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Single-cell RNA sequencing in *Drosophila*: Technologies and applications

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Abstract

Single-cell RNA sequencing (scRNA-seq) has emerged as a powerful tool for investigating cell states and functions at the single-cell level. It has greatly revolutionized transcriptomic studies in many life science research fields, such as neurobiology, immunology, and developmental biology. With the fast development of both experimental platforms and bioinformatics approaches over the past decade, scRNA-seq is becoming economically feasible and experimentally practical for many biomedical laboratories. *Drosophila* has served as an excellent model organism for dissecting cellular and molecular mechanisms that underlie tissue development, adult cell function, disease, and aging. The recent application of scRNA-seq methods to *Drosophila* tissues has led to a number of exciting discoveries. In this review, I will provide a summary of recent scRNA-seq studies in *Drosophila*, focusing on technical approaches and biological applications. I will also discuss current challenges and future opportunities of making new discoveries using scRNA-seq in *Drosophila*.

This article is categorized under:

Technologies > Analysis of the Transcriptome

Keywords

drosophila ; single-cell RNA sequencing

1 | INTRODUCTION

Transcriptomic analysis has been widely used to study gene expression patterns for understanding the functions of a tissue or a population of cells. The advent of next-generation sequencing has transformed transcriptomic studies (Schuster, 2008; Soon, Hariharan, & Snyder, 2013). Most of what we have learned from transcriptomic research about fundamental principles underlying biological processes comes from bulk RNA-seq, which provides averaged gene expression from a whole tissue or from a large number of individual cells at scale of the entire transcriptome. However, the bulk approach can mask

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CONFLICT OF INTEREST

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meaningful differences between molecularly similar cell types within a tissue. The analysis of single cells has the potential to overcome these limitations and can provide unprecedent molecular resolution for understanding cell functions at the single-cell level. Meanwhile, the existing high throughput datasets from bulk RNA-seq, such as modENCODE (Consortium, 2010), provide good resources for validating the sequencing data of scRNA-seq studies.

The past decade has witnessed the emergence and rapid development of a host of single-cell sequencing technologies (Gawad, Koh, & Quake, 2016; Labib & Kelley, 2020; Schwartzman & Tanay, 2015; Shapiro, Biezuner, & Linnarsson, 2013; Stuart & Satija, 2019; Tanay & Regev, 2017), including single-cell RNA sequencing (scRNA-seq), which allows the survey of transcriptomes of individual cells. scRNA-seq offers unique opportunities for both basic and clinical research, such as identifying new cell types, exploring cell heterogeneity, revealing developmental trajectories, studying drug resistance, and investigating cancer relapse (Haque, Engel, Teichmann, & Lönnberg, 2017; Hwang, Lee, & Bang, 2018; Liu & Trapnell, 2016; Potter, 2018). scRNA-seq has already significantly impacted our conceptual understanding of diverse biological processes.

The fruit fly, *Drosophila melanogaster*, is a premier model organism to study fundamental and evolutionarily conserved biological mechanisms ranging from development to aging, largely owing to the availability of sophisticated genetic tools. Combining scRNA-seq with powerful genetic tools holds a great potential for making new discoveries. Indeed, recent scRNA-seq studies in *Drosophila* have revealed novel biological findings, such as characterizing new cell types in different tissues including the whole embryo, whole brain, ventral nerve cord, gut, blood, abdominal cuticle, testis, and ovary (Allen et al., 2020; Brunet Avalos, Maier, Bruggmann, & Sprecher, 2019; Cattenoz et al., 2020; Cho et al., 2020; Croset, Treiber, & Waddell, 2018; Davie et al., 2018; Fu, Huang, Zhang, Leemput, & Han, 2020; Ghosh et al., 2019; Guo et al., 2019; Hung et al., 2020; Jevitt et al., 2020; Karaiskos et al., 2017; Rust et al., 2019; Shin, Jones, Petkau, Panteluk, & Foley, 2019; Slaidina, Banisch, Gupta, & Lehmann, 2020; Tattikota et al., 2020; Witt, Benjamin, Svetec, & Zhao, 2019), revealing unrealized mechanisms underlying neural development and brain aging (Davie et al., 2018; Konstantinides et al., 2018; Kurmangaliyev, Yoo, LoCascio, & Zipursky, 2019; Li et al., 2017, 2020), and uncovering transcriptional regulation or signaling pathways controlling development and tumorigenesis (Ariss, Islam, Critcher, Zappia, & Frolov, 2018; Deng et al., 2019; Genovese et al., 2019; Ji et al., 2019). So far, scRNA-seq profiling has been performed in various *Drosophila* tissues from multiple stages (Figure 1 and Table 1), providing valuable resources for future studies of those individual tissues or tissue-tissue interactions.

In this review, I will summarize scRNA-seq technologies in general with an emphasis on those that have been used in *Drosophila* and discuss how scRNA-seq is employed as a tool for exciting biological discoveries in this model. For a broader view of single-cell analysis beyond scRNA-seq in *Drosophila* research, see (Gawad et al., 2016; Labib & Kelley, 2020; Packer & Trapnell, 2018; Schwartzman & Tanay, 2015; Tanay & Regev, 2017).

2 | SINGLE-CELL RNA-SEQ IN *DROSOPHILA*: TECHNOLOGIES

The first scRNA-seq method was reported in 2009 (Tang et al., 2009). Since then many different scRNA-seq platforms have been developed (Chen, Ning, & Shi, 2019). scRNA-seq faces a number of challenges. The two primary challenges are the low cell capture efficiency and the low amount of input RNA material from individual cells. *Drosophila* cells are much smaller than mammalian cells with fewer RNA transcripts per cell, further magnifying the second challenge.

Depending on biological systems or study purposes, different scRNA-seq protocols have been used. Almost all of these protocols can be divided into five major steps (Figure 1a): (a) preparing single-cell suspension, (b) capturing individual cells, (c) making cDNA and barcoded libraries, (d) sequencing, and (e) analyzing data. With the maturation of scRNA-seq technologies and bioinformatics, commercially available kits and standardized data analysis platforms are rapidly increasing. Here, I will focus on the first three steps of these protocols and will not discuss sequencing, as it is relatively standardized. Data analysis methods will be discussed in the following section.

