Taking Insect Immunity to the Single-Cell Level

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13   **Abstract** (120 words)

14

15   For decades, insect immunology has contributed groundbreaking discoveries on the

16   intricacies of innate immunity. It has profoundly impacted our understanding of innate

17   immunology in mammalian systems and improved human therapeutic interventions,

18   from the composition of vaccines to cell-based immune therapies. Our current

19   knowledge of insect immunity mainly encompasses detailed molecular mechanisms and

20   systemic responses to pathogen infection. However, the organs and specific cell

21   populations involved in immune responses remain elusive. Here, we highlight the need

22   for a better understanding of insect immune responses at the level of organs and cell

23   populations. Not only will it improve our current understanding of tissue or cell-specific

24   immune processes across species, but it will also pave the way for spatial modeling of

25   within-host infection dynamics.

26   **(Re)-discovering insect immunity using single-cell technologies**
27

28   Over the last few decades, intense research efforts exploiting the power of the model
29   organism *Drosophila melanogaster* have led to fruitful discoveries in the field of insect
30   immunology and impacted the field of innate immunity at large [1]. Innate immune
31   responses constitute the first line of defense against microbial agents and are defined
32   by their rapid activation, efficiency, and lack of memory. Pathogen recognition is
33   mediated by germline-encoded receptors recognizing pathogen-associated molecular
34   patterns (**PAMPs**). Innate immunity is often distinguished from adaptive immunity,
35   which emerged approximately 500 million years ago in vertebrates. Adaptive immune
36   responses are based on pathogen recognition by highly specific antibodies generated
37   by somatic gene rearrangements and long-term protective memory to counter
38   pathogen re-exposures [2].
39

40   Detailed insights into innate antibacterial, antifungal, and antiviral immunity have been
41   obtained in *Drosophila*, notably because of the vast genetic toolbox available in this
42   organism. Early studies focused on the mechanisms of pathogen recognition and
43   subsequent signal transduction. The discovery of an inducible antibacterial immune
44   response in the fruit fly, first reported in 1972, led to the discovery of **antimicrobial**
45   **peptides** (AMPs) that are not only conserved from insects to mammals, but are also
46   controlled by highly conserved nuclear factor-κB (NF-κB) signaling cascades [3]. In
47   1997, the discovery of the Toll receptor as a key factor in *D. melanogaster* antifungal
48   immunity [4] was a turning point for the field, as this signaling pathway turned out to be
49   homologous to the myeloid differentiation primary response protein 88 (MYD88)-
50   dependent **Toll-like receptor (TLR)** pathway in mammals [1].
51

52   Since those early days, critical molecular mechanisms for pathogen recognition have
53   been elucidated, and the coordination of immune responses at the organismal level
54   have been largely characterized [5]. Briefly, in insects (*Drosophila sp*)**., humoral**
55   **immunity** is controlled by a set of highly conserved signaling pathways. The **Toll** and
56   the **Immune deficiency** (IMD) pathways mainly control bacterial and fungal infections,
57   and the Janus kinase-signal transducer and activator of transcription (**Jak-Stat**) and

58 RNA interference (**RNAi**) pathways are primarily involved in antiviral defense [6]. In
59 reality, these pathways are **pleiotropic**, and their roles against a specific class of
60 pathogens are not always clearly distinguishable. Toll and IMD pathways also
61 participate in antiviral immune responses [6], and the Jak-Stat pathway mediates the
62 response to tissue damage that occurs upon bacterial, fungal, and parasitic infections
63 [7].

65 While humoral immunity is relatively well-characterized, **cellular immunity** in insects
66 (*Drosophila sp*.) remains more enigmatic. The best characterized immune cells are
67 **hemocytes**, which circulate in the insect hemolymph and differentiate in four main
68 subtypes: **plasmatocytes**, **granulocytes**, **lammelocytes**, and **oenocytoids** [8].
69 Our understanding of the immune pathways activated in those cells remains a black
70 box. Moreover, little is known about their movement and communication within immune
71 organs, somewhat due to the technical challenges linked to their study. In fact,
72 harvesting hemocytes from an insect's body cavity requires great technical skills [9] and
73 their number ranges from ≈500 to 3,000, depending on the age, genetic strain,
74 developmental stage, and infection status of the specific insect species [10, 11].

