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MINI REVIEW

Decoding nervous system by single-cell RNA sequencing

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***Background*: Mammalian brain are composed of a large number of specialized cell types with diverse molecular composition, functions and differentiation potentials. The application of recently developed single-cell RNA sequencing (scRNA-seq) technology in this filed has provided us new insights about this sophisticated system, deepened our understanding of the cell type diversity and led to the discovery of novel cell types.**
***Results*: Here we review recent progresses of applying this technology on studying brain cell heterogeneity, adult neurogenesis as well as brain tumors, then we discuss some current limitations and future directions of using scRNA-seq on the investagation of nervous system.**
***Conclusions*: We believe the application of single-cell RNA sequencing in neuroscience will accelerate the progress of big brain projects.**

Keywords: single cell RNA-seq; brain transcriptome; brain cell types

INTRODUCTION

The mammalian brain is the greatest achievement of evolution, with a vast number of cells tangling together in a conserved way to perform complex functions [1]. It is insufficient to study the function of brain regions on bulk cells due to the fact that great diversity of cell types exists, and they are wired into sophisticated cortical circuits and under the process of self-renew with the help of neuronal regeneration system. Thanks to the recent progress, through transcriptomic profiling of individual cell, single-cell technics provide us an unbiased view of the classification of those cells at cell level resolution and have gained widespread popularity [2].

Single-cell RNA sequencing (scRNA-seq) is more and more accessible due to the application of new experimental protocols and lower sequencing costs, which make the experiment much easier and more scalable. Currently there are several scRNA-seq protocols in use, e.g., SMART-seq2, CELL-seq and Drop-seq [3–5]. These protocols share general workflows, including single cell capturing, cell lysis, reverse transcription of RNA into cDNA, cDNA amplification and library preparation. In order to increase the efficiency of each step, commercial platforms such as the Fludigm C1 and the 10× Genomics Chromium are now commercially available. As a result, the range of dataset in those scRNA-seq experiments is growing exponentially, which has reached to 10^5 cells in a recent study [6]. The number of sequenced cells is still increasing every year.

Through the application of scRNA-seq technology, which uncovers cell-specific transcriptomic changes, it becomes possible to elucidate how many cell types exist in the brain, discover the novel and rare adult neural stem cells and further explain intratumor heterogeneity. In this review, we will focus on the major applications of scRNA-seq in those biological researches, and explore its future directions in neuroscience.

CLASSIFICATION OF NEURONAL CELL TYPES

The mammalian brain is composed of billions of neurons with different molecular identities and contributes to a variety of functions. Up to date, it is unclear how many neuronal cell types generated during development, which is

a basic bottleneck to the bottom-up approach of understanding the whole brain. Different type of neurons distinct greatly in their morphology, number of synapse, electrophysiological and molecular properties [7]. For example, excitatory neurons which are located at different layers of cerebral cortex express different transcriptional characteristics [8,9], while inhibitory neuron in the cortex is far more heterogeneous, expressing different kinds of neuropeptides as marker genes [10]. However, identifying different cell types is particularly challenging in the heterogeneous brain tissue as they are highly interconnected. This problem is partly solved by accessing the gene expression profile of individual cells, which can be captured by the high throughput and high sensitivity scRNA-seq technology.

By using scRNA-seq, different cells from particular heterogeneous brain regions could be characterized and classified. Over the past few years, a large number of single cell transcriptomic experiments have been carried out in different brain regions, including somatosensory cortex, visual cortex, hippocampus, stratum, hypothalamus and other peripheral sensory neurons [11–16]. Known cell types could be characterized according to their distinguished gene expression pattern, while more and more novel cell types have been identified in each region. A recent study have classified 62 neuronal subtypes with different transcriptional identities in the hypothalamus [16]. Earlier studies using microfluidic C1 platform have identified more than 40 types of neurons and glial cells in both mouse somatosensory cortex and visual cortex [12,15].

