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# Back to the future: Forgotten protocols for optimizing the isolation of arthropod haemocytes

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ABSTRACT

Consideration is given to previous and more recent protocols for harvesting arthropod haemocytes from *Galleria*, *Drosophila*, mosquitoes, *Limulus* and crustaceans. The optimal harvesting of these cells is essential for meaningful studies of invertebrate immunity *in vitro*. The results of such experiments, however, have often been flawed due to a lack of understanding of the fragile nature of arthropod haemocytes on exposure to bacterial lipopolysaccharides, resulting in the aggregation and loss of cell types during haemolymph clotting. This article emphasizes that although there are similarities between mammalian neutrophils and arthropod haemocytes, the protocols required for the successful harvesting of these cells vary significantly. The various stages for the successful harvesting of arthropod haemocytes are described in detail and should provide invaluable advice to those requiring both high cell viability and recovery of the different cell types for subsequent experimentation.

## 1. Introduction

Recently, impressive progress has been made in understanding the development, classification and functions of arthropod haemocytes and immunity using modern molecular and functional techniques such as single cell RNA sequencing, gene silencing, and advanced molecular labelling *in vivo* and in *vitro* (Campbell et al., 2024; Gomes et al., 2022; Hultmark and Andó, 2022; Koiwai et al., 2021; Kwon et al., 2021; Raddi et al., 2020; Severo et al., 2018; Söderhäll et al., 2022; Tattikota et al., 2020). Such studies are invaluable due to:

1. The continued use of *Drosophil*a as a source for understanding many of the mammalian biological, physiological, and neurological processes and for testing new therapeutic agents (Pandey and Nichols, 2011; Sheehan et al., 2018).
2. The adoption of *Galleria* as an alternative model to rhodents for the understanding of mammalian innate immunity and testing toxicological, microbial and pathological interactions (Campbell et al., 2024; Kavanagh and Reeves, 2004; Wojda et al., 2020; Wright and Kavanagh, 2022).
3. The need to investigate in more detail the role of mosquito cellular immunity in *Plasmodium* infections (Smith et al., 2016), and during other parasite life cycles, with the possibility of detecting new targets for control methods.
4. The new information revealed on the differentiation and functions of arthropod haemocytes (Hultmark and Andó, 2022; Koiwai et al., 2021; Kwon et al., 2021; Raddi et al., 2020; Severo et al., 2018; Söderhäll et al., 2022; Tattikota et al., 2020).

Many of these studies already utilise *in vivo* assays but the importance of understanding more about these processes and interactions by isolating and testing arthropod haemocytes *in vitro* with sophisticated labelling techniques has now been recognized (Campbell et al., 2024).

## 2. Haemocytes are not mammalian neutrophils!

Many studies have been published describing the similarities of the *Drosophila* and *Galleria* innate immune systems to that of mammals (Kavanagh and Reeves, 2004; Pandey and Nichols, 2011; Sheehan et al., 2018; Wojda et al., 2020; Wright and Kavanagh, 2022). The mammalian neutrophils, in particular, have been compared to insect haemocytes (Browne et al., 2013; Renwick et al., 2007). For example, the recognition pathways, Toll, IMD, JNK, and JAK-STAT and NFκB-like transcription factors in these cells are similar, as are the antimicrobial peptides and the superoxide generated via p47 and p67 proteins (Browne et al., 2013; Renwick et al., 2007). Mammalian leucocyte surface CD molecules have also been detected on the outside of *Galleria* haemocytes (Gallorini et al., 2024). These similarities therefore appear to have misled some scientists

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to assume that insect haemocytes can be harvested and manipulated in similar ways to mammalian neutrophils. Having worked with both mammalian neutrophils and arthropod haemocytes, however, the author very much emphasizes that to obtain optimal viabilities of these cells *in vitro* requires different harvesting protocols (Brookman et al., 1988; Pickaver et al., 1972). This fact has frequently been overlooked in many recent studies and as a result, arthropod haemocytes have often been incorrectly treated. Furthermore, comprehensive searches of the literature reveal, in the greatest detail, the correct protocols for isolating arthropod haemocytes and avoiding damage to the cells (see below). The necessity, cannot be overemphasized, of optimizing the basic and all-important haemocyte isolation processes for using the cells in i*n vitro* testing. Sometimes incorrect protocols from previously published papers are also cited which only serves to compound the spread of misinformation and further mislead subsequent workers. The consequences of this may result in the loss of haemocyte types so that experiments may generate incomplete data sets.