76 Beyond immune cells, immune organs are also poorly characterized. In adult insects,
77 the fat body tissue has, in addition to its metabolic function, a central role in immunity
78 [5]. It is a secretion site for antimicrobial peptides, but also a central signaling hub for
79 the activation of cellular immunity [12]. In *Drosophila*, the fat body activates hemocytes,
80 through a yet to be discovered mechanism that is Toll-dependent [13]. The exact
81 cellular composition of the fat body remains unknown, as is the identity of the cells that
82 can communicate with hemocytes, or coordinate immune processes.

84 In addition to *Drosophila*, non-model insect species are more challenging to study but
85 also medically more relevant. This review mainly highlights recent studies in mosquitoes
86 (*Anopheles* and *Aedes* genera) that contributed seminal discoveries to the field. The
87 completion of genome sequencing and assembly in important vector species, as well as
88 the advent of **RNAi-mediated knock-down** and **CRISPR-Cas9** technologies, have
89 been instrumental in the study of mosquito immunity [14-16]. Comparative genomics

90 reveal a high degree of conservation of immune signaling pathways, combined with
91 specific adaptations to particular pathogens or lifestyles. Overall, the core signaling
92 pathways are well conserved between *Drosophila* and mosquitoes, whereas upstream
93 signaling factors and downstream effectors diverged during evolution [17, 18]. The
94 opinions expressed in this paper are mostly based on work in *Drosophila* and
95 mosquitoes but are widely applicable to the study of ticks (*Ixodes* genus), sandflies
96 (*Phelobotominae* subfamily) and further arthropods that are the focus of remarkable
97 research efforts (**Box 1**).

98

99 Single-cell technologies encompass a vast array of methods to study genomics,
100 epigenomics, proteomics and metabolomics at the single-cell level [19-21]. Here, we
101 focus on single-cell RNA sequencing (scRNA-seq) and single-cell transcriptomics,
102 which are on their own, revolutionary and paradigm-shifting. In 2009, the first study
103 using single-cell transcriptome analysis based on a high-throughput sequencing
104 platform led to the characterization of cells from early-stage murine embryos [22].
105 Several protocols have been developed over the last decade, leading to an impressive
106 increase in throughput and data quality [21]. Historically, for many technical reasons,
107 transcriptomic studies have typically been performed on large groups of cells originating
108 from multiple individuals, multiple tissues, or pools of individual or tissues, therefore
109 referred to as **bulk RNA-seq**. Hence, several levels of biological variation, including
110 organismal, tissular and cellular differences, could be "hidden" in one transcriptome
111 data set (**Figure 1**). Single-cell transcriptomics allow for the first time, to study cell-to-
112 cell transcriptional heterogeneity in insects, including the gene expression stochasticity
113 that was first reported over 15 years ago [23].

114

115 How can single-cell technologies help to accomplish a leap forward for studies of insect
116 immune systems? Could these technologies enable, in the near future, the acquisition
117 of a more comprehensive picture of insect immunity? Can they integrate into the same
118 model, our knowledge of molecular mechanisms and systemic responses by bridging
119 those components with a better knowledge of cellular and tissular immunity? In this
120 opinion article, we argue that the increasing availability and applicability of scRNA-seq

