



Research Techniques Made Simple: Single-Cell RNA Sequencing and its Applications in Dermatology

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RNA sequencing is one of the most highly reliable and reproducible methods of assessing the cell transcriptome. As high-throughput RNA sequencing libraries at the single cell level have recently developed, single cell RNA sequencing has become more feasible and popular in biology research. Single cell RNA sequencing allows investigators to evaluate cell transcriptional profiles at the single cell level. It has become a very useful tool to perform investigations that could not be addressed by other methodologies, such as the assessment of cell-to-cell variation, the identification of rare populations, and the determination of heterogeneity within a cell population. So far, the single cell RNA sequencing technique has been widely applied to embryonic development, immune cell development, and human disease progress and treatment. Here, we describe the history of single cell technology development and its potential application in the field of dermatology.

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Description: This article, designed for dermatologists, residents, fellows, and related healthcare providers, seeks to reduce the growing divide between dermatology clinical practice and the basic science/current research methodologies on which many diagnostic and therapeutic advances are built.

Objectives: At the conclusion of this activity, learners should be better able to:

- Recognize the newest techniques in biomedical research.
- Describe how these techniques can be utilized and their limitations.

- Describe the potential impact of these techniques.

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Abbreviation: scRNA; single cell RNA

BENEFITS

- One of the most efficient approaches to detect cell-to-cell variation
- Identification of rare cell populations
- Specific identification of the cells that are responsive to treatments
- Identification of heterogeneity in a population by clustering the cells based on their transcriptomics
- High throughput: it can sequence thousands of cells in one sample and visualize the transcriptomics of all cells individually
- Identification of new mutations in individual cells

LIMITATIONS

- ScRNA sequencing has higher unavoidable bias compared with bulk sequencing because of the small amount of starting RNA
- The cost of each sequencing is very high and could easily exceed \$5,000 from single cell isolation to library sequencing
- Lack of a standard to judge the accuracy of the data
- Most library preparation platforms lack visualization capacity before library generation, and visualization is essential to increase single cell library quality and success rate
- Has inherent defect of unavoidable doublets in most currently available scRNA sequencing platforms

INTRODUCTION

Single cell RNA (scRNA) sequencing is a powerful technique used to assess the transcriptomics at the single cell level. Although high-throughput RNA sequencing has become one indispensable tool for profiling gene expression at the mRNA level (Grada and Weinbrecht, 2013), scRNA sequencing has started to show its advantages (Table 1) and has become increasingly popular in life science fields (Whitley et al., 2016). scRNA sequencing can be used to assess cell-to-cell variation and has already been used to discover rare populations that are usually hidden in the background of the regular bulk RNA sequencing (Li et al., 2017; Yan et al., 2017). In addition, scRNA sequencing technology shows promise in distinguishing between drug effective and non-effective cells when applied to human diseases, which will be extremely useful in precision medicine, such as guiding treatment selection and drug development (Shalek and Benson, 2017). However, this technology is generally challenging because of the low amount of RNA present in a single cell. In the past few years, scRNA sequencing technology has dramatically improved, which allows more investigators to use this advanced tool in their studies, including embryonic development, cancer biology, and immunology (Engel et al.,

Table 1. Difference between scRNA sequencing and bulk RNA sequencing

RNA Sequencing Capability	Bulk RNA Sequencing	scRNA Sequencing
Comparison of the transcriptome between WT and mutants	Yes	Yes
High throughput and high sensitivity	Yes	Yes
Identifying novel transcripts and genes	Yes	Yes
Identifying rare cell populations	No	Yes
Identifying mutations in individual cells	No	Yes
Transcriptome profiling in individual cells	No	Yes
Antigen-specific cell tracking	No	Yes
Determining heterogeneity in a cell pool	No	Yes
Identifying antigen-specific T-cell or B-cell receptors	No	Yes

Abbreviations: scRNA, single cell RNA; WT, wild type.

2016; Islam et al., 2011; Patel et al., 2014). Even though relatively fewer studies in the field of dermatology have used this technology, there is no doubt that scRNA sequencing is a promising method by which to make discoveries beyond the capability of other methods. In this review, we describe the history and development of scRNA sequencing technology, general principles of performing this technique, and its application within dermatology.