2.1 | Tissue dissociation and single-cell suspension

For all large-scale scRNA-seq experiments, preparing the single-cell suspension is the first step. If starting materials are cultured cell lines or circulating blood cells (called hemocytes in *Drosophila*), making single-cell suspension is relatively easy (Cattenoz et al., 2020; Fu et al., 2020; Tattikota et al., 2020). In most other cases, dissected tissues need to be dissociated using either specific set of enzymes or mechanical force or both. Widely used dissociating enzymes in *Drosophila* include trypsin, collagenase, papain, liberase, and elastase, and in most cases these enzymes are used in combination to improve the dissociation efficiency (Table 1). When choosing a dissociation method, it is best to test multiple methods as their efficiency can vary significantly, depending on the cell type, tissue type, and developmental stage. Another important factor that needs to be considered is the cell viability. Since tissue dissociation is a harsh process, it can cause cellular stress and transcriptional changes. Thus, if two methods can both adequately dissociate the desired tissue, the less damaging one with higher cell viability should be used.

As stated above, cell dissociation can lead to endogenous transcriptional alterations, and minimizing these changes is important for downstream analyses. Tissue fixation can preserve transcriptome integrity, and recent studies have begun to explore the feasibility of adding a fixation step for scRNA-seq. For example, paraformaldehyde (PFA) was used to fix human radial glial cells for single-cell transcriptomic analysis (Thomsen et al., 2016). It is worth mentioning that PFA fixation-induced cross-linking prevents primer annealing in the reverse transcription step and reversal of cross-linking may cause RNA degradation. Alles et al. (2017) showed that methanol fixation could preserve dissociated cells for several weeks without compromising scRNA-seq data quality, and this method was validated in both mouse brain cells and *Drosophila* embryos. Attar et al. (2018) reported that the reversible cross-linker, dithiobis(succinimidyl propionate) (DSP), could be used to preserve cells for subsequent single-cell suspension and that the scRNA-seq data quality from these fixed cells is similar to fresh cells. Additionally, fixation steps increase the flexibility of sample

handling, especially for samples that cannot be processed immediately. In addition to tissue fixation, another promising method is to add transcription inhibitor. For instance, the general transcription inhibitor actinomycin D (ActD) was recently used for mouse brain tissues to reduce transcriptional alterations resulting from dissociation (Hrvatin et al., 2018; Wu, Pan, Zuo, Li, & Hong, 2017). This method has not been applied to *Drosophila* but is an important consideration in future studies. In general, the cell dissociation should be done as quickly as possible to minimize the transcriptional changes introduced in this step. In addition, single-nucleus RNA-seq allows researchers to start with frozen tissues so that the *in vivo* transcriptional profiles are better preserved. This will be discussed in the later section.

2.2 | Single cell capture

Next step is to capture individual cells. Currently, there are several approaches available for single cell capture: limiting dilution, micromanipulation or micropipetting, laser capture microdissection, fluorescence-activated cell sorting (FACS), and microfluidics. Limiting dilution, a traditional method that is commonly used for isolating monoclonal cell lines, employs statistical strategy to isolate single cells by diluting cells into a concentration of less than one cell per aliquot (Fuller, Takahashi, & Hurrell, 2001). Micromanipulation allows manual cell picking with micropipettes via microscope observation followed by transfer of cells to lysis buffer to preserve RNA molecules, which has been used for early embryos and cardiomyocytes in mice and *Drosophila* mushroom body neurons (Crocker, Guan, Murphy, & Murthy, 2016; Flynn, Santana, & Melov, 2011; Guo et al., 2019). Laser capture microdissection method combines a laser system with a computer system to isolate single cells from solid tissues (Nichterwitz et al., 2016). These three methods, although very useful in certain applications, are time-consuming or low-throughput and thus are not widely utilized in scRNA-seq studies.

FACS is a powerful tool for purifying or enriching specific cells if they can be labeled by fluorescent markers. Two of the most frequently used labeling strategies are genetic labeling (e.g., Cre-loxP system in mice and GAL4-UAS system in *Drosophila*) (Brand & Perrimon, 1993; Schwenk, Baron, & Rajewsky, 1995) and antibody staining (e.g., CD cell-surface proteins for immune cells). In addition, multi-color and negative selection is also possible for desired cells. FACS-based method possesses several unique features for scRNA-seq studies. First, dead cells can be removed. Combining live/dead florescent dye staining with cell-type specific marker is sufficient to reduce cell damage effects that are introduced by the tissue dissociation procedure. This has been proven to be useful for isolating *Drosophila* neurons (Li et al., 2017). Second, single cells can be isolated from doublets or cell aggregates according to the cells size and fluorescence intensity, which can be visualized during cell sorting. Third, cell debris, usually generated in the single-cell suspension step and consisting of small pieces of membranes or other parts of a cell, will decrease the capture efficiency if collected as cells. They can be removed through FACS because they lack fluorescence labeling and show different forward scatter (FSC) and side scatter (SSC) signals from intact cells. The advantage of this feature is more profound for *Drosophila*, because most *Drosophila* cells are very small and cannot be easily distinguished from debris by size. Through FACS, individual cells can be either collected into single wells of 96- or 384-well plates for plate-based scRNA-seq or collected as a whole in one tube

for droplet-based scRNA-seq (see below). The disadvantages of the FACS method include longer waiting time and additional stress introduced by the FACS procedure.

Microfluidics-based methods for capturing single cells are commonly used. Microfluidic technology was initially used in biochemical assays for quantifying DNA and protein molecules, and later was adopted for long-term monitoring of single bacteria and for gene expression profiling of single cells (Balagaddé, You, Hansen, Arnold, & Quake, 2005; Marcus, Anderson, & Quake, 2006). The first widely used commercial microfluidic system is Fluidigm C1, which provides automated single-cell capture, lysis, and reverse transcription and cDNA amplification (Pollen et al., 2014). For each run, Fuidigm C1 can capture and process 96 individual cells and the later upgraded version (high-throughput integrated fluidics circuits, HT IFCs) can process up to 800 cells. However, the average cost per cell is high and a large number of cells is required as input, limiting the adoption of Fluidigm C1. In 2015, two high-throughput droplet-based microfluidics methods were developed, called inDrop and Drop-seq (Klein et al., 2015; Macosko et al., 2015). These two methods, together with the commercialized 10× Genomics Chromium system (Zheng et al., 2017), have tremendously boosted the scRNA-seq field in recent years, owing to their high-throughput and low-cost features. inDrop, Drop-seq, and 10x Genomics share a similar workflow: individual cells are captured with uniquely barcoded beads in water-in-oil droplets; cells are lysed and cDNAs are generated; cDNAs are amplified and gene expression libraries are constructed. The pros and cons of plate-based and droplet-based scRNA-seq methods will be further discussed below.

