Video Article

A Simple Protocol for Extracting Hemocytes from Wild Caterpillars

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Abstract

Insect hemocytes (equivalent to mammalian white blood cells) play an important role in several physiological processes throughout an insect's life cycle [1]. In larval stages of insects belonging to the orders of Lepidoptera (moths and butterflies) and Diptera (true flies), hemocytes are formed from the lymph gland (a specialized hematopoietic organ) or embryonic cells and can be carried through to the adult stage. Embryonic hemocytes are involved in cell migration during development and chemotaxis regulation during inflammation. They also take part in cell apoptosis and are essential for embryogenesis [2]. Hemocytes mediate the cellular arm of the insect innate immune response that includes several functions, such as cell spreading, cell aggregation, formation of nodules, phagocytosis and encapsulation of foreign invaders [3]. They are also responsible for orchestrating specific insect humoral defenses during infection, such as the production of antimicrobial peptides and other effector molecules [4, 5]. Hemocyte morphology and function have mainly been studied in genetic or physiological insect models, including the fruit fly, *Drosophila melanogaster* [6, 7], the mosquitoes *Aedes aegypti* and *Anopheles gambiae* [8, 9] and the tobacco hornworm, *Manduca sexta* [10, 11]. However, little information currently exists about the diversity, classification, morphology and function of hemocytes in non-model insect species, especially those collected from the wild [12].

Here we describe a simple and efficient protocol for extracting hemocytes from wild caterpillars. We use penultimate instar *Lithacodes fasciola* (yellow-shouldered slug moth) (**Figure 1**) and *Euclea delphinii* (spiny oak slug) caterpillars (Lepidoptera: Limacodidae) and show that sufficient volumes of hemolymph (insect blood) can be isolated and hemocyte numbers counted from individual larvae. This method can be used to efficiently study hemocyte types in these species as well as in other related lepidopteran caterpillars harvested from the field, or it can be readily combined with immunological assays designed to investigate hemocyte function following infection with microbial or parasitic organisms [13].

Video Link

The video component of this article can be found at http://www.jove.com/video/4173/

Protocol

1. Material Preparation

1. Prepare needles (1-2 µm tip and 3-4 mm taper) using borosilicate glass capillary tubes and a micropipette puller. Instrument settings: ramp: 561; velocity: 20; heat: 560; delay: 1; pull: 100; pressure: 500.
2. Prepare the collection solution: 60% of Grace's Medium (GM) supplemented with 10% of Fetal Bovine Serum (FBS) and 20% of Anticoagulant Buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid, pH 4.5). The above solution is prepared fresh under sterile conditions and kept on ice at all times.
3. Prepare the incubation buffer solution: 90% of GM supplemented with 10% of FBS. This solution is also prepared fresh and kept on ice at all times.
4. Prepare a total of at least 15 µl × the number of insects for both the collection and the incubation solutions.

2. Injection of Caterpillars with Collection Solution

1. Anesthetize penultimate instar larvae by chilling them on ice for 20 min prior to hemocyte extraction.
2. Fit the blunt end of a clean capillary needle (16-gauge) into the tubing that is connected to a 20 ml glass syringe (**Figure 2**). Leave the tube containing the collection solution open on the lab bench and slowly fill the capillary with the buffer. The capillary is usually filled with approximately 10-15 µl of collection solution, depending on the size of the caterpillar (approximately 1 µl solution per 10 mg of caterpillar total body mass = 0.01% of total body mass). Rest the needle on a ball of clay that is placed next to the dissecting microscope.
3. Place the caterpillar in a Petri dish. The insect should be positioned on its side so that the spiracles are clearly visible (**Figure 3**).
4. While viewing under a dissecting microscope, hold the caterpillar in place with a pair of forceps and place the needle close to one of the spiracles, where the insect cuticle is generally softer. Gently press the capillary needle against the body of the insect. Once the needle has

penetrated the cuticle, inject the total volume of solution into the caterpillar (**Figure 3**). It is important to avoid injecting air bubbles into the caterpillar. The caterpillar's body will swell (*e.g.*, increase in volume by 20%) following injection but should not burst or ooze.
5. Return the caterpillar to the ice, positioning the insect dorsally to prevent buffer from leaking out of the wound (**Figure 3**).
6. Repeat steps 2.1-2.4 for each caterpillar used for hemolymph extraction. At this stage, allow all insects to chill on ice for 30 min before proceeding.