OVERVIEW OF THE METHODOLOGY

History of scRNA sequencing development

scRNA sequencing research was first used in a four-cell-stage blastomere by Tang et al., 2009 (Figure 1). The purpose of their study was to compare the efficiency of single cell sequencing versus microarray. At that time, scRNA sequencing libraries were generated manually in individual tubes, but it lacked the capacity to increase multiplex numbers. In 2011, the first multiplexed scRNA sequencing libraries were created by Islam et al. (2011) using a mouse embryo. Isolated single cells were barcoded individually in a 96-well plate, then transferred into a single tube, called single-cell tagged reverse transcription sequencing (STRT-seq). Oligonucleotides from each cell were then amplified by PCR. This increased the scale of scRNA sequencing libraries to hundreds, setting the foundation for future high-throughput methodology. Soon, another group introduced cell expression by linear amplification and sequencing (CEL-seq) to generate scRNA sequencing libraries (Hashimshony et al., 2012). Both studies showed that individual cells can be clustered based on their transcriptomic changes using scRNA sequencing. However, all of these procedures were very time and labor consuming, which limited the scale increase of scRNA sequencing. In 2014, a platform that made single cell isolation and library generation a two-step process was made commercially available, greatly reducing the time and labor required. In 2015, Klein et al. and Macosko et al. (Klein et al., 2015; Macosko et al., 2015). independently reported drop-seq (or drop-like) techniques through use of microfluidics. This procedure allowed a single cell and a functional bead to be compacted into one droplet in an oil

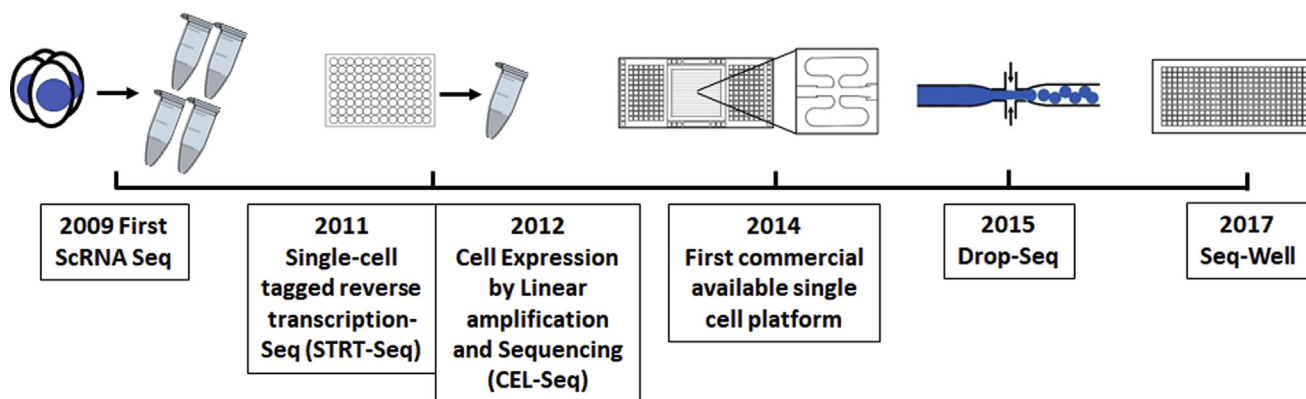


Figure 1. The development history of single cell sequencing. ScRNA sequencing was first performed in a mouse 4-cell stage blastomere in 2009. A few years later, the first multiplexed scRNA sequencing methodology was developed. In 2014, a commercial single-cell platform became available. Drop-Seq and Seq-Well, more efficient single cell library preparation platforms, were recently invented. Seq, sequencing; ScRNA, single cell RNA.

emulsion, so that the cell lysing, barcoding, and reverse transcription could be completed in individual sub-microliter droplets. Companies further optimized this technique to manufacture their own scRNA sequencing platforms (Zheng et al., 2017), allowing more investigators to use this technique. Even though the cost of DNA sequencing remains relatively high, the capacity to generate single cell libraries on a large scale with relatively low cost and high efficiency is an appealing feature of this methodology. In early 2017, Gierahn et al. (Gierahn et al., 2017) reported a single cell library preparation procedure called seq-well, which was the first portable single cell library generation platform. This is a simple, powerful, and affordable tool for the generation of single cell libraries, which could be a useful and widely available technique in the future.