121 present a new opportunity to deepen our knowledge of insect immunity and impact our
122 global knowledge of innate immunology across species.
123
124 **How can single-cell transcriptomics be instrumental to the study of insect**
125 **immunity?**
126
127 *Deciphering the cellular composition of organs*
128
129 The cellular composition of organs, including immune sites, remains to be investigated
130 in insects. Early studies based on cellular morphology helped decipher the composition
131 of tissues and led to the classification of hemocytes [24] and digestive tract cells [25].
132 However, a recent study revealed that *Anopheles gambiae* mosquito hemocytes display
133 a high level of complexity, given that two highly distinct cellular "populations" -- as
134 defined by their transcriptional profile -- actually share morphological and functional
135 features, such as their phagocytotic capacity [26]. Therefore, the classification of cell
136 types historically based on morphology or function might need to be revisited. In
137 addition, little is known about the cellular re-composition of organs after an infectious
138 episode. In *Drosophila*, infection of the intestinal tissue with the Gram-negative
139 bacterium *Erwinia caratovora* has resulted in dramatically increased stem cell
140 proliferation and epithelial renewal, as shown by the increase of mitotic recombination in
141 the intestinal tissue, accompanied by a higher number of stem cells (expressing the
142 *escargot* marker gene) upon infection [27]. However, it remains to be determined
143 whether the cellular content of the tissue pre and post pathogen exposure is
144 comparable, or if infection-induced changes of immune site composition could impact
145 subsequent immune responses. In fact, *Plasmodium berghei* (rodent malaria)-
146 challenged mosquitoes constitutively secrete a hemocyte differentiation factor into their
147 hemolymph that induces **immune priming** when experimentally transferred into naive
148 mosquitoes [28]. The hemocyte differentiation factor was first discovered in mosquito
149 hemolymph by mass spectrometry, and is a complex consisting of a protein (called
150 Evokin) that is a lipid carrier of lipoxins [28]. Immune priming is characterized by an
151 increased number of granulocytes (a subpopulation of hemocytes) and can be
152 measured by extracting hemolymph from mosquitoes and counting the number of cells

present while assessing their morphology and binding properties [29]. *P. berghei*-challenged mosquitoes mount a strong immune response when the parasite breaks through the gut and, by doing so, brings the gut bacteria in contact with injured epithelial gut cell. This stimulation creates a state of systemic immune surveillance upon which the immune system is primed to respond again to a new exposure. Upon re-infection, the immune system mounts a stronger antibacterial response that is also detrimental to the survival of *Plasmodium sp.* parasites [29]. It will be interesting in the future to analyze the transcriptome of these cell populations at the single-cell level and understand the depth of immune priming processes. In fact, this could yield new insights into the mechanisms of immune memory that are so far poorly understood in insects.

Discovering new immune cell populations

In addition to discovering new cell populations that are induced by infection, single-cell sequencing can also enable identification of cells that hold an immune function based on their transcriptional similarity with existing immune cells, but have not yet been characterized as such. In fact, it is likely that most tissues contain cells that mediate their defense and viability upon infection but are rare, non-canonical, or difficult to characterize. For instance, the immune capacity of glial cells in the *Drosophila* brain was only discovered recently, with the observation that they were able to secrete AMPs [30]. Additionally, infection of nervous tissues such as the brain by Zika virus has recently be modeled in *Drosophila* and pointed to a role for the IMD pathway in glial cells [31]. In fact, mutant flies that have a deficient IMD signaling failed to induce autophagy, an antiviral process that helps reducing Zika virus infection [31]. Single-cell sequencing, which is virtually applicable to any tissue or organs, is likely to contribute exciting discoveries of new immune cell types in insects that were so far unreachable using traditional methods.