3. Extraction of Hemocytes

1. Place the caterpillar in a Petri dish under the dissecting microscope.
2. Make a shallow (2-3 mm long) incision in the posterior region of the caterpillar using a sterile scalpel (**Figure 3**). Take care not to damage the gut or other internal organs; the goal is to puncture the cuticle carefully and collect the buffer/hemolymph mix containing hemocytes from individual insects.
3. Squeeze the caterpillar gently using flexible plastic forceps while collecting the hemolymph mix (approximately 10-20 µl) with a 10 µl pipette tip, again taking care not to injure the internal tissues (**Figure 3**). Hemolymph is collected from the droplet squeezed from the caterpillar's body. Discard the caterpillar's remains.
4. Collect hemolymph from each individual caterpillar into separate labeled siliconized 0.5 ml microcentrifuge tubes.
5. Add equal volume of the incubation solution (approximately 10-20 µl) to each hemolymph sample collected from an individual caterpillar. Keep hemolymph samples on ice at all times.
6. Repeat steps 3.1-3.5 for each caterpillar used in the experiment.

4. Quantification of Hemocytes

1. Mix the hemolymph sample and the incubation solution immediately prior to counting hemocytes. This treatment allows for proper separation of hemocytes and prevents clumping.
2. Use a clean pipette tip to transfer 10 µl of hemolymph sample mixed with the incubation solution onto a hemocytometer.
3. Using a compound microscope, count and record the number of hemocytes in at least 15 of the large squares (area of one square: 0.04 mm^2, depth: 0.1 mm) of the central grid of the hemocytometer (**Figure 3**). Add these values to obtain a total sum of the number of hemocytes in the 15 squares.
4. Rinse the hemocytometer and its cover slide thoroughly using a wash bottle with distilled and deionized water and wipe dry with Kimwipes (Kimberly-Clark) between each sample.
5. Repeat steps 4.1-4.4 for each hemolymph sample.
6. Calculate the mean number of hemocytes per ml of hemolymph volume for each caterpillar, as follows:

Total volume of each square of the hemocytometer grid: (0.04 mm^2 area) × (0.1 mm depth) = 0.004 mm^3 × 15 squares = 0.06 mm^3 = the total volume of all 15 counted squares.

(Sum of the total number of hemocytes in the 15 squares counted / the total volume of all 15 counted squares) x 2 dilution factor = the number of hemocytes/mm^3 x 1,000 = the number of hemocytes/ml of hemolymph.

Representative Results

The protocol described herein allows the collection of a minimum volume of 10-20 µl of insect hemolymph from individual caterpillars. Hemocytes collected using this method are free of cell clumping, melanization defects, tissue debris or other contaminants. Therefore hemocytes can be readily observed and counted under the microscope, and several insects can be observed within a few hosur. We observed that the majority of hemocytes in our samples consisted of plasmatocytes [3] (hemocytes spreading asymmetrically) (**Figure 4**). We are currently characterizing the different hemocyte types found in these caterpillars in detail (Stoepler *et al.*, unpublished data). The method described here provides a quick and accurate means for counting total hemocyte numbers for individual caterpillars collected from field experiments.

Although hemocyte density can vary greatly between and within insect species [1], we found that hemocyte numbers for *L. fasciola* caterpillars range from 1.10 × 10^6 - 8.23 × 10^6 cells/ml of hemolymph (mean: 3.51 × 10^6; n = 31 caterpillars), and are in the range of previously reported values in other lepidopteran species [14].

Figure 1. Photograph of a penultimate instar *Lithacodes fasciola* (Lepidoptera: Limacodidae) caterpillar. Typical body length of these caterpillars ranges from 10-15 mm in penultimate to ultimate instars. Photo credit: John T. Lill.