Library construction and data analysis for scRNA sequencing

As shown in Figure 2, the first step of scRNA sequencing sample preparation is to isolate single cells from desired tissues or disease models, such as whole skin, epidermis, dermis, lesional skin, or skin tumors. The cell type of interest can be stained with antibodies of signature surface markers and isolated by fluorescence activated cell sorting (FACS) or other isolation methods, such as magnetic-activated cell sorting (MACS). For example, if skin resident immune cells are the desired cell type, fluorescence-conjugated CD45 antibodies can be used to stain the whole immune cell population, and then the CD45⁺ cells can be isolated by FACS or MACS. The isolated cells are then lysed and barcoded. The barcoding, reverse transcription, and library construction procedures depend on the library generation platform used.

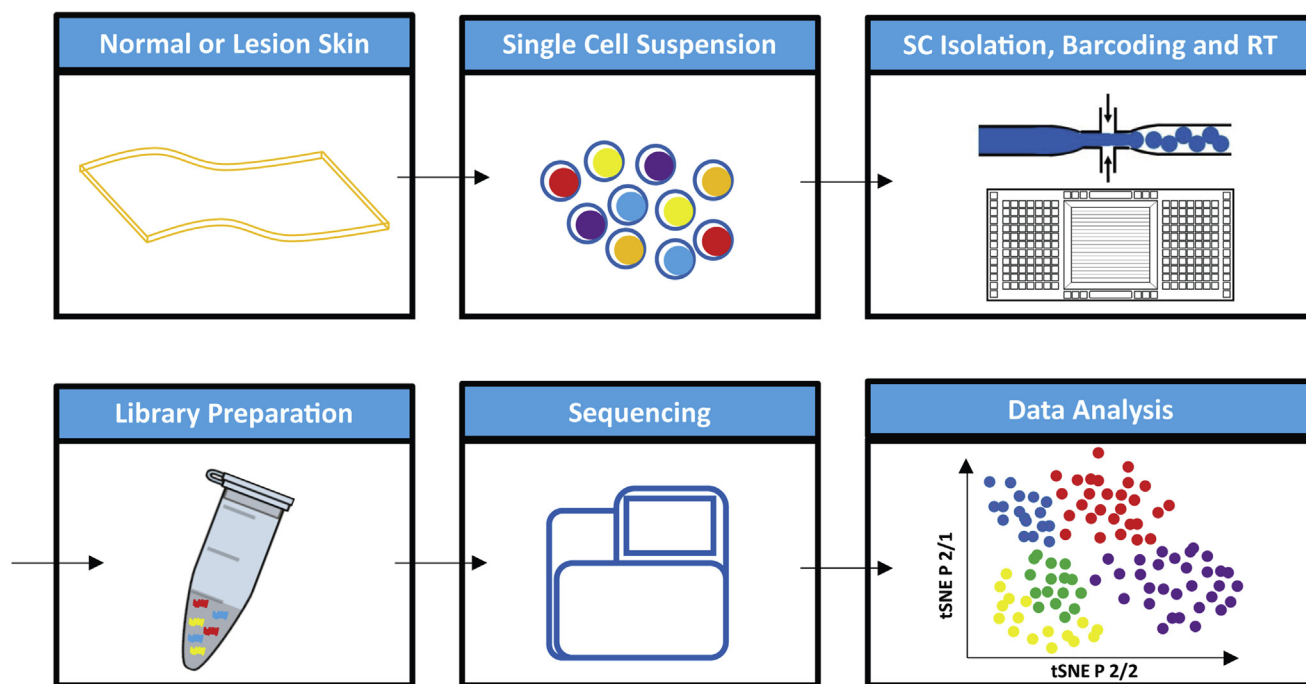


Figure 2. General single cell RNA sequencing procedure. Normal or lesion skin is freshly harvested and then the epidermis and dermis are further separated to generate a single cell suspension. Sorted single cells of interest are loaded onto a single cell isolating device. The barcoded and amplified cDNA is used to generate libraries for sequencing. The sequencing data is analyzed based on available analysis pipelines. RT, reverse transcription; SC, single cell; tSNE, t-distributed stochastic neighbor embedding.