## 3. Characteristics of arthropod haemocytes

Prior working knowledge of arthropod immune systems and haemocytes would have revealed that in important ways arthropod haemocytes are very different to mammalian neutrophils. This fact has been confirmed many times previously (see below). For example, one common characteristic of arthropod haemocytes is their rapid instability *in vitro* after harvesting and this has frequently been described as in the following:

i. "When haemolymph is exposed to air, immediate blood coagulation takes place, as is the case for many insects—"(Admella and Torrents, 2022).
ii. In *Galleria*, "The first phase is modified clot formation resulting from granular cell lysis—" (Schmit and Ratcliffe, 1977).
iii. In dipterans, "GRs contained numerous small to medium size cytoplasmic granules and were often extremely fragile cells as compared with PRs and PLs. They were rarely found intact and often their presence in haemolymph samples was evidenced only by masses of discharged granules and naked nuclei" (Kaaya and Ratcliffe, 1982)
iv. "It is well known that hemocytes from crayfish are very sensitive and easily aggregate and lyse" (Söderhäll et al., 2022).
v. In the shrimp, *Sicyonia ingentes,* "At 1 min after withdrawal, spheres of clotted haemolymph were seen, each surrounding a lysed deposit cell" (Omori et al., 1989).
vi. In *Drosophila,* "plasmatocytes degranulate within seconds after wounding (or bleeding) and crystal cells rupture within minutes" (Theopold et al., 2014).

## 4. Blood clotting in arthropods and mammals

The reason for this difference between the stability of harvested arthropod haemocytes and mammalian neutrophils has been explained and is related to the nature of the circulatory systems in these animals (Sheehan et al., 2018). Mammals not only have a closed circulatory system but also adaptive immunity and blood clotting so that following wounding any invading microbes can be effectively localized and prevented from spreading. In arthropods with open circulatory systems, however, there are few if any blood vessels and instead large fluid-filled hydrostatic skeletons under pressure are present so that wounding could potentially result in a massive loss of haemolymph and a rapid spread of parasites and pathogens throughout the body. To avoid these problems, the clotting system in arthropods is more efficient than in mammals, sealing wounds and localizing the potential entry site almost immediately. In addition, insects have transglutaminases, that are homologous to human clotting factor XIIIa, but appear to be involved in the clotting cascade at a much earlier stage (Sheehan et al., 2018). It has been shown in the larvae of *Manduca sexta* that clotting is a two-step process involving cell aggregation followed by rigidification of the aggregate. This occurs in a few minutes, which is many, many times faster than wound plugging and scab formation in vertebrates (Aprelev et al., 2019). In crustaceans, clotting is also a very rapid and efficient process in which haemocytes release the clotting enzyme, a transglutaminase (TGase), into plasma and the clotting protein (CP) is crosslinked into polymers and forms a clot by the TGase. The CP was first characterized in crayfish, *Pacifastacus lenisusculus* (Hall et al., 1999) as was the clotting enzyme TGase (Wang et al., 2001). In addition, peroxinectin (Johansson et al., 1995) will be released from the haemocytes upon slight degranulation and this protein will cause haemocyte aggregation and cell adhesion of hemocytes to internal tissues. One interesting observation is that CP in addition to being a clotting protein is also an extracellular matrix protein in the hematopoietic tissue (HPT), maintaining the HPT stem cells in their stem cell stage inside the HPT (Junkunlo et al., 2018). Thus, in crustaceans, haemocyte stability is a must to avoid clotting and haemocyte aggregation and their attachment to internal tissues.

In conclusion, the clotting reaction is still not completely deciphered in insects as opposed to crustaceans. One possible reason for this is that the clotting reaction in insects is initiated very early due to the sensitivity of the haemocytes, so that potential cell adhesion and aggregation factors will be released already during manipulation, even in AC (anticoagulant), and the clots will therefore contain several different proteins.