How do we make sure captured cells are single cells, but not doublets or multiplets? This is a frequently asked question in the scRNA-seq field. There is no perfect method to completely solve this issue. However, here are several steps to help minimize the issue. First, after tissue dissociation, the single-cell suspension can be validated under microscope using cell counting slides. We find this step is very useful to determine if a tissue dissociation protocol should be further optimized. Next, if FACS is used, doublets can be distinguished by cell size and fluorescent intensity. However, it is worth mentioning that if cell sizes are largely varied in the single cell suspension, this can be challenging because large cells may show similar sizes or florescent intensity to doublets of small cells. Fuidigm C1 system has an imaging step to check if captured cells are single cells. Finally, multiple bioinformatic methods have been developed to detect and remove doublets, for example, DoubletFinder (McGinnis, Murrow, & Gartner, 2019), Scrublet (Wolock, Lopez, & Klein, 2019), and DoubletDecon (DePasquale et al., 2019), Solo (Bernstein et al., 2020).

2.3 | scRNA-seq platforms: Smart-seq2 and 10× genomics chromium system

For most established scRNA-seq platforms, the cell capture process is integrated with downstream steps: reverse transcription, cDNA amplification, and sequencing library preparation. So far, numerous scRNA-seq platforms have been developed, such as CEL-seq (Hashimshony, Wagner, Sher, & Yanai, 2012), Smart-seq2 (Picelli et al., 2013), MARS-seq (Jaitin et al., 2014), inDrop (Klein et al., 2015), Drop-seq (Macosko et al., 2015), 10× Genomics (Zheng et al., 2017), Sci-RNA-seq (Cao et al., 2017), MATQ-seq (Sheng, Cao, Niu, Deng, & Zong, 2017), SPLIT-seq (Rosenberg et al., 2018), and SEQ-well (Aicher et

al., 2019). Key differences between these approaches include cDNA coverage (full-length or 5′/3′ counting), the use of unique molecular identifier (UMI), handling platforms (plate- or droplet-based), targeted read depth, throughput, and cost. Detailed comparison has been discussed by other reviews (Haque et al., 2017; See, Lum, Chen, & Ginhoux, 2018).

Among those platforms, four have been used in *Drosophila* scRNA-seq studies: plate-based Smart-seq2 and droplet-based inDrop, Drop-seq, and 10× Genomics. The three droplet-based methods share many key features (Table 2), and 10× Genomics is becoming popular (15 out of 23 published *Drosophila* studies used this method) (Table 1), owing to its high accessibility. Here I will focus on Smart-seq2 and 10× Genomics system.

Smart-seq was developed to increase read coverage across transcripts for scRNA-seq studies (Ramsköld et al., 2012). Smart-seq2 is an improved version of Smart-seq, featuring the generation of full-length cDNAs using template switching in the reverse transcription step (Picelli et al., 2014). A Smart-seq2 based scRNA-seq protocol follows following steps: (a) After tissue dissociation, individual cells are FAC-sorted into single wells of 96- or 384-well plates, with lysis buffer preloaded. Sorted plates can be either stored in −80°C for long-term storage or processed immediately. (b) The first strand full-length cDNA is synthesized with the customized oligo-dT primer and the template switching oligo. (c) Full-length cDNAs are PCR-amplified for 18–25 cycles depending the amount of starting RNA materials. We have used 25 cycles for *Drosophila* neurons (Li et al., 2017; Li, Li, et al., 2020). (d) Sequencing libraries are made by Tn5 tagmentation according to standard procedure (Adey et al., 2010). (e) Libraries are pooled and sequenced.

The 10× Genomics Chromium system takes advantage of rapid droplet-based encapsulation of single cells with a gel bead in emulsion (Zheng et al., 2017). Each gel bead is tagged with millions of oligonucleotides containing a bead-specific barcode, different unique molecular identifiers (UMIs), and the oligo-dT with sequencing primer. The bead-specific barcode is used to index individual cells; UMIs can be used to index individual mRNA molecules, allowing transcripts to be directly counted to reduce PCR-introduced amplification bias; oligo-dT primers allow cDNA generation from poly(A) mRNAs. 10× Genomics provides different kits that allow to profile either 3′ or 5′ end of mRNAs. Up to eight different samples can be processed simultaneously, and about 10k cells can captured from each sample. The downstream processing for reverse transcription and library preparation is very simple because all cells from one sample are processed together in one tube.

As described above, both Smart-seq2 and 10× Genomics have advantages and disadvantages (Table 2). Compared to 10x Genomics, Smart-seq2 method allows full-length cDNA coverage thus enabling isoform analysis, provides higher sequencing depth allowing better detection of lowly-expressed transcripts, and offers a stable, long-term storage option after cell capture which increases experimental flexibility. Another advantage of Smart-seq2 is the capability to capture large polyploid cells, which is a challenge for 10× Genomics. This is a significant factor for *Drosophila* research because polyploid cells are very common for many *Drosophila* tissues. On the other hand, 10× Genomics provides much higher throughput, does not require cell sorting by FACS, and utilizes UMIs to remove PCR-amplification bias. Meanwhile, the cost per cell for 10× Genomics is much lower than that of Smart-seq2. As

a result, all these parameters should be taken into account when choosing a scRNA-seq platform.

3 | SINGLE-CELL RNA-SEQ IN *DROSOPHILA*: APPLICATIONS

scRNA-seq enables direct comparison of transcriptomes among individual cells. Therefore, an immediate application of scRNA-seq is to characterize the cellular heterogeneity within a complex tissue, for example the fast-developing embryo or complex brain regions (Guo et al., 2017; Karaiskos et al., 2017; Tasic et al., 2016; Zeisel et al., 2018). By cluster analysis, single cells can be classified into different groups according to transcriptomic similarity. This allows the identification of rare cell populations, and also permits comparison of cell states in a number of biological contexts, such as development, aging, stem cell differentiation, and disease. In addition to revealing transcriptomic differences of individual cells, scRNA-seq can also provide critical information about fundamental features of gene regulation. For example, characterizing the gene co-expression patterns in single cells allows the identification co-regulated gene modules and gene-regulatory networks that may underline cellular heterogeneity (Wagner, Regev, & Yosef, 2016). Next, I will discuss those applications in general and then in *Drosophila* scRNA-seq studies (Figure 2).