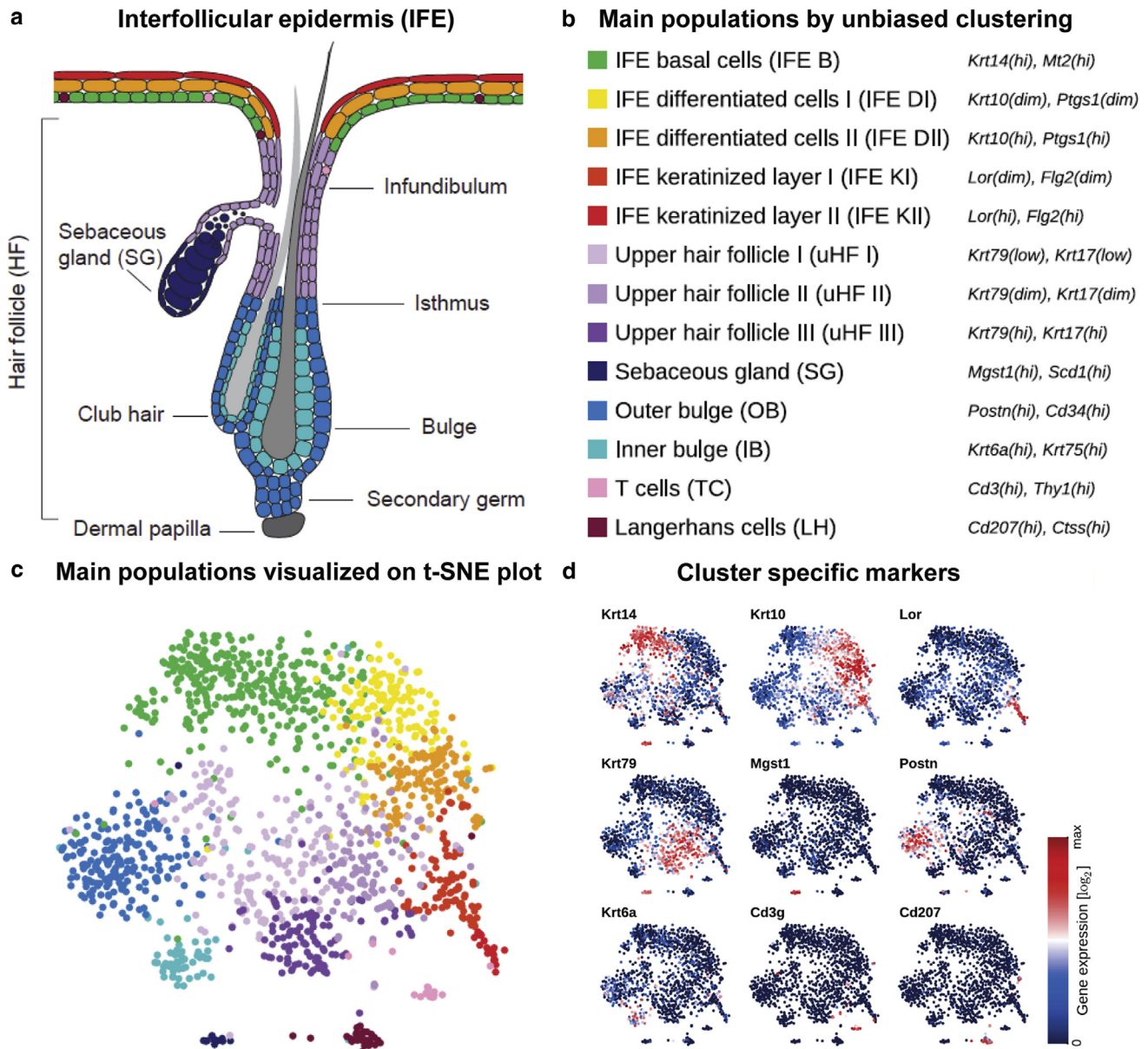


Figure 3. Defining the main epidermal cell populations. Adapted from Joost et al. (2016). max, maximum; tSNE, t-distributed stochastic neighbor embedding.