## 5. The example of horseshoe crabs

There is also an extensive literature on the harvesting, not only of insect and crustacean blood cells, but also of horseshoe crab amoebocytes (haemocytes). In research on *Limulus polyphemus* amoebocytes, clues to the efficiency of the arthropod clotting system following exposure of the blood to the air has been widely recognized (Armstrong and Conrad, 2008; Pettipher et al., 2005). This animal contains numerous amoebocytes which are full of granules. Upon bleeding or wounding, these cells immediately release their granules and instigate a rapid clotting process to aggregate the cells and entrap and kill any invading bacteria. All the clotting components are in the amoebocyte granules. The trigger for this degranulation process is the lipopolysaccharide (LPS, endotoxin) produced by Gram-negative bacteria. Such is the sensitivity of the *Limulus* amoebocytes to LPS that a clinical test was developed, the *Limulus* amoebocyte lysate (LAL) assay, and used for over 30 years for testing for endotoxin in drugs, medical devices and food (Novitsky, 2009; Virzì et al., 2023). The sensitivity of this test is astounding and can detect as little as $10^{-12}$, sometimes $10^{-15}$ g, LPS per milliliter with a single Gram-negative bacterium containing ca. $10^{-14}$ g of LPS (Pettipher et al., 2005). For the LAL test, amoebocytes are harvested and prevented from degranulating and clotting following a detailed protocol to avoid endotoxin (Armstrong and Conrad, 2008). This LPS is ubiquitous in the environment, including in the water from stills and deionising columns which may not fully remove this toxin (Armstrong and Conrad, 2008; Gorbet and Sefton, 2004).

Like *Limulus*, most insects also have rapid blood coagulation in response to endotoxin and for which they have pattern recognition receptors (PRRs) such as the Gram-negative bacteria binding proteins (GNBPs) (Kim et al., 2000). In contrast to *Limulus* clotting, however, in which all the necessary components are confined to the amoebocytes, in different insect species clotting factors have been reported from the plasma, fat body cells, crystal cells, granulocytes, oenocytoids and plasmatocytes (eg. Kaaya and Ratcliffe, 1982; Moyetta et al., 2021; Schmid et al., 2019). In addition, clotting reactions in insects and crustaceans are commonly accompanied by lysis of the haemocytes in the clots (Rowley, 1977; Omori et al., 1989), as indicated above.

In summary, there is adequate published material to warn about the fragile nature of arthropod haemocytes and to emphasise the importance of developing protocols to stabilize the cells before use in

experiments. Ignoring this problem will probably result in both loss of cell types and quantities of cell debris in the harvestings.

## 6. Developing optimal protocols for harvesting arthropod haemocytes

It is highly recommended to consult one or more of the following and related articles to provide a basic background to this subject:

- Johansson, M.W., Keyser, P., Sritunyalucksana, K., Söderhäll, K., 2000. Crustacean haemocytes and haematopoiesis. Aquaculture 191 (1–3), 45–52. ISSN 0044–8486. https://doi.org/10.1016/S0044-8486(00)00418-X.
- Smith, V.J., Söderhäll, K., 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, Astacus by components of the prophenoloxidase activating system *in vitro*. Cell Tissue Res, 233 (2), 295–303. https://doi:10.1007/bf00238297.
- Söderhäll, K. and Smith, V. J. 1983. Separation of the hemocyte populations of *Carcinus maenas* and other marine decapods and prophenoloxidase distribution. Dev. Comp. Immunol. 229–239. https://doi.org/10.1016/0145-305X(83)90004-6
- Mead, G. P., Ratcliffe, N. A., Renwrantz, L. R., 1986. The separation of insect haemocyte types on Percoll gradients; methodology and problems. J. Insect Physiol. 32(2), 167–177. https://doi:10.1016/0022-1910(86)90137-x
- Armstrong, P., Conrad, M., 2008. Blood collection from the American horseshoe crab, *Limulus polyphemus*. J. Vis. Exp. 20, 958. https://doi.org/10.3791/958.

Initially, attention should be given to the culture methods of the arthropods used for experimentation. Previous results obtained with arthropod immune research have sometimes been variable from laboratory to laboratory due to differences in the methods used for maintaining the experimental animals. For example, with *Galleria* larvae and crustaceans, the animals are sometimes purchased from local pet shops and suppliers or online with little control over their nutrition or environmental conditions prior to use (Serrano et al., 2023). Diet, rearing temperature, weight, and light/dark ratio are all factors likely to affect the stress, immune efficiency and mortality of animals. Scientists working with vertebrates are usually in institutions with animal rearing facilities that are carefully monitored by government regulations. Likewise, invertebrates should ideally also be bred and maintained in-house although for horseshoe crabs and some crustaceans this may be problematic (Maurelli, 2022). Also, in contrast to some published recommendations and before use, animals should not be maintained at low temperatures or without food as this will stress and effect immunoreactivity (Serrano et al., 2023).