Assessing heterogeneity of immune responses to infection

184 Recent studies on mammalian cells such as the human lung epithelial carcinoma line
185 A549, the human hepatoma line (Huh7), or the murine fibroblast L929 line, utilized
186 scRNA-seq to assess the diversity of the transcriptional responses to infections by
187 influenza, dengue, Zika and West Nile viruses [32-35]. Taken together, these studies
188 revealed astonishing heterogeneity at the level of host transcriptomes and viral
189 abundance from cell to cell. The measurement of both host and viral RNA transcripts
190 (also called visc-RNAseq) in the same cells can reveal correlations between gene
191 expression, and make possible the detection of strong pro- or antiviral candidate genes
192 [33]; indeed, this was previously done in a bulk RNA-seq study on dengue virus-
193 infected midguts of *Aedes aegypti* mosquitoes, that identified the sterol regulatory
194 element-binding protein (SREBP) as a host factor with a proviral role in the mosquito
195 midgut [36]. Moreover, A scRNA-seq study performed on peripheral blood mononuclear
196 cells isolated from dengue virus-infected human patients identified a set of genes that
197 might be used as biomarkers for disease progression to severe dengue fever [37]. For
198 example, the expression of MX2 within naive B cells is strongly upregulated before the
199 onset of severe dengue fever [37]. The predictive power of such markers discovered by
200 visc-RNAseq remains to be tested with larger study cohorts. Finally, quantifying the
201 heterogeneity of immune responses to infection in relation to pathogen presence might
202 also help identify by-stander effects of cells near infection sites that are not infected
203 themselves, but which deploy immune responses linked to the detection of infection-
204 linked signals in their environment. For example, in *Drosophila*, reactive oxygen species
205 are released from necrotic cells and act as an immediate danger signal which can
206 induce he recruitment of hemocytes to the injured tissue [38]. Indeed, it will be
207 interesting to compare immune profiles of infected cells versus uninfected cells that
208 have been alerted of an ongoing infection by danger signals in their intra-host
209 environment.
210
211 *Assessing heterogeneity in pathogen genetic diversity upon infection*
212
213 Pathogen-inclusive scRNA-seq opens possibilities to assessing pathogen genetic
214 diversity such as viral quasispecies at the intra-cellular level, as has already been
215 done for hepatitis C virus, human immunodeficiency virus and vesicular stomatitis virus

216   [39]. In insect vectors of viruses, it will be possible to identify cellular subtypes that are
217   responsible for increasing viral diversity, or on the contrary, act as **population**
218   **bottlenecks**. In fact, the organs and cell types that are sites of viral replication and
219   enable the virus to genetically diversify or homogenize, are not characterized despite
220   being important targets for future vector-control strategies. Intra-host viral diversity can
221   impact **viral fitness**, and perhaps the transmission efficacy of a given virus population,
222   as well as potentially, the epidemiology of an outbreak [40, 41]. For example, it was
223   shown that the fitness of West Nile virus decreased as it spread from one mosquito
224   organ to another: the virus replicated poorly in avian cells after a passage through *Culex*
225   *quinquefasciatus* mosquitoes [41]. Single-cell technologies might help identify cellular
226   sites and molecular mechanisms of viral diversity within the insect host and potentially
227   support genetic interventions aiming at reducing pathogen transmission.
228
229   Beyond viral infections, a recent scRNA-seq study that focused on *Plasmodium* sp.
230   parasites revealed discrete transcriptional signatures during the parasite's life cycle, as
231   well as unexpected sex-specific roles for contingency gene families (*var* in *P. falciparum*
232   and *pir* in *P. berghei*) that are known for enabling parasite adaptation to new
233   environments [42]. In addition, scRNA-seq enabled the Malaria Cell Atlas to characterize
234   thousands of single-parasite transcriptomes across the entire *Plasmodium* sp. life cycle
235   [43]. Those studies are relevant as they may open multiple new research avenues --
236   from the analysis of nonculturable life stages of *Plasmodium* species (such as *P. vivax*),
237   to the analysis of cell-to-cell variability in response to drugs and vaccines, as well as the
238   development of blocking strategies against infection in humans. Overall, pathogen-
239   inclusive scRNA-seq offers exciting research avenues that will allow to assess the
240   impact of the host cells on pathogen evolution and adaptation.
241

242   **What are the main challenges of single-cell sequencing implementation in**
243   **insects?**
244

245   *Dissociation and fixation*
246

247   The first step of any single-cell sequencing experiment consists of the preparation of the

248   cell suspension. With the exception of hemocytes that circulate in the insect's

249   hemolymph, cells are embedded in tissues and organs that have to be dissociated in

250   order to obtain a suspension of single cells. Dissociation methods are varied, ranging

251   from the use of a diverse set of enzymes to the use of mechanical force (**Table 1**).