However, it is still unclear how many cell types exist in the nervous system, and even for the discovered cell types, their neurological function are yet to be fully explored. Technological improvements are also needed for scRNA-seq in order to discover more novel cell types. Tasic *et al*. reported that 40% additional cell types in the visual cortex are found after increasing the sequencing depth from 100,000 to 1,000,000 mapped reads per cell [15]. On the other hand, in order to cover more rare cell types and reduce technical noise caused by batch effects, it is necessary to increase the input cell number from individual experiment. Drop-seq, wherein single cells could be captured and paired in thousands of droplets with single barcoded beads in a single reaction, has dramatically reduced the labor and reagent costs. Taking advantage of this technology, Shekhar *et al*. profiled almost 50,000 retina cells and defined 39 transcriptionally distinct clusters [6]. Regrettably, this study only sequenced on average 8,200 mapped reads per cell, which partly explains why only two novel cell types are characterized. Therefore, both the throughput and sequencing depth should be improved in the experiments in order to better study brain regional heterogeneity.

MOLECULAR CHARACTERIZATION OF NEURAL STEM CELLS

It is generally accepted that there are two germinal niches containing neural stem cells (NSCs) and generating new neurons throughout the lifespan in adult mammalian brain: the wall of the lateral ventricles (including the subventricular zone (SVZ) and the lateral ventricle (LV) and the subgranular zone in the dentate gyrus of hippocampus [17]. Quiescent neural stem cells generate new neurons through sequential steps including quiescent to active state transitions, entering the cell-cycle, and neuronal differentiation [18]. However, due to the rarity of NSCs and the complexity of their surrounding environment, the molecular characterization of NSC has been particularly challenging [19]. In addition, conventional bulk cell RNA-seq is not sufficient to elucidate the dynamic nature of neurogenesis by using only a few selected time points. As scRNA-seq could profile stem cell gene expression at cell level resolution and reveal dynamic molecular signals driving their development and neurogenesis, it has been widely applied in investigating adult neurogenesis.

Recent studies using scRNA-seq have successfully characterized heterogeneous stages of NSC and captured their transcriptional changes during neurogenesis in both SVZ and hippocampus areas. Shin *et al.* reconstructed the stem cell dynamics of NSC homeostasis in mouse hippocampus and revealed common features among different adult NSCs [20]. Dulken *et al.* investigated adult NSC populations in SVZ region and examined the molecular surface makers of *in vivo* NSC populations that are in intermediate states [21]. The application of scRNA-seq also allowed researchers to identify genes expressed in activating stem cells during endogenous NSCs regeneration after the injury or disease stimulation. Luo *et al*. identified a subset of quiescent NSCs in the lateral and fourth ventricles that could be activated and differentiated into neurons and glia cells upon VEGF stimulation [22]. And Llorens-Bobadilla *et al.* analyzed NSCs in the SVZ and identified genes that drive NSC activation and proliferation after ischemic brain injury [23]. In the second study, they were also able to detect different states of NSC from quiescent to activation and the heterogeneous response of dormant NSC during activation. Moreover, although emerging evidences indicated that there are new generated neurons after birth in cerebral cortex, it is still debated whether novel neurons are generated in the postnatal cerebral cortex at similar stage. By using scRNA-seq and lineage tracing technology, Bifari *et al.* proved the existence of neural progenitor cells in the meninges and meningeal substructures, these radial glia like NSC could migrate

through the caudal ventricular zone and integrate functional new neurons into cerebral cortex postnatally [24].

Notably, unlike in the cell classification studies which brain cells in specific regions were profiled in an unbiased fashion, almost in all of those NSC studies the cells have to be enriched by using a combination of known markers. Although this strategy allows the evaluation of certain rare NSC population, it is inevitably biased for the samples and potentially lead to the missing of some useful information. This problem might be partly solved as more marker genes are reported for each cell type. Future studies should focus on identifying more cell type specific marker genes and improving the sensitivity of scRNA-seq technology to increase the accuracy of distinguishing rare cell types from other cells. Overall, single cell transcriptomics could reveal pervasive molecular heterogeneous in NSCs and uncover dynamic state transitions during both homeostasis and injury process.