3.1 | Classifying cell types

Before the molecular biology era, cell types were defined and classified typically by their morphology, and later by their function and physiology (Fischbach & Dittrich, 1989; Kepecs & Fishell, 2014; Waddintong, 1957). These characteristics are largely determined by a cell's molecular signature or gene expression pattern. Thus, the advent of scRNA-seq has pushed the cell type clarification to new heights in recent years (Brbić et al., 2020; Darmanis et al., 2015; Hung et al., 2020; Jaitin et al., 2014; Li et al., 2017; Zeisel et al., 2015).

Most scRNA-seq studies in *Drosophila*, as well as in other model organisms, start with classifying cells according to transcriptomic similarity. Numerous computational platforms have been developed for cluster analysis, for example Monocle (Trapnell et al., 2014), ASAP (Gardeux, David, Shajkofci, Schwalie, & Deplancke, 2017), Seurat (Butler, Hoffman, Smibert, Papalexi, & Satija, 2018), Scope (Davie et al., 2018), SCANPY (Wolf, Angerer, & Theis, 2018), MARS (Brbić et al., 2020), CellFindR (Yu et al., 2019), and many others. Cluster analysis is a common and efficient strategy for investigating complex tissues, for example the brain. Davie et al. (2018) profiled the entire adult *Drosophila* brain and revealed 87 primary cell clusters, many of which can be further divided when the clustering resolution is enhanced. Specific parts of the *Drosophila* nervous system from different stages have also been profiled, including pupal olfactory projection neurons and olfactory receptor neurons (Li et al., 2017; Li, Li, et al., 2020), pupal and adult optic lobes (Konstantinides et al., 2018; Kurmangaliyev et al., 2019), adult central brain (Croset et al., 2018), larval brain (Brunet Avalos et al., 2019), and adult ventral verve cord (Allen et al., 2020).

The *Drosophila* midgut is a great model system for studying adult stem cell biology and aging mechanisms (Jasper, 2020; Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). Four major epithelial cell types in the midgut have been assumed from a traditional view, intestinal stem cells, enteroblasts, enterocytes, and enteroendocrine cells (Li & Jasper,

2016). Recently, Hung et al. (2020) provided the first cell atlas of the adult *Drosophila* midgut and revealed 22 clusters representing these four cell types, suggesting a high heterogeneity of these cells. Guo et al. (2019) performed scRNA-seq analysis of FAC-sorted enteroendocrine cells which formed 10 clusters, confirming the enteroendocrine cell heterogeneity. From the intestinal immunity view, scRNA-seq was performed in wild-type midgut and in midguts where IMD innate immune signaling was inactivated, and it was found that IMD inactivation resulted in appearance of a new enterocyte population and absence of one enteroendocrine cell population (Shin et al., 2019). These studies provide useful resources for understanding intestinal stem cell function and gut physiology.

How is a cell type's identity encoded in the transcriptome? There are two possibilities: each cell type is defined by a unique marker, or each cell type is specified by a combinatorial code. It is clear that most major cell types from different tissues, such as neurons, glia, muscles, hemocytes, or distinct cell types from the same tissue, such as the four epithelial cells in the fly midgut discussed above, can be distinguished from one another using unique markers. However, often these cell types can be further divided into functionally distinct subtypes, which typically requires the use of multiple markers rather than a single unique marker. For example, the identity of fly olfactory projection neurons can be easily distinguished from astrocytes using one neuronal marker but differentiation of each of the 50 projection neuron subtypes requires the use of a combinatorial code (Li et al., 2017), and in the midgut each enteroendocrine cell is specified by 2–5 different classes of hormone peptides (Guo et al., 2019).

3.2 | Characterizing rare cells

Following cluster analysis, one immediate question is whether these transcriptomic clusters represent meaningful cell types (or subtypes). There are three commonly used strategies to address this question: (a) using previously characterized marker genes, (b) sequencing specific subtypes of cells and re-clustering them with the large population to see if they form expected clusters, and (c) identifying novel markers for specific clusters and validating their expression pattern *in vivo*. These can be achieved in *Drosophila* without too much difficulty, because for most genes there are available GAL4 lines for direct validation and generating a new transgenic fly line is relatively simple, especially with combination of CRISPR technology and fly genetics (Diao et al., 2015; Jenett et al., 2012; Kanca et al., 2019; Lee et al., 2018).

During the validation, many clusters can be assigned to different known cell types and some uncharacterized cell clusters may reflect rare cell types. For example, in the adult *Drosophila* brain, analysis of dopaminergic neurons revealed a *Fer2*+ cluster of protocerebral anterior-medial dopaminergic cells, and analysis of peptidergic neurons, another rare cell type, revealed multiple specific subtypes of petidergic neurons (Davie et al., 2018). scRNA-seq of adult *Drosophila* testis and ovary have revealed transcriptomes of rare germline stem cells, allowing detailed characterization of spermatogenesis and oogenesis at the single cell level (Jevitt et al., 2020; Rust et al., 2019; Witt et al., 2019). scRNA-seq of the developing larval ovary enabled the identification of a new cell type corresponding to the elusive follicle stem cell precursors (Slaidina et al., 2020). *Drosophila* blood cells (hemocytes) from the larval

stage have recently been profiled from four independent studies and they all reported the high heterogeneity of plasmatocytes, the major cell type of *Drosophila* hemocytes (Cattenoz et al., 2020; Cho et al., 2020; Fu et al., 2020; Tattikota et al., 2020). Interestingly, Fu et al. (2020) revealed two new blood cell types, which were named as thanacytes and primocytes. Tattikota et al. (2020) discovered rare subsets within crystal cells and lamellocytes, two other less frequent hemocytes. These studies provide a rich resource for understanding *Drosophila* blood cell types and physiologies.

3.3 | Developmental trajectory

Another important application for scRNA-seq is to construct the cellular trajectory from one state to another (Packer & Trapnell, 2018). In tissues where cell fates are not fully terminated, individual cells undergo dynamic processes, such as stem cell proliferation and differentiation, in response to internal developmental clock or external environmental stimuli. This dynamic process is partially encoded in a cell's transcriptome. Thus, scRNA-seq data can be utilized to map cell developmental trajectory from early states to terminal states along a pseudotime axis. Multiple methods have been invented to visualize the developmental trajectory, such as Monocle (Trapnell et al., 2014), SCUBA (Marco et al., 2014), Wanderlust (Bendall et al., 2014), Waterfall (Shin et al., 2015), and Wishbone (Setty et al., 2016). Detailed comparison of these methods has been discussed (Hwang et al., 2018). RNA velocity, which can be calculated by comparing unspliced and spliced mRNA ratios from scRNA-seq data, is a powerful indicator of future state of individual cells (La Manno et al., 2018). It is conceivable that combination of RNA velocity with single-cell trajectory analysis will provide complementary insights into cellular dynamics during tissue development and regeneration. It is worth mentioning that current RNA velocity analysis tools do not perform very well for 10x data from *Drosophila* cells (personal communication with the Reviewer).