252   Most often, several methods need to be tested, as their efficiency is often dependent on

253   the cell type, tissue, organism, and insect species. Dissociation is an aggressive

254   process that causes cellular stress and therefore transcriptional changes related to

255   stress responses. Accordingly, if feasible, it is preferable to fix cells and thus preserve

256   the transcriptome before dissociation. Several fixation agents are available

257   (paraformaldehyde, acetone, methanol, Lomant's reagent, RNAlater™, vivoPHIX™) ;

258   some have been tested in side-by-side studies with live cells and do not seem to bias

259   or alter the quality of the transcriptome [44, 45]. In all cases, a set of quality indicators

260   should be measured: (i) dissociation efficacy, (ii) cell viability and (iii) cell integrity (**Box**

261   **2**). Importantly, dissociation methods can potentially affect the transcriptome of cells or

262   a subset of cells [46]. It was recently shown that a subset of cells detected in several

263   single-cell studies, for example in mouse muscle cells or zebra fish osteoblasts, was

264   over expressing genes associated with tissue injury (i.e. heat shock proteins such as

265   *Hspa1a* and *Hspa1b*). However, those cells were not found as a functional subset *in*

266   *vivo*, pointing to a likely experimental artifact [46]. Noteworthy, these 'injured' cells might

267   also be present as potential contaminants in bulk RNA-seq studies. However, such

268   transcriptional signals would be harder or likely impossible to identify, as they would be

269   averaged at the level of an entire tissue or individual. By contrast, In a single-cell study,

270   'injured' cells could be identified and excluded from subsequent analyses.

271

272   Insect cells present additional challenges at the dissociation step due to their limited

273   number and reduced size. Insect cells are often smaller in size than their mammalian

274   counterparts [47, 48]. For instance, *Drosophila* neurons are ten times smaller than

275   mammalian neurons [49]. Moreover, insect organs and tissues are small in size. The

276   *Drosophila* brain contains approximately 100,000 cells [48], while the human brain

277   contains 100 billion cells [50]. While it is possible to dissect high numbers of organs for

278   single-cell experiments in insects, never will it be possible to reach the order of

magnitudes of larger organisms. This dramatically complicates the execution of single-cell experiments, not only because of the sheer number of cells available but also because cell recovery after dissociation and cell viability have to be extraordinary. Once dissociation and potential fixation are optimized, RNA sequencing can take place. Apart from cellular sizes that might not be adapted to all microfluidic platforms, which are generally designed for mammalian cells, no specific considerations need to be taken for the sequencing of insect cells.

Data analysis and interpretation

Amongst the significant applications of scRNA-seq is the identification of novel cell types and states, founded on the detection of co-expression patterns and clustering of cells based on their transcriptomic similarity. Cell clusters can be analyzed further by identifying gene sets characteristic of each cluster, also called markers. Whereas markers for mammalian immune cells are generally well known, they remain elusive for most insect cells. Cluster analysis and marker discovery is, therefore, more challenging for insect cells, as preliminary knowledge about sub-groups to be detected is scarce. Recently, an unbiased classification of *An. gambiae* hemocytes was performed using a combination of scRNA-seq, high-content imaging flow cytometry, and single-molecule RNA hybridization [26]. It revealed an unexpected degree of complexity in the hemocyte population together with the existence of cellular cross-talk via exchange of mRNA between cells [26]. This combination of required tools highlights the need for orthogonal validation methods, especially in non-model insect systems for which there is little preconceived knowledge about cell populations, and almost no cell markers available. Overall, scRNA-seq data analysis and interpretation is challenging for insect cells and, given the poor availability of markers for many cell populations, will require cross-validation with other experimental methods.

How can single-cell sequencing data be exploited to model immune responses in insects?

311

312 Single-cell sequencing data sets are comparatively more complex and multi-

313 dimensional then classical bulk RNA-seq data sets. Beyond the standard analysis

314 methods, consisting of reducing dimensional space (by PCA and t-SNE), functional

315 clustering or trajectory analysis [21], even more sophisticated data analysis methods

316 could help informing insect immunity.

317

318 Subjecting multiple tissue samples over an infection's time course to scRNA-seq could

319 help inform infection dynamics in a deeper manner than has ever been possible. Each

320 data set might describe degree of infection, correlating it with transcriptional responses

321 at the cellular level in different tissues over time. Given the high dimensionality of such

322 data, integration into a mathematical modeling framework is highly desirable. Such

323 information might help identify key immune cells and processes, as well as model the

324 progression of the pathogen through the host's body. Once built, such a modeling

325 framework could ideally be used to predict infection outcomes, severity, or further

326 pathogen transmission potential.