ScRNA sequencing generates a large amount of data. Commercially available scRNA sequencing library preparation platforms typically come with specific data analysis software. Data analysis pipelines typically include cell barcode and unique molecular identifier recognition, sequencing read mapping, and cell population clustering based on their transcriptomics (Figure 2). There are also other data analysis pipelines available for scRNA sequencing (Poirion et al., 2016).

APPLICATIONS

In the past decades, considerable progress has been made to determine the pathogenesis of skin diseases. The relevant cell types in skin associated with these diseases have been discovered by advanced modern technologies. However, whether the disease pathogenesis is related to certain cell subpopulations has not been determined because of the

limitation of previously used technologies. Rare cell populations and subpopulations tend to be lost in the analyses of bulk RNA sequencing or microarrays. The application of scRNA sequencing will enable investigation of skin development and function under the healthy and disease conditions at single cell resolution. We may uncover previously undetermined new subpopulations of keratinocytes and hair follicle stem cells and potential new subsets of skin immune cells such as dendritic cells and T cells, and we will gain an overall better understanding of skin biology. In inflammatory and autoimmune skin diseases, including psoriasis, atopic dermatitis, vitiligo, and bullous skin diseases, we may be able to define the specific target cell populations to revolutionize their classifications and uncover new immune cells related to the disease development, including autoreactive T cells or B cells. In skin cancer, such as squamous cell carcinoma, basal cell carcinoma, mycosis fungoides/cutaneous T-cell

lymphoma and melanoma, scRNA sequencing will help us define new subtypes of skin cancer and tumor-infiltrating cells, which will assist in new tumor immunotherapy design. Furthermore, the development of T-cell receptor/B-cell receptor scRNA sequencing allows us to identify specific T-cell receptors or B-cell receptors and their targets, which will be essential for developing chimeric antigen receptor-associated immunotherapy and defining specific tumor antigens and autoantigens related to skin cancer and autoimmune diseases. In addition, using scRNA sequencing, drug-susceptible cells or disease subtypes can be identified by their transcriptomic differences, and their gene expression profiles can further explain why they are better targeted by certain drugs than other cells, which will help in the design of better precision medicine.

SCRNA SEQUENCING IN PRACTICE

scRNA sequencing uncovers 25 populations of murine epidermis

Although there have not yet been many studies using scRNA sequencing in the field of dermatology, investigators have started to explore the structure and composition of skin using this technique. Joost et al. (2016) performed scRNA sequencing to cluster cell populations in the murine epidermis and identified 25 distinct cell types among the 1,422 single cells they sequenced based on their transcriptomics (Joost et al., 2016) (Figure 3). In contrast to previous bulk RNA sequencing studies in skin biology, the authors sequenced a relatively large number of single cell libraries and clustered the populations in normal murine epidermis based on the signature genes. For example, sebaceous gland cells were characterized by *Scd1/Mgst1*; inner and outer bulge keratinocytes were marked by *Krt6a/Krt75* and *Cd34/Postn*; and two immune cell populations, Langerhans cells and resident T cells, were marked by *Cd207⁺* and *Cd3⁺*, respectively. To confirm the existence of these clustered cell populations, the authors used immunohistochemistry and/or single-molecule fluorescence in situ hybridization, which allowed them to locate the cell populations spatially. More detailed subclustering analysis divided large populations into subpopulations, allowing discovery of previously unreported subpopulations (Figure 3). They also distinguished stem cells that expressed stem cell and progenitor markers that share the same signature genes as *Lgr5-EGFP⁺* cells, predominately located in the basal compartments. This study showed the capacity of scRNA sequencing to identify and characterize new skin subpopulations. Given that keratinocytes were the dominant cell types among the studied cell populations, there were not enough immune cells sequenced to investigate the potential subsets of Langerhans cells and T cells in this study.