Equally important is the absolute need to avoid activating immunity during harvesting of the haemocytes by reducing exposure of the cells to endotoxins and other possible microbial components (PAMPS). The same applies during use of the haemocytes in *in vitro* experiments. The following should help in avoiding endotoxins and stabilize the haemocytes:

i. Clean the surfaces of animals with alcohol or disinfectant prior to use.
ii If perfusing animals with buffers, anticoagulants (AC) or particulates/beads then these should be made up in endotoxin-free water available from drug stores/pharmacists. Otherwise, the immune system will be activated and some cells will become sticky and remain stuck to internal tissues or filtration devices. Remember, water from stills and deionising columns may still contain endotoxin (Armstrong and Conrad, 2008; Gorbet and Sefton, 2004).
iii. All glass pipettes, slides etc should be treated at 180 °C for at least 3hr to remove endotoxin. Plastic items can be treated with E-Toxa-Clean (Sigma) (see, Mead et al., 1986; Söderhäll and Smith, 1983 for more details). Many more recent articles on the isolation of arthropod haemocytes indicate that the authors are unaware of the fragile nature of the cells or of the need to avoid endotoxin contamination. The use of an AC will stabilize the cells but making it up in sterile water and by filtration will not guarantee it is endotoxin-free. The best example is the horse-shoe crab amoebocytes which are extremely sensitive to degranulation and aggregation in response to LPS. This is why it is necessary to use LPS free solutions and to autoclave all material at 180 °C for ca 3 h to degrade LPS before it is in contact with the amoebocytes.
iv. If bleeding animals without prior perfusion with an AC, then immediately immerse the emerging haemolymph into an AC. Delays sometimes occur when harvesting from several small insects such as *Drosophila,* mosquitoes and even *Galleria* during which the blood is pooled. Allowing untreated harvested cells to attach to non-sterile but "clean" glass slides or in Petri dishes before AC treatment may activate degranulation, clotting and cell lysis. If in doubt, spend some time studying the haemocytes under high power phase contrast in which degranulation can often be observed even in AC.
v. The AC should be adjusted with NaCl to the osmolarity of the haemolymph of the arthropod used. In the citrate-EDTA buffer, the citric acid prevents cell lysis and the EDTA inhibits prophenoloxidase activation and clotting (Johansson et al., 2000).
vi. Centrifuging the harvested cells will inevitably result in some cell degranulation, lysis and loss of cells. Again, this effect is usually not taken into account in many papers. One exception is the work of Campbell et al. (2024) with *Galleria* who showed by flow cytometry that centrifugation reduced numbers of living cells and generated cell debris. Many other protocols published include centrifuging the haemocytes 2, 3, 4 or even 5 times, with no checks made of total or differential cell recovery numbers or of viability in comparison to untreated controls. Even AC treated cells will probably be activated by excess centrifugation and smearing on slides under any circumstances is not recommended.
vii. When developing any new protocol for harvesting the cells, rapid checks in a haemocytometer, or other means, of cell numbers, cell types and viabilities should be made at each stage of processing and compared with unprocessed or PBS controls. Some papers do viability checks at the end of processing and not surprisingly quote very high numbers as the more fragile cells have been lost during the protocol. In addition, the fragility of the haemocytes may vary from animal to animal and certainly from species to species.

## 7. Conclusion

The above includes advice for harvesting the haemocytes of any arthropod species including mosquitoes, *Drosophila, Galleria,* horseshoe crabs, shrimps, crabs and crayfish in which rapid clotting and cell lysis have been observed. The protocols used can be streamlined according to the results obtained. For example, Percoll separation of haemocyte types from different arthropods involves centrifugation during which, according to the species, some limited degranulation and lysis occurs (Mead et al., 1986; Söderhäll and Smith, 1983). Careful monitoring of the gradients will reveal the extent of these so that modifications to the protocols can be made. Above all, it is vital to optimize viable haemocyte recovery in order to ensure that the cells utilized for study represent the full complement of those in circulation.

## CRediT authorship contribution statement

**Norman A. Ratcliffe:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

## Data availability

No data was used for the research described in the article.

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