327

328 For instance, when an *Aedes* mosquito bites a human infected with an arbovirus such

329 as Zika virus, the bloodmeal containing the virus is first ingested in the mosquito midgut.

330 The virus initially replicates in midgut cells before disseminating throughout the

331 mosquito's body. Eventually, the virus reaches the salivary glands and can be

332 transmitted again through a new bite [51]. The exact progression of the virus

333 throughout the mosquito's body remains elusive. How does the virus circulate in the

334 hemolymph? Is it carried by hemocytes or does it circulate freely? Which organs does

335 the virus replicate in, and when? Are similar immune pathways triggered in the midgut,

336 fat body, and salivary gland? Can we predict the transmission potential of a certain virus

337 based on the host immune response and viral levels in certain organs? Such

338 fundamental questions might be answered, at least in part, by the use of single-cell

339 technologies and modeling of infection dynamics.

340

341 Concluding remarks

342

343 For decades, the study of innate immune processes in insects has greatly contributed
344 to our overall knowledge of innate immunology. Here, we propose that the recent
345 developments of single-cell transcriptomics represent an opportunity to make a
346 significant leap forward in this exciting research field. Improving our knowledge of
347 immunity at the cellular and tissular level has the potential to be paradigm shifting at the
348 fundamental level (see outstanding questions). Indeed, it may also have a wider impact
349 on novel strategies deployed to control vector-borne diseases.
350
351 Nowadays, performing single-cell transcriptomics remains challenging technically, but
352 also financially. Although the protocols and technologies evolve rapidly, the costs of the
353 technology remain high. Hopefully, the constant decrease of sequencing costs and
354 commercialization of scRNA-seq by more industrial actors will make it more accessible.
355
356 Spatial transcriptomics are a recent and exciting technological development [52]. In
357 addition to obtaining single-cell transcriptomes, this technology allows to map the
358 transcriptional activity to its tissular location and place it in a morphological context. In
359 fact, it relies on the deposition of tissue slices on arrays covered with probes that
360 capture RNA. After sequencing of the array, the transcriptomes are reconstituted in
361 space and mapped to their location of origin on the tissue slice [53]. This will help
362 identification of immune cells, infected cells, by-stander cells, rare cell types on a 3D
363 map, and may contribute to a more holistic understanding of immune responses at a
364 cellular, tissular and organismal level.
365
366 Here, we focused on the promise of single-cell transcriptomics to improve our
367 knowledge of insect immunology, without mentioning the development of other single-
368 cell based technologies. Performing DNA sequencing at the level of single cells can also
369 help identify cell-to-cell heterogeneity in single-nucleotide polymorphisms and **copy**
370 **number variations**. Moreover, **DNA mosaicism** remains an unexplored territory in
371 insects although it has been extensively investigated in the context of human health
372 [54]. Single-cell proteomics and epigenomics have also been developed more recently,
373 offering multiple and diverse research possibilities in the near future [19, 20].
374

375   Finally, we posit that single-cell transcriptomics will need integration at the systems
376   level, i.e. using a holistic approach to capture emerging properties of cells and tissues
377   functioning as a system. This will be possible with the complementary single-cell based
378   technologies aforementioned, and more generally, with various approaches of
379   genomics, proteomics, epigenomics, metabolomics and lipidomics [55]. For now, it
380   remains challenging to perform experiments and analyze samples in such a multi-
381   dimensional manner for technical and financial reasons. However, we predict that within
382   the next decade, the development of sophisticated computational methods will enable
383   seamless data integration and analysis at a systems level. Alexander von Humboldt was
384   the first scientist to suggest that, while each element in nature demanded specialized
385   analytical attention in order to establish its own specific rules of order and organization,
386   the most important challenge was that of integrating all this knowledge. With his 250th
387   birthday, his vision remains as relevant and applicable to systems biology in the 21st
388   century.

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421   **Figure legend**

422

423   **Figure 1**. Schematic overview of the advantages of scRNA-seq over bulk RNA-seq.