ScRNA sequencing in skin cancer

ScRNA sequencing technology has also been used in skin cancer models, such as melanoma. Tirosh et al. (2016) sequenced a total of 4,645 single cells from tumor tissues of 19 patients with melanomas. They showed the heterogeneity among the tumor cells by clustering the malignant cells. Nonmalignant cells inside the tumors were also clustered and analyzed for understanding the tumor

MULTIPLE CHOICE QUESTIONS

- Which of the following is an advantage of scRNA sequencing over bulk RNA sequencing?
 - Identifying rare cell populations
 - Assessing genetic heterogeneity in a cell population
 - Tracking pathogen-specific cells
 - All of the above
- The drop-seq single cell isolation and barcoding systems were adapted from which of the concepts below?
 - RNA sequencing
 - Enzymatic DNA fragmentation
 - Microfluidics
 - Single cell mass cytometry
- What is the general procedure of scRNA sequencing in the right order?
 - Single cell isolation, DNA fragmentation, library amplification, sequencing, data analysis
 - Single cell isolation, cell barcoding and reverse transcription, library preparation, sequencing, data analysis
 - Single cell isolation, RNA fragmentation, cDNA pre-amplification, sequencing, data analysis
 - Single cell isolation, PCR amplification, DNA fragmentation, library amplification, sequencing, data analysis
- Which of the following is a potential application of single cell sequencing in precision medicine in the clinic?
 - Tracing of drug-susceptible cells during treatments
 - Determination of heterogeneity within a tumor mass
 - Comparison of gene expression profiles at the single cell level between responders and nonresponders
 - All of the above

microenvironment. In the malignant cells, they specifically investigated MITF-high and/or AXL-high populations, because they were likely to be essential for melanoma cell survival and drug resistance. AXL-high cells have been previously linked to RAF and MEK inhibition resistance. More important, a subpopulation of AXL-high melanoma cells in MITF-high tumors was found to be undetectable through bulk RNA sequencing, indicating the importance of investigating tumor subpopulations through scRNA sequencing. Nonmalignant cells in the tumor microenvironment play an important role in supporting tumorigenesis and responding

to treatments. They found that multiple T-cell exhaustion markers, such as PD1, TIGIT, TIM3, LAG3, and CTLA4, were co-expressed across individual cells. In addition, the exhaustion genes were highly correlated with cytotoxicity markers and T-cell activation status, indicating an activation-dependent T-cell exhaustion program. The results of the study are highly valuable for characterizing melanomas and guiding future precision tumor therapy.

DRAWBACKS AND FUTURE DIRECTIONS

ScRNA sequencing shares basic principles with regular bulk RNA sequencing, but it requires more advanced techniques and observes detailed transcriptomics in individual cells, as previous studies have addressed (Whitley et al., 2016). Although scRNA sequencing technology is a remarkable milestone, there are drawbacks. For example, scRNA sequencing data is noisier than bulk sequencing data, because it uses a very limited amount of RNA. Therefore, accurate annotation and transcript quantification can be challenging. Live/dead cell visualization is critical to ensuring the quality of libraries generated because the viability of single cells used for library generation largely determines the success rate and data accuracy. Drop-seq, currently being one of the most powerful, affordable, and labor-efficient single cell library preparation methods, has a high multiplex number, but it does not support live cell visualization before library generation. Drop-seq also has the inherent issue of an unavoidable doublet rate, which needs to be resolved by either excluding doublets before library generation or during data analysis in the future. Although the cost of processing samples from single cell isolation to library sequencing has declined in the past several years, it is still a prohibitively costly procedure. Finally, more powerful and reliable computational data analysis tools must be developed to more accurately and efficiently analyze data from scRNA sequencing.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

XW, BY, LZ, and Q-SM drafted the manuscript. IU, SJ, and OZ were involved in manuscript discussion, writing, and editing. Q-SM and LZ outlined and finalized the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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