In *Drosophila*, scRNA-seq analysis of the adult ovary and testis allowed the reconstruction of developmental trajectories of germ cells during oogenesis and spermatogenesis, respectively (Jevitt et al., 2020; Rust et al., 2019; Witt et al., 2019). In the ovary, pseudotime analysis revealed germ cell trajectory with three branches, one representing early stages of differentiation and other two representing the paths to oocytes and nurse cells (Jevitt et al., 2020; Rust et al., 2019). In the testis, such analysis revealed de novo gene expression bias during spermatogenesis (de novo genes represent new genes that evolve from DNA sequences that were ancestrally nongenic). For example, the top five most differentially expressed de novo genes tend to be biased toward early and middle pseudotime (Witt et al., 2019). Developmental trajectory analysis of neuroblast tumors in *Drosophila* larvae identified a subset of genes, responsible for temporal patterning of normal neuroblasts, that are redeployed in tumors to generate a differentiation trajectory leading to tumor cell heterogeneity (Genovese et al., 2019). scRNA-seq in adult *Drosophila* midgut allowed the lineage analysis to reveal the differentiation trajectory of intestinal stem cells, allowing future characterization of stem cell functions in homeostasis and in response to injuries (Hung et al., 2020).

Another interesting question is how transcriptomic differences of two cell types evolve during development. We (Li et al., 2017) performed scRNA-seq analysis of two types of *Drosophila* olfactory projection neurons from five stages covering middle pupa to adulthood and found that these two transcriptomes are quite distinct in developmental stages, but become indistinguishable in adulthood. Similar findings of transcriptomic convergence from development to adulthood were reported in the mouse lateral geniculate nucleus (Kalish et al., 2018), as well as in mouse retinal ganglion cells (Tran et al., 2019). These findings suggest that using scRNA-seq to characterize cell types from just one stage, such as adulthood, may be not sufficient to reveal the differences between two functionally distinct cell types. Although this principle may not be applicable to other cell types, it is advised to take it into account when performing scRNA-seq studies for classifying cells and characterizing cell types.

3.4 | Mechanisms of development, aging, and disease

Besides trajectory analysis-related applications, scRNA-seq can also provide valuable insights into additional mechanisms controlling development. For example, single-cell transcriptomic profiling of the *Drosophila* embryo, consisting of about 6,000 cells, revealed a new mechanism underlying the embryo pattern formation (Karaiskos et al., 2017). The *Drosophila* embryo is an excellent model for studying pattering principles that specify cellular identities. By combining scRNA-seq and a computational mapping strategy to predict spatial gene expression, Karaiskos et al. (2017) obtained a 3D virtual in situ hybridization map of the embryo. This 3D in situ map enabled the researchers to identify the expression of multiple Hippo pathway components in an anterior region of the embryo and to reveal a new role of Hippo signaling in embryo patterning.

How does aging affect cell-identity at the transcriptomic level? By comparing scRNA-seq data from young and old *Drosophila* brains, Davie et al (Davie et al., 2018) obtained several interesting observations. First, it was found that mRNA abundance of almost all brain cell types, including neurons and glial cells, displayed a decline with age. Second, the decline of mRNA abundance did not affect cell identity, because most cell type clusters that were characterized in young brains remained in old flies. Third, genes involved in oxidative phosphorylation and mitochondrial turnover showed most significant decline. These characterizations provide a valuable resource to study brain aging. It will be of interest for future studies to characterize how aging impact other cell types at the single-cell level beyond the brain.

scRNA-seq has also greatly contributed to our understanding of numerous disease processes, such as tumorigenesis (Potter, 2018). Tumors usually consist of a heterogenous mix of multiple cell types, including cancer, vascular, immune, and fibroblast cells, each of which can be further divided into subtypes. Thus, scRNA-seq can be used to dissect tumor heterogeneity to understand tumor development and to devise treatment approaches. *Drosophila* wing disc has served as a model system for studying conserved mechanisms underlying tumorigenesis (Morimoto & Tamori, 2017). Ji et al. (2019) performed scRNA-seq on *scrib* mutant-induced wing disc tumors and found that dynamic MAPK signaling activity control the transition from growth arrest to cell proliferation during tumorigenesis.

3.5 | Gene regulatory network

Deciphering the gene regulatory network is another nice feature of scRNA-seq (Shalek et al., 2013; Xue et al., 2013). At the transcriptomic level, two cell types may carry expression differences of tens or hundreds of genes. However, at the transcriptional regulation level, these differences can be attributed to a limited number of transcription factors (TFs) or cofactors. In other words, genes can be grouped into co-regulated modules based on their shared upstream regulators. Inferring gene regulatory network from scRNA-seq data is not only a strategy to refine cluster analysis, but also a powerful tool to discover regulatory mechanisms driving cellular heterogeneity. Recently, multiple methods have been developed to reconstruct the gene expression network from scRNA-seq data (Aibar et al., 2017; Chan, Stumpf, & Babtie, 2019; Matsumoto et al., 2017). For example, Aibar et al. (2017) developed SCENIC, which utilizes a computational strategy combining gene co-expression and cis-regulatory motif information to infer gene regulatory network, and they showed that SCENIC can accurately predict the interactions between TFs and their targets.

In the *Drosophila* nervous system, gene regulatory network analysis revealed that different modular transcriptional programs regulate distinct neural wiring features during development (Kurmangaliyev et al., 2019). In this study, scRNA-seq profiling was performed on developing T4 and T5 neurons, two cell types in the *Drosophila* visual system involved in motion detection, and modular analysis identified eight transcriptional programs that represent eight T4/T5 subtypes defined by a combination of dendrite and axon wiring patterns. Importantly, analysis-instructed gain- and loss-of-function experiments revealed a new role of the TF, *grn*, in controlling T4/T5 axon targeting. In the *Drosophila* immune system, gene regulatory network analysis identified hemocyte cluster-specific modular signatures that are associated with either a unique TF or a combination of TFs, which could be validated *in vivo* (Cattenoz et al., 2020). These data provide useful resources for generating more targeted genetic tools to study immune cell functions during homeostasis and upon infection.