424   Over the last few decades, RNA sequencing technologies have evolved from using

425   pools of individual insects, to single individuals, to single organs. At every step, the

426   transcriptome obtained is an "averaged" picture of gene expression. It reflects several

427   individuals, several organs, or several cell types. Only using single-cell sequencing is it

428   possible to get a unique cellular transcriptome.

429

430   **Table 1**. Summary of the published work applying scRNA-seq to insect cells.

Organism	Cell type	Dissociation method	Fixation method	Sequencing method	Reference
D. melanogaster	Total embryonic cells	Douncer homogenizer (mechanical)	Methanol	Drop-Seq	[56]
D. melanogaster	Olfactory projection neurons	Papain Liberase™	/	Smart-Seq2	[57]
D. melanogaster	Eye disc cells	Collagenase Trypsin	/	Drop-Seq	[58]
D. melanogaster	Total brain cells	Dispase Collagenase I	/	Chromium Smart-Seq2	[48]

		Trypsin		Cel-Seq 2 ATAC-seq	
D. melanogaster	Midbrain cells	Papain Collagenase I	/	Drop-seq	[49]
A. gambiae	Blood cells	/	/	Smart-Seq2	[26]

431

432 **Box 1.** scRNA-seq for studying immunity in arthropod vectors beyond mosquitoes.

433 The majority of published work describing the use of scRNA-seq in insects has been

434 performed in *Drosophila* sp. and mosquitoes (Table 1). However, scRNA-seq is a

435 technology that is applicable to arthropod vectors beyond mosquitoes, such as fleas

436 (*Siphonaptera* order), ticks (*Ixodes* genus), tsetse flies (*Glossina* genus) and sandflies

437 (*Phlebotominae* subfamily). Most current knowledge on insect immune signaling

438 pathways has been acquired in *Drosophila*, and served as a roadmap for studying

439 immunity in other arthropods. However, recent studies have revealed that immune

440 signaling pathways in…. are mechanistically more distinct than anticipated,

441 emphasizing the need for in-depth studies in other arthropods [59, 60]. For example,

442 ticks have a smaller repertoire of Toll receptors compared to *Drosophila* [61] and have

443 also undergone gene losses in the IMD pathway, despite both pathways remaining

444 functional [62, 63]. This suggests that unidentified immune pathway components might

445 be replacing predicted canonical factors to mediate the recognition of microbial

446 invaders in ticks, although this remains to be further investigated [64]. It might be

447 possible in the future to isolate individual cells in which those pathways are active (e.g.

448 using flow cytometry) and analyze their transcriptome by scRNAseq to find the

449 unknown components that keep the pathway functional. Another recent study [65]

450 analyzed the role of a mutualist bacterial symbiont on the adult immune response of the

451 tsetse fly, *Wigglesworthia* [65] . It revealed that the symbiont induced the upregulation

452 of an odorant binding protein (Obp6) in the gut of intrauterine tsetse larvae relative to….,

453 which was necessary and sufficient to induce the subsequent production of **crystal**

454 **cells** mediating the **melanotic immune response** [65]. In the future, it might be

455 possible to determine, using scRNA-seq, exactly which cell types secrete Obp6, and

456 which tissues are targeted by this molecule. Beyond these examples, we argue that

457 scRNA-seq will help elucidate fascinating biological processes, and enable in-depth

458 studies in a broad range of arthropods that have so far been understudied because of
459 the limited toolbox available.
460

461 **Box 2.** Essential questions to ask during sample preparation for scRNA-seq.
462 • How many cells are recovered after dissociation? Is that number consistent?
463 • Is the tissue fully dissociated?
464 • What is the percentage of cell death?
465 • Are the live cells intact and healthy? Are there apoptotic or necrotic cell bodies in
466 the cellular suspension?
467 • Are the cells metabolically active during dissociation potentially leading to
468 changes in the transcriptome?
469 • Does the cell suspension contain cellular debris to be filtered?
470 • Are cells recovered after fixation?
471 • Are the cells potentially infectious?
472 • Do the cells need to be used immediately or can they be stored before use?
473

474 **Glossary** (450 words)
475

476 **Antimicrobial peptides:** In insects, AMPs are a class of small peptides with potent
477 antimicrobial activity.
478

479 **Humoral immunity**: Immune responses mediated by factors present in extracellular
480 fluids, mainly the hemolymph in insects.
481

482 **Toll pathway**: Immune pathway of insects mainly triggered by Gram-positive bacteria
483 leading to antimicrobial peptide production.
484

485 **IMD pathway**: Immune pathway of insects mainly triggered by Gram-negative bacteria
486 leading to antimicrobial peptide production.
487

488 **Jak-Stat pathway**: Immune pathway of insects triggered by the Unpaired cytokines
489 mainly upon viral infection.

