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Single-Cell Transcriptome Analysis of Neural Stem Cells

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Abstract

Neural stem cells (NSCs) in the adult central nervous system play essential roles in both normal homeostasis and repair of damaged tissue after injury. The study of adult NSCs is hampered by the heterogeneous NSC population. In this review, we describe recent progresses in using single-cell RNA-sequencing (scRNA-seq) technique for the investigation of NSCs. The first part of this review focuses on the scRNA-seq techniques and bioinformatic analysis. The second part emphasizes the applications of scRNA-seq analysis in NSC research. Finally, we discuss the challenges and future directions of scRNA-seq technique for both basic research and regenerative medicine.

Keywords

Neural stem cells; Single-cell sequencing; Transcriptome; Bioinformatics; Homeostasis; Injury and diseases

Introduction

Neural stem cells (NSCs) are found in two regions of adult mammalian brains, the subventricular zone (SVZ) of the lateral ventricles in the forebrain, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus [30, 34, 44]. These adult NSCs are essential for adult neurogenesis during normal homeostasis and repair of damaged cells after injury [17]. NSCs are believed to support learning and memory [12, 52]. Studies have demonstrated that NSCs are heterogeneous cell population containing both quiescent and active cells [3]. Both intrinsic and extrinsic pathways have been identified for the adult neurogenesis process [7]. The underlying molecular mechanisms that activate quiescence NSCs into an active state may provide therapeutic benefits for nervous system regeneration.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Traditionally, the study of NSCs is hampered by the heterogeneity of NSCs and technology limitations.

In recent decade, technological advances have brought breakthrough and revolutions to scientific research. Since the first publication in 2009 [45], single-cell RNA-sequencing (scRNA-seq) has become an important method for dissecting and analyzing cells. It has accelerated our knowledge of molecular events that occur in each individual cell and amended several prevailing concepts such as the existence of adult NSCs in non-ventricular zone locations [31]. In the past 7 years, both the sequencing and analysis methods for scRNA-seq have dramatically improved [19]. scRNA-seq has been used to study epigenomes, exomes and transcriptomes of viruses, bacteria, immune cells, oocytes, sperms, and neurons [50]. The scRNA-seq results have been employed to understand the dynamics of differentiation or lineage reprogramming [9, 43, 49], assess the endogenous response to injuries and disease [31], discover rare cell types [31, 32], profile tumor heterogeneity and hierarchy [47, 50], and address the vulnerability of human cells to viruses [36, 46].

In comparison to bulk-sequencing methods, scRNA-seq can capture the cellular state of individual cells within a heterogeneous population which are undergoing proliferation or differentiation. Its measurement can represent each single cell within the sample, and the signal will not be averaged out through the population. This feature is especially beneficial for NSC studies, in which the quiescent semi-active and active stem cells co-exist within a niche of certain tissues and cannot be easily separated. scRNA-seq can be used to determine distinct population of NSCs as they transition into different cellular states. Pseudotemporal ordering of the cellular states provides inferred transitions within these cellular states. Understanding how changes in gene expression drives proliferation or differentiation in heterogeneous population of cells is necessary for understanding stem cell biology. In this review, we discuss the current advances and challenges of scRNA-seq and bioinformatic analysis methods in NSC research, and propose future directions of scRNA-seq technique for both basic research and regenerative medicine (see Fig. 1 and Table 1).

Single-cell sequencing and bioinformatic analysis methods

Isolating cells and library preparation

Single-cell isolation is the first and the most essential step for single-cell based genome-wide sequencing analysis. Technologies have been developed to allow researchers to pick individual cells instead of using small populations of heterogeneous cells. To perform single-cell sequencing, individual cells need to be isolated from the tissue or culture of interest, with its identity being tagged and traced. Many groups have been developing tools and standards, including CellSearch [55], Nanofilter [2], and Robotic micromanipulation [10]. In addition, three prevailing methods are discussed below due to their robustness and ease of carrying out.

Fluorescence-activated cell sorting (FACS) has been used for sorting a large number of cells for traditional sequencing methods, and it is also applicable to the single-cell sequencing. With sorting gates set based on scatter properties such as morphology, composition of cells or fluoresce, FACS can isolate a small population or individual cells [9, 31, 32, 35]. Up to 20

different parameters can be set up to select for individual cells. However, FACS usually requires large number of cells to start with and is thus not suitable for the study of rare cells. Moreover, the resulting cells from FACS may be biased by the pre-determined sorting parameters and the knowledge of the researchers. Despite these drawbacks, FACS is easy to perform and does not require purchase of specific equipment or expensive supplies.

Other types of automated cell isolating methods, the microfluidic and bead-based methods are also widely used. Many of them have been commercialized and services are provided to researchers to help with their single-cell sequencing, such as Drop-seq [33] and Fluidigm C1 autoprep system [24]. Drop-seq is advantageous because of unbiased sequencing of individual cells with a unique barcode provided for each library, while Fluidigm C1 system allows unsupervised cell sorting. However, for both systems, the lack of selecting cells of a certain size window usually result in a cluster of two or more cells in each compartment and can cause an averaging of the true single-cell signal.