4 | CHALLENGES, OPPORTUNITIES, AND PERSPECTIVES

Currently, *Drosophila* researchers still face a number of challenges for conducting specific scRNA-seq studies. However, challenges usually lead to new opportunities in scientific fields. Here, I will focus on three challenges and discuss potential opportunities (Figure 3).

How to perform scRNA-seq when intact cells cannot be isolated?

Performing scRNA-seq in some adult *Drosophila* tissues has proven to be difficult, as many cell types are strongly associated with surrounding cuticles, for example sensory neurons in the *Drosophila* antenna, wing, and body. It is extremely difficult to isolate these intact cells, because mild dissociation methods cannot break the tough cuticles while harsh dissociation methods will destroy cuticle as well as attached cells. Single-nucleus RNA-seq (snRNA-seq) methods provide a great opportunity to overcome this issue. Recently, snRNA-seq has been successfully applied to profile adult mouse and human brain cells and proven to be sensitive, efficient and unbiased for classifying cells (Habib et al., 2016, 2017; Lake et al., 2016). Importantly and encouragingly, direct comparison between snRNA-seq and scRNA-seq of

mouse visual cortex cells suggest although the nuclear content and proportion varies among cell types, nuclear transcripts carry adequate information to identify highly related neuronal cell types with a resolution similar to whole cells (Bakken et al., 2018). snRNA-seq has other advantages: (a) tissues can be stored, long-term, at −80°C before nucleus extraction, which is very helpful when sample collection and downstream sequencing preparation are not in the same location, such as for clinical samples; (b) snRNA-seq will reduce sampling bias introduced in the tissue dissociation step. For scRNA-seq, specific cell types may be susceptible to damage-induced cell death and will be removed before cell capture, while for snRNA-seq, all nuclei will be applied; (c) large and fragile cells, such as adipocytes, may not easily flow into microfluidics-based channels, including 10× Genomics, but their nuclei should be easily captured. Ghosh et al. (2019) has recently reported the application of snRNA-seq for profiling adult fly abdominal cuticles. We have recently developed a reliable snRNA-seq protocol in *Drosophila* through both Smart-seq2 and 10× Genomics for profiling adult olfactory neurons (Li, McLaughlin, Luo, unpublished) and expect more snRNA-seq to be performed in other adult *Drosophila* tissues.

How to profile nonpolyadenylated transcripts?

So far, almost all published scRNA-seq studies have focused on profiling poly(A) mRNAs because all current scRNA-seq protocols get first-stand cDNAs using the oligo-dT based primers. However, many regulatory noncoding RNAs, such as microRNAs, long noncoding RNAs (lncRNAs), circular RNAs, are not polyadenylated, but have essential functions in lots of biological processes, including development, aging, and disease (Batista & Chang, 2013; He & Hannon, 2004; Memczak et al., 2013; Yang, Duff, Graveley, Carmichael, & Chen, 2011). Note that in some cases nonpolyadenylated RNAs can be detected in oligo-dT primer-based profiling, and this is presumably due to internal poly(A) priming (Nam et al., 2002). Systematic investigation of those nonpolyadenylated RNAs in single cells is still greatly needed. Random hexamer priming is a potential strategy to capture all RNAs with or without poly(A) tails (Fan et al., 2015; Kang et al., 2011). Incorporating such a strategy into existing scRNA-seq technologies will allow us to explore the functions of those critical RNA species at the single-cell level. Encouragingly, the recently developed MATQ-seq method nicely demonstrates that nonpolyadenylated RNAs can be profiled by utilizing primers based on multiple annealing and looping-based amplification cycles in a small number of single cells (Sheng et al., 2017; Zong, Lu, Chapman, & Xie, 2012). It is anticipated that further development of MATQ-seq will allow high-throughput profiling of nonpolyadenylated RNAs in a large scale.

How to integrate other complementary single-cell technologies with scRNA-seq?

Although scRNA-seq is a powerful tool for studying cell states and functions, it has some limitations. For example, scRNA-seq does not carry the spatial information of profiled cells, it cannot infer epigenetic landscapes, and it does not directly reflect the protein level of genes whose post-transcriptional modifications alter their translation. Recently, several methods have been developed to detect spatial transcriptomes at single-cell resolution, including FISSEQ (Lee et al., 2014), MERFISH (Chen, Boettiger, Moffitt, Wang, & Zhuang, 2015), seqFISH (Shah, Lubeck, Zhou, & Cai, 2016), and STARmap (Wang et al., 2018). The development of single-cell ATAC-seq allows researchers to measure chromatin

accessibility in the genomic level of single cells (Buenrostro et al., 2015; Cusanovich et al., 2015). Peterson et al (Peterson et al., 2017) developed REAP-seq which allows simultaneous measurement of mRNAs and certain proteins with barcoded antibodies in single cells. *in vivo* tissue-specific proteomic profiling methods have been recently applied to *Drosophila*, providing valuable information that cannot be revealed by transcriptomic analysis (Droujinine et al., 2020; Li et al., 2020). High throughput single-cell proteomic profiling is still a dream, but not far from being achieved (Aebersold & Mann, 2016; Labib & Kelley, 2020). Several integration algorithms have been developed to either integrate different scRNA-seq datasets or integrate scRNA-seq data with data from scATAC and spatial transcriptomes (Korsunsky et al., 2019; Stuart et al., 2019). Combining scRNA-seq with these complementary single-cell technologies will help us to draw a complete picture of *Drosophila* cells, as well as in other systems.

5 | CONCLUSION

Biological findings are largely driven by technology development. In the past decade, scRNA-seq emerged as one of the most important techniques in biomedical fields and has profoundly changed our comprehension of many biological phenomena. Due to the smaller size of *Drosophila* cells, the application of scRNA-seq to *Drosophila* fell slightly behind compared to mammals. However, recent scRNA-seq studies in *Drosophila*, researchers have gained numerous insights into mechanisms underlying embryo cell patterning, neural development, germ cell development, intestinal stem cell differentiation, brain aging, tumorigenesis, immune cell specification and many others to come. Combining scRNA-seq with other single-cell technologies hold a high potential for making new exciting discoveries in the next decade.