490

RNAi: RNA interference refers to a set of gene-silencing mechanisms induced by double-stranded RNA among which the small interfering RNA (siRNA) pathway is considered a major antiviral defense of insects.

Pleiotropic: A gene or pathway is called pleiotropic when it affects two or more seemingly different phenotypic traits.

Cellular immunity: Immune responses mediated mainly by hemocytes.

CRISPR-Cas9: Prokaryotic immune system based on Clustered Regularly Interspaced Short Palindromic Repeats and the endonuclease Cas9, which was converted into a powerful a gene-editing technique targeting specific sequences recognized by a complementary guide RNA.

Bulk RNA-seq: Conventional RNA sequencing technique that probes the transcriptome of a large number of pooled cells.

Immune priming: analogous to trained immunity, it describes the phenomenon by which a first pathogen exposure enhances host resistance to a subsequent challenge.

viscRNAseq: virus-inclusive RNA sequencing probes the host transcriptome together with intra-cellular viral RNA at the single-cell level.

Viral quasispecies: Cloud of diverse virus variants that are genetically linked through mutation and collectively contribute to the characteristics of the viral population.

Population bottleneck: In evolutionary biology, it is defined as an event that drastically reduces the size of a population.

Viral fitness: Usually equated to replicative fitness, it is defined as the capacity of a virus to produce infectious progeny in a given cellular environment.

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PCA: Principal component analysis is a linear dimensionality reduction method commonly used to identify cell subpopulations by clustering their features in 2D or 3D.

t-SNE: t-distributed stochastic neighbor embedding is a dimensionality reduction method that helps identifying cell subpopulations and sometimes provides clearer visualization of clusters then PCA.

RNAi-mediated knock-down: Experimental gene-silencing technique using double-stranded RNA or siRNAs to neutralize target RNA molecules by RNAi.

Plasmatocytes: Macrophage-like cells involved in phagocytosis and encapsulation that are the major constituents of the insect hemocyte population.

Granulocytes: Adhesive cells mediating phagocytosis, encapsulation and degranulation in insects.

Lammelocytes: Large cells involved in encapsulation of invading pathogens in insects.

Oenocytoids: Large cells responsible for the production of prophenoloxidase in insects.

Crystal cells: Cells responsible for humoral melanization that accompanies some immune reaction in insects.

Pathogen-Associated Molecular Patterns (PAMPs): Diverse set of molecules that share a set of general "patterns," or structures, that alert immune cells to destroy invading pathogens. One example is lipopolysaccharides present in the membrane of Gram-negative bacteria.

Toll-like receptor (TLR): A class of pattern recognition receptors that initiate the innate immune response by sensing pathogen-associated molecular patterns.

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Hemocytes: A group of several cell types that are found in the hemolymph of insects.

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Copy number variation: Phenomenon in which the number of copies of a particular gene varies from one individual to the next.

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DNA mosaicism: Phenomenon that involves the presence of two or more populations of cells with different genotypes in one individual that is called a mosaic.

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Melanotic immune response: A branch of the innate immune response that involves the synthesis of melanin, a dark pigment that can encapsulate invading pathogens or unhealthy cells.

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716

Figure 1

Pool of multiple individuals

Gene X expression

Pool 1 Pool 2 ....

Individual 1
Individual 2
Individual 3
Individual 4
....

Single individual

Gene X expression

Individual 1 Individual 2 ....

Organ 1
Organ 2
Organ 3
Organ 4
....

Single organ

Gene X expression

Organ 1 Organ 2 ....

Cell 1
Cell 2
Cell 3
Cell 4
....

Single cells

Gene X expression

Cell 1 Cell 2 ....