TRANSCRIPTOME ANALYSIS OF BRAIN TUMOR BIOLOGY

Tumor development involves in both clonal evolution from the mutated normal cells and differentiation hierarchy from cancer stem cells. Clonal diversity is a hallmark of cancer, and plays an important role in cancer invasion, metastasis, and the evolution of resistance to therapy [25–27]. Cancer stem cells (CSCs) are a group of rare stem cell-like progenitor cells that give rise to the major tumor cell populations [28]. Brain tumor may cause unexpected brain related symptoms such as headache and seizure, and tumors that locate in specific area of the brain can cause the lost of function of that brain region. As some brain tumor related genes prefer to be expressed in nerves system, the functional investigation of those genes can decode how important of those genes in the brain. Traditional bulk cell RNA-seq experiments only measure the average transcriptional profile of the whole cell population and therefore have limitations in understanding the intratumor heterogeneity and tumor stem cells. Single-cell brain cancer transcriptome has the advantage of characterizing cellular heterogeneity and thus provides us great insights about rare tumor stem cells and their role in tumor progression.

With the improvement of sequencing coverage and quantification accuracy, single cell transcriptomics have been widely used to study brain tumors. Patel *et al*. leveraged scRNA-seq to characterize heterogeneous gene expression programs within five glioblastoma tumors and reveal their transcriptional, functional, and genetic diversity [29]. They successfully distinguished malignant cells from normal surrounding cells by estimating copy number variation (CNV) from the average expression of genes in large chromosomal regions within each cell as well as distinct genetic clones. This pioneer study has broaden the application of single cell transcriptomics in cancer biology. Another recent study performed unbiased scRNA-seq to profile the transcriptome in thousands of single tumor cells from *IDH1/IDH2* mutated human oligodendroglioma patients and discovered a small subset of cancer stem cells containing signs of proliferation [30]. This may be the first time of identifying cancer stem cells and their differentiated progeny in human brain tumor samples, supporting the hypothesis that cancer stem cells are important drivers in the tumor.

Single cell transcriptomics are able to detect large scale chromosome deletion/duplication as well as tumor specific mutations, which were validated by fluorescence *in situ* hybridization (FISH) and whole-exome sequencing. However, as current scRNA-seq technology still suffers from 3′ terminal bias and the dropout problem during reverse transcription, the accurately estimation of tumor genomic instability is still challenging [31]. In addition to making effort to reduce technical noise in scRNA-seq technology, we should also seek to complement with other single cell omics approaches such as single-cell genomics in the experimental design [32].

CONCLUSION REMARKS

Single-cell transcriptomic technology is invaluable in creating a census of cell diversity, neurogenesis and cancer mechanisms in nervous system. Recent progress have used scRNA-seq to examine detailed biology processes in newborn neuron generation and synaptic regulation [33,34]. However, there are still challenges for the application of scRNA-seq in the brain, given the highly myelinated and complicated cellular-cellular connection of the tissue. For instance, the making of fully expression profile of neural mRNA including dendritic and axonal mRNA is still difficult [35–37], although a previous TIVA approach based on a light-activated mRNA-capture reagent was designed to harvest mRNA from cells in their natural environment [38]. Another replacement strategy is to represent whole neuron RNA by their neuronal nuclei RNA as several studies have demonstrated that the nucleis contain enough amount of mRNA for analysing [34,39,40]. Additionally, as most cells in the body are under control of the circadian transcriptional system, it would introduce unwanted biological variations if this factor is ignored in the experimental design. A potential solution of this potential challenge might be to strictly harvest the cells at similar time points during experiment. Beyond these technical challenges, a central direction in this field would be how to perform systematic analysis to understand the biology underlying the data. For instance, although various cell

types are discovered in each well desinged single cell experiment, including excitatary neuron, inhibitory neuon and non-neuronal cells (oligodendrocytes, microglia, astrocytes, etc.), what are the metabolic and transcriptional differences of those cell types? And are they under different selective pressures during the evolution of the nervous system? Additionally, neuronal cells are connected into neuronal circuits, with different cells have different connection properties. How are these connections reflected at the single cell level? The combination of neuronal morphology and single cell transcriptimics, which is single cell connectomics, would provide the opontunnity of understanding the whole brain connctomics at single cell level. We believe the answer of all above questions would accelerate the progress of big brain projects.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors Ganlu Hu and Guang-Zhong Wang declare that they have no conflict of interests.

This article is a review article and does not contain any studies with human or animal subjects performed by any of the authors.

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