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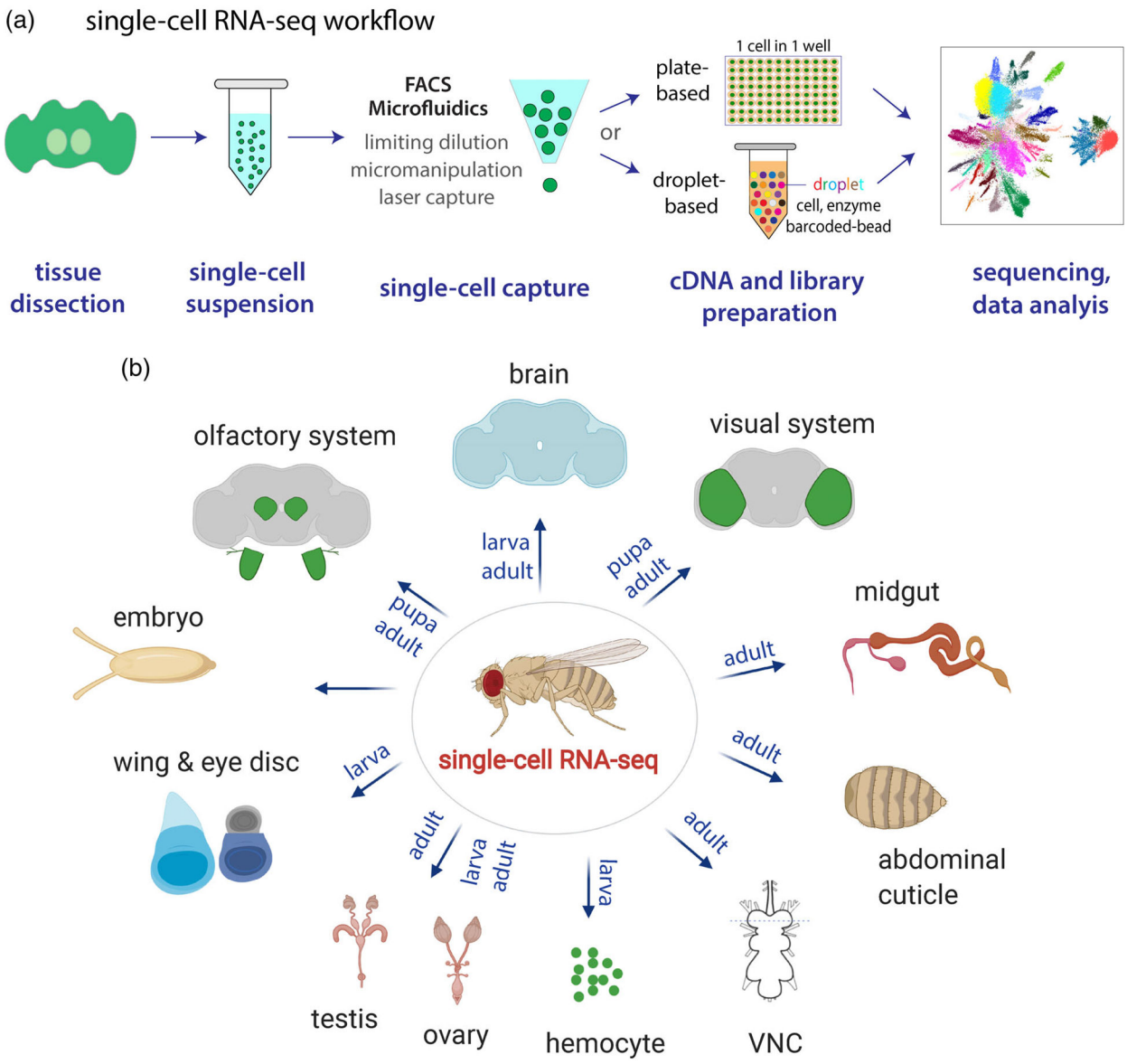

FIGURE 1.

(a) Single-cell RNA-seq workflow. It contains five major steps: tissue dissection and dissociation, single-cell capture, cDNA and library preparation, sequencing, and data analysis. FACS- and microfluidics-based methods are two most commonly used methods for single-cell capture. In plate-based methods, each individual cell is captured in one well. In droplet-based methods, cells are captured in droplets with enzymes and barcoded-beads. (b) Summary of scRNA-seq studies in *Drosophila* (see Table 1 for details). Tissue stages are indicated. The abdominal cuticle is profiled through single-nucleus RNA-seq, and all other tissues are sequenced by single-cell RNA-seq. VNC, ventral nerve cord

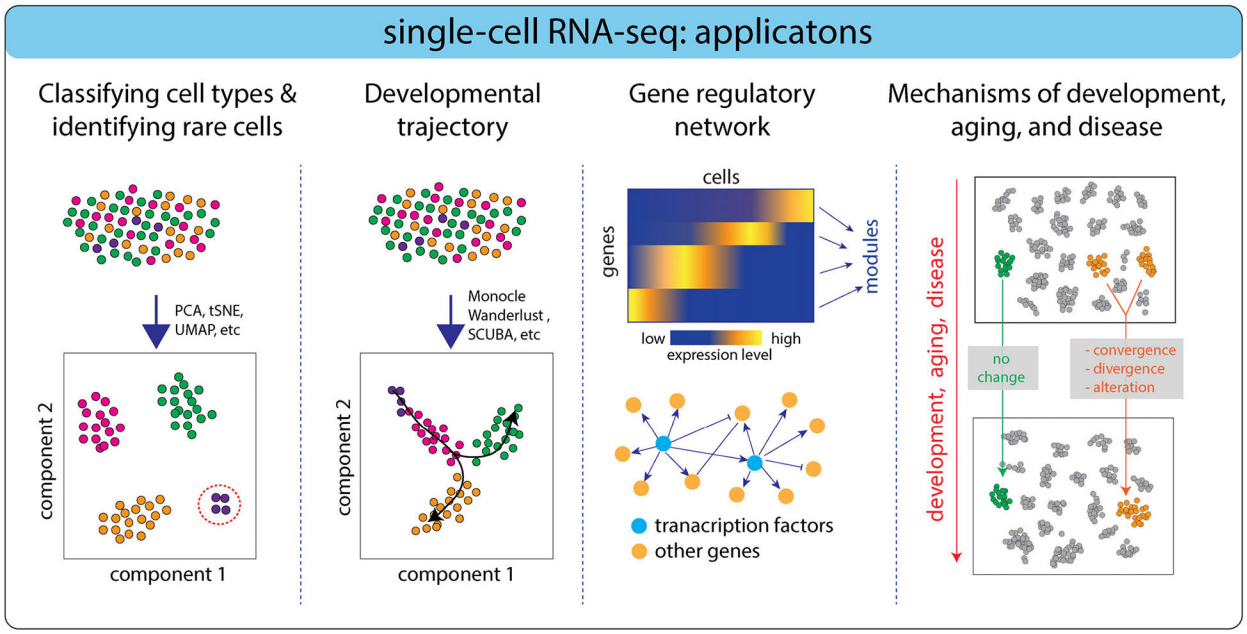

FIGURE 2.

