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Intracellular cytokine detection based on flow cytometry in hemocytes from *Galleria mellonella* larvae

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ABSTRACT

Invertebrates are becoming increasingly popular models for research on the immune system. The innate immunity possessed by insects shows both structural and functional similarity to the resistance displayed by mammals, and many processes occurring in insect hemocytes are similar to those that occur in mammals. However, the use of insects as research models requires the development of methods for working with hemocytes. Therefore, a protocol for intracellular cytokine detection in *Galleria mellonella* larvae hemocytes based on flow cytometry has been developed. It describes the anticoagulant composition of the buffer, the optimal conditions for hemocyte permeabilization and fixation, as well as the conditions of cell centrifugation to prevent cell disintegration. A key element is the selection of staining conditions, especially the length of the incubation time with the primary antibody, which turned out to be much longer than recommended for mammalian cells. The development of these individual steps allowed for the creation of a reproducible protocol for cytokine detection using flow cytometry in wax moth hemocytes. This will certainly facilitate the development of further protocols allowing for wider use of insect cells in immunological research.

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KEYWORDS

cytokine detection , flow cytometry, *Galleria mellonella*, hemocytes

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MATERIALS TEXT

PBS+AB: PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄) with 0.1 mM
PTU (phenylthiourea) and anticoagulant buffer (AB): 10mM EDTA
(ethylenediaminetetraacetic acid), 30mM sodium citrate

Fixation buffer: 4% paraformaldehyde in PBS+AB.

Permeabilization buffer: 0.1% Triton X-100 in PBS+AB.

SAFETY WARNINGS

Paraformaldehyde is:

- flammable solid
- carcinogen (IARC Group 1 – confirmed human carcinogen)
- sensitizer (skin and pulmonary)
- irritant (skin, eye, and respiratory tract.
- reproductive toxin (as a result of the formation of formaldehyde which is a decomposition product of paraformaldehyde)
- toxic (by skin contact and inhalation)


Breathing paraformaldehyde powders will irritate the nose and throat after prolonged exposure causing a cough, shortness of breath and possible lung damage.

Sample preparation 40m




- 1 Before bleeding, wash the larvae with distilled water (15 seconds) and then immerse briefly (5^{2m} seconds) in 70% (v/v) ethanol to sterilize their surfaces, thus reducing the chance of contamination of hemolymph samples.
- 2 Collect the hemolymph into sterile tube from the larvae through the incision made in the last^{10m} proleg
- 3 100 µl of fresh hemolymph collected from ten larvae suspend in 100 µl of PBS+AB buffer^{3m}
- 4 Centrifuge cells 🌀**300 x g, 4°C, 00:05:00**^{5m}
- 5 Carefully remove the supernatant and resuspend the cell pellet in 100 µl of PBS+AB buffer.^{20m} Repeat this step three times.

Fixation 35m








- 6 Carefully remove the supernatant and resuspend the cell pellet in 500 µl of fixation buffer.^{10m} Incubate for 🕒**00:10:00** 🌡️ **Room temperature**
- 7 Centrifuge at 🌀**300 x g, 4°C, 00:05:00** and remove the fixation buffer.^{5m}

- 8 Wash fixed cells with PBS+AB buffer. Centrifuge at  **300 x g, 4°C, 00:05:00** and discard the supernatant. Repeat this step three times. 20m

Permeabilization 30m

- 9 Resuspend the cell pellet in 500 µl of permeabilization buffer with 0.5% BSA (bovine serum albumin). Incubate for  **00:10:00**  **Room temperature** 10m
- 10 Wash the fixed/permeabilized cells with permeabilization buffer. Centrifuge at  **300 x g, 4°C, 00:05:00** and discard the supernatant. Repeat this step three times. 20m

Staining 18h 20m

- 11 Dilute the primary antibody with permeabilization buffer to an optimal working concentration and resuspend the fixed/permeabilized cells with primary antibody solution. Incubate  **Overnight**  **4 °C** in the dark. 16h
- 12 Centrifuge at  **300 x g, 4°C, 00:05:00** and remove the supernatant. 5m
- 13 Wash with permeabilization buffer and centrifuge at  **300 x g, 4°C, 00:05:00** and discard the supernatant. Repeat this step three times. 20m
- 14 Dilute the fluorescent-conjugated secondary antibody with permeabilization buffer for an optimal working concentration and resuspend the cell pellet with secondary antibody solution. Incubate for  **01:30:00**  **Room temperature** in the dark. 1h 30m
- 15 Wash with permeabilization buffer and centrifuge at  **300 x g, 4°C, 00:05:00** and discard the supernatant. Repeat this step three times. 20m
- 16 Resuspend cells in 1000 µl PBS+AB buffer. Before cytometric analysis, filter the resuspended cells using cell strainers, mesh size: 30µm. 5m