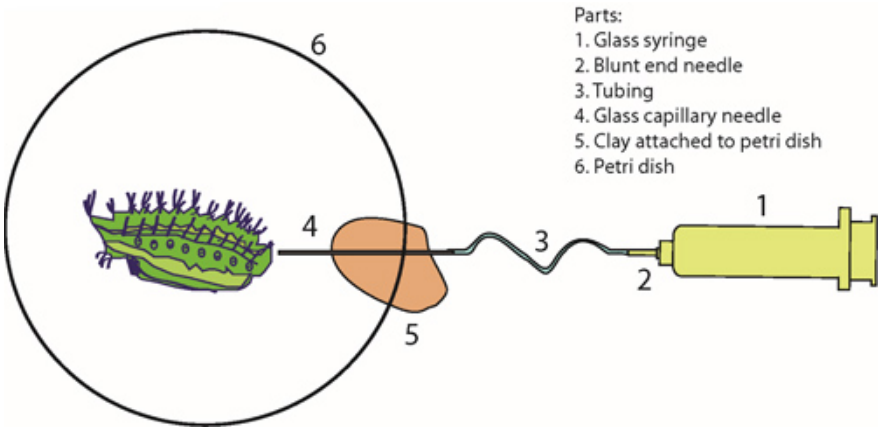

Figure 2. Injection needle setup. A glass syringe is connected to a capillary needle via a blunt end needle and plastic tubing (panels 1-4). A piece of plastic clay is used to rest the capillary needle (5). The caterpillar is placed in a Petri dish for injection with the collection solution (6).

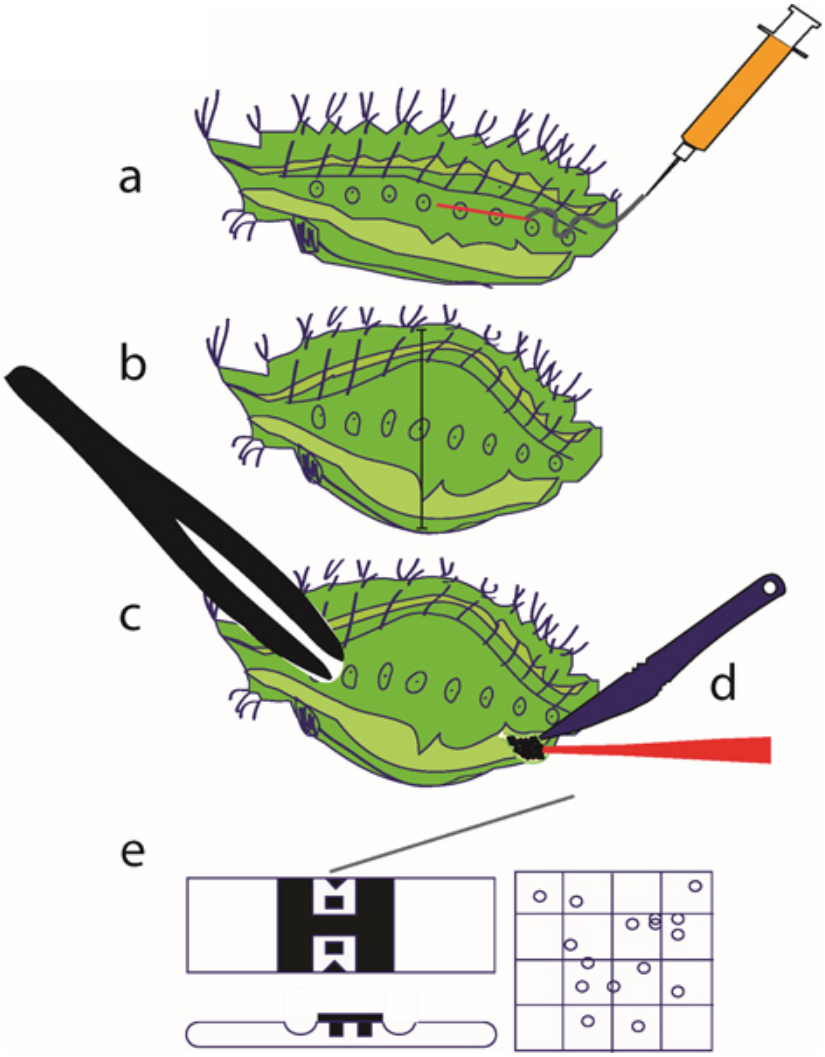

Figure 3. Hemocyte extraction method. (a) The chilled caterpillar is injected with the collection solution; (b) following injection, the caterpillar's body will swell slightly, and the injected insect is allowed to rest on ice for 30 min; (c, d) a cut is made using a clean scalpel, the caterpillar is squeezed using a pair of plastic forceps to force the hemolymph out of the body through the incision, and hemocytes are collected with a pipette; (e) hemocytes are counted using a hemocytometer.

