience, using a water bath to heat larvae for crystal cell visualization resulted in widely variable results, especially if the larvae are heated in the vials in which they are raised. Heating larvae in individual PCR tubes yielded less variable and more reproducible results. The lymph gland dissection method described here, though previously outlined in a written protocol[26], provides an alternative to existing visual references[28]. Finally, if access to a confocal microscope is limited, we suggest that deconvolution of standard fluorescence images might be a suitable substitute for confocal images. Deconvolution algorithms either remove or reassign out-of-focus light captured by conventional fluorescence microscopy, improving resolution and contrast similar in principle to the elimination of out-of-focus light by confocal microscopy, and can be applied to 2-dimensional and 3-dimensional (Z-stack) images. For some applications, as demonstrated here, deconvolution can dramatically improve images taken with conventional fluorescence microscopes.

Disclosures

The authors declare that they have no competing or financial interests.

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