Summary of current applications of single-cell RNA-seq in *Drosophila*, including classifying cell types and identifying rare cells, constructing cellular developmental trajectories, deciphering gene regulatory networks, and discovering mechanisms that control development and aging and that contribute to diseases

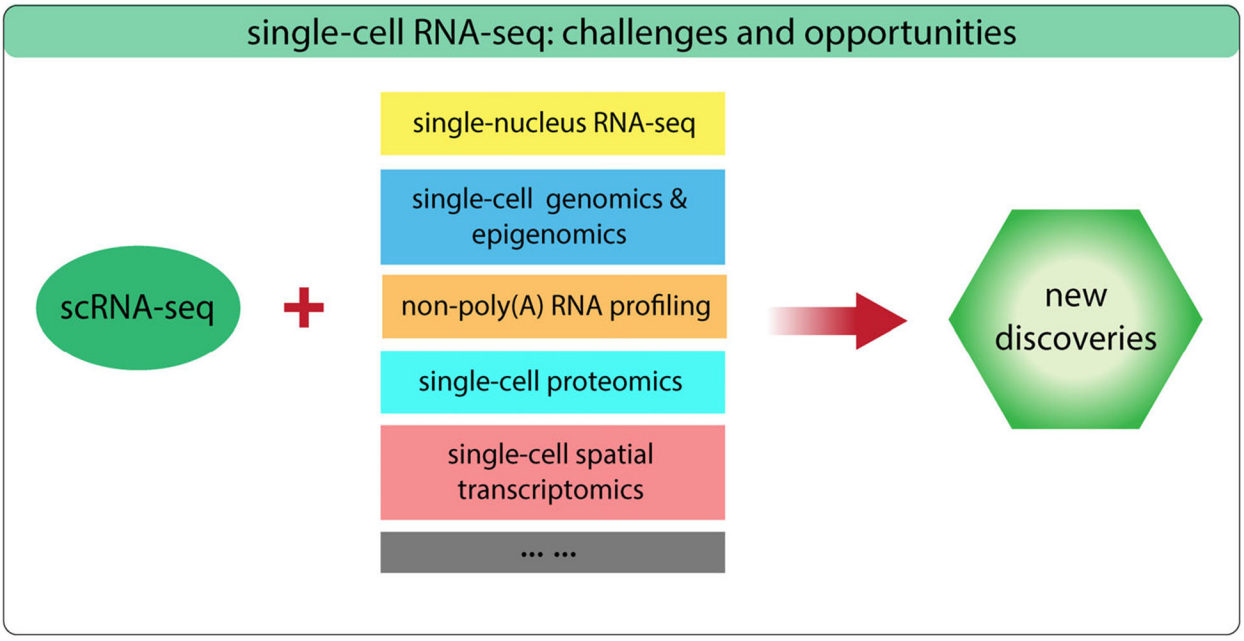

FIGURE 3.

Challenges and future opportunities to extend the applications of scRNA-seq by combining scRNA-seq with other technologies, including single-nucleus RNA-seq, single-cell genomics, and epigenomics, nonpoly(A) RNA profiling, single-cell proteomics, and single-cell spatial transcriptomics

TABLE 1

Summary of scRNA-seq studies in *Drosophila*

Tissue	Stage	Dissociation	Technology	Reference
Olfactory projection neuron	Pupa	Papain, liberase	Smart-seq2	Li et al. (2017)
Olfactory receptor neuron	Pupa	Papain, liberase	Smart-seq2	Li, Li, et al. (2020)
Embryo	Embryo	Dounce homogenizer	Drop-seq	Karaiskos et al. (2017)
Whole brain	Adult & aging	Dispase, collagenase	10×	Davie et al. (2018)
Midbrain	Adult	Papain, collagenase	Drop-Seq	Croset et al. (2018)
Whole brain	Larva	Collagenase	10×	Brunet Avalos et al. (2019)
Optical lobe	Adult	Dispase, collagenase	Drop-Seq	Konstantinides et al. (2018)
Optical lobe (T4/T5)	Pupa	Papain, liberase	10×	Kurmangaliyev et al. (2019)
Abdominal cuticle	Adult	Dounce homogenizer	10×	Ghosh et al. (2019)
Blood	Larva	NA	inDrop; 10×	Tattikota et al. (2020)
Blood	Larva	NA	10×	Fu et al. (2020)
Blood	Larva	NA	10×	Cattenoz et al. (2020)
Lymph gland	Larva	Papain, liberase	Drop-seq	Cho et al. (2020)
Eye disc	Larva	Typsin, collagenase	Drop-seq	Ariss et al. (2018)
Wing disc	Larva	TrypLE	Drop-seq	Bageritz et al. (2019)
Wing disc	Larva	Typsin	10×	Ji et al. (2019)
Gut (EEs)	Adult	Elastase	10×	Guo et al. (2019)
Gut	Adult	—	—	Shin et al. (2019)
Gut	Adult	Elastase	inDrop	Hung et al. (2020)
Ovary	Adult	Elastase, collagenase	10×	Rust et al. (2019)
Ovary	Adult	Papain	10×	Jevitt et al. (2020)
Ovary	Larva	Trypsin, collagenase	10×	Slaidina et al. (2020)
Testis	Adult	Typsin, collagenase	10×	Witt et al. (2019)
VNC (tumor model)	Adult	Papain, collagenase	10×	Genovese et al. (2019)
VNC	Adult	Papain collagenase	10×	Allen et al. (2020)

TABLE 2

Comparison between plate-based Smart-seq2 and droplet-based scRNA-seq platforms

	Smart-seq2	**inDrop**	**Drop-seq**	**10× genomics**
cDNA coverage	Full length	3′ end	3′ end	3′ or 5′ end
Plate or droplet	96- or 384-well plate	Droplet	Droplet	Droplet
UMI	None	Yes	Yes	Yes
Throughput (number of cells)	96 or 384	1k–10k	1k–10k	1k–10k
Sequencing depth (read per cell)	10^6	10^4–10^5	10^4–10^5	10^4–10^5
Feature	FACS sorting, isoform analysis	Emulsion, low cost	Emulsion, low cost	Emulsion, low cost
Long-term storage	Yes, cells sorted into lysis buffer	No, must process immediately	No, must process immediately	No, must process immediately