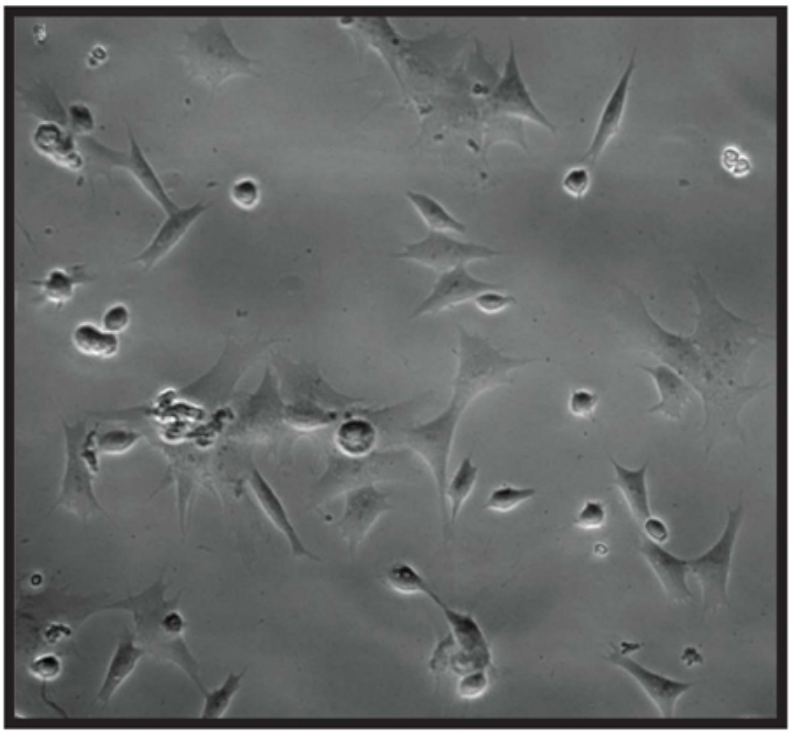

Figure 4. Microscopy picture showing extracted hemocytes from *Euclea delphinii* (Lepidoptera: Limacodidae) caterpillars (magnification: 20x).

Discussion

Methods for extracting hemocytes from medically important insects and lepidopteran model insects have previously been reported [9, 14]. Hemocyte extraction methods are adapted according to the insect species, the developmental stage of the insect and its morphological features. For example, hemolymph isolation from *Manduca* larvae can be readily performed by snipping the curved horn near the end of the abdomen [15]. Because slug caterpillars (Limacodidae) lack this abdominal structure, we have developed a distinct protocol for hemolymph extraction from small-sized field-collected caterpillars. This method proves efficient for isolating sufficient hemolymph volumes containing large numbers of hemocyte cells that can be stored or processed for further experimentation. In addition, hemocyte collection from adult mosquitoes includes a time-consuming step that involves the careful removal of legs, wings and the tip of the abdomen [16]. A similar step is not required in our protocol, thus isolation of hemocytes from slug caterpillars is a simpler and quicker process. Limitations of our technique may include: 1) as with all hemocyte extraction methods, insects must be sacrificed on the day of the assay, and 2) hemocyte numbers may vary significantly between larvae of the same species and developmental stage, which necessitates the use of several individuals in order to calculate mean numbers of hemocytes per volume of extracted hemolymph.

We intend to utilize this method in future studies to morphologically characterize hemocyte cell types from different developmental stages of forest-harvested caterpillars. This assay can also provide the first critical step for isolating healthy hemocytes to examine in detail several physiological aspects of the insect cellular immune response, including the effect of various developmental stages and nutritional conditions on the total number of hemocytes in naïve or infected caterpillars obtained from their natural environments. Given the chronic exposure of lepidopteran larvae to a range of different pathogens in nature, as well as the potentially continuous presence of their microbial symbionts, it is anticipated that these insects possess types of hemocytes with different properties compared to immune cells found in laboratory-reared insects. In addition, this hemocyte extraction protocol can also be applied to studies with other groups of insects, allowing immunological-related assays to be incorporated into ecological field studies, thus increasing our understanding of cellular defense mechanisms in non-model insect species of agricultural or ecological importance. In conclusion, our method will provide a valuable resource to insect biologists, ecologists and those interested in understanding the functional interplay between hemocyte abundance and the ability of wild insect populations to withstand microbial or parasitic infections.

Disclosures

No conflicts of interest declared.

Acknowledgements

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