In addition to automated cell isolation methods, several manually picking methods are still employed as an aid for selectively isolating particular individual cells. Such methods include serial dilution, micropipetting, and laser capturing [16]. Serial dilution and micropipetting are often used to isolate abundant cells and provide a cost effective alternative. Laser capture is especially useful for isolating rare cells or cells from a specific location in the tissue. However, all manual methods are low throughput. The number of individual cells to be included in one set of sequencing may be limited if the researcher uses manual methods for selecting cells.

Whole genome transcriptome amplification and sequencing methods

After isolating individual cells, researchers face another challenge in amplifying transcriptome for sequencing without introducing noise and artifacts. Genome loss, mutation, chimaeras, allelic dropouts, false-positives, and false-negatives often occur during the amplification and sequencing steps. The whole transcriptome amplification (WTA) is less challenging than the whole genome amplification (WGA), since a single cell only contains two copies of individual genes while there might be thousands of copies of RNA. The most frequently used WTA methods are oligo dT-anchoring method and template-switching method. Both methods have been thoroughly reviewed elsewhere [19, 50]. Several recently published works are compared and summarized (Table 1). For sequencing, the Smart-seq protocol [38] or its advanced version [39] has frequently used because of its ability to fully cover the transcriptome. Other commonly used sequencing methods include CEL-seq and STRT-seq, which provide unique molecular identifier (UMI) and strand information but suffer from poor coverage [20]. Thus, in studies seeking full transcriptome information, Smart-seq is usually used. In studies attempting to identify new sub-population of known cell types or new cell populations, CEL-seq and STRT-seq are mostly commonly used.

Quality control

After acquiring the sequencing data and before analysis, quality filtering should be performed by trimming the sequencing reads or removing low yield cells. In fact, all the

current single-cell isolation methods induce cellular stress that often results in necrotic or apoptotic cells, which consequently contribute to misleading results. Usually, criteria can be set to remove such cells by filtering out cells with low transcript number, low amplification efficiency, or low input material [1, 8]. Several standard tools are available, such as fastqc (Babraham Bioinformatics), which can perform a quality analysis of sequenced library, or bwa [28], which can be employed for trimming the low quality bases from each reads. However, simply setting a cutoff value is not sufficient to remove all low quality cells or may sacrifice high quality cells. Microscopic imaging of individual cells or staining with viability dyes may also be used to aid the quality filtering before sequencing. Several packages for evaluating the quality of single-cell sequencing are developed, including RSeQC [51], RNA-SeQC [11], and a machine-learning-aided tool [22]. These pipelines support the analysis of sequencing data generated from different platforms in a comprehensive manner and are open sourced for future development.

Analyzing single-cell sequencing data

After quality filtering, the sequencing data can be mapped and analyzed. Standard mapping tools are available for bulk analysis [18], and the cDNA sequences of most of the model organisms can be obtained from several online databases such as the UCSC genome browser. After mapping the sequencing reads to the genome, these data could be further analyzed to determine the similarity and differences.

Dimension-reduction techniques—The most prevailing method for analyzing raw single-cell sequencing data usually involves the dimension-reduction such as independent component analysis (ICA), principle component analysis (PCA), multi-dimensional scaling (MDS), or t-distributed stochastic neighbor embedding (t-SNE). With such techniques, the multi-dimensional properties of sequenced library can be represented by a few simplified vectors. For example, in PCA, the variance/standard deviation and covariance are calculated, and data sets for each individual cell are placed in clusters base on their “distances”, which represent the quantitative measurement of the difference between each pair of cells. From there, model-based grouping methods can be used to assign each cell a biological “pseudotime” and generate hierarchical structure of cells and identify the different populations these individual cells fall in. Several pipelines were developed under this principle: Monocle [48], Waterfall [43], Sincell [25], Oscope [27], Seurat [42], and Wanderlust [5]. For most of these pipelines, prior-knowledge of the cell populations is usually required, such as cell-type specific or stage-specific markers [31, 32]. However, such requirements limit the ability to identify new cell populations and are not sufficient to map out trajectories at a high resolution. Thus, a system without requirement of prior-knowledge, such as Waterfall [43], may be of more value for a blind analysis. In the Waterfall algorithm, unsupervised clustering and reconstruction of developmental trajectory was achieved by calculating the Euclidian distance, a quantification of the whole transcriptomic difference from each cell to the next. This method does not rely on identifying cells with known markers, but similar to other single-cell sequencing analyzing methods, it may overestimate cellular heterogeneity and provide false-positive results.

CellTree—Dimension-reduction techniques are limited by over-representing the main component and limited data processing of cells from multiple lineages. In overcoming these drawbacks, a better method adapted from Bayesian models called CellTree was developed [14]. In CellTree, a statistical approach (Latent Dirichlet Allocation) borrowed from natural language processing was used to identify the group structure of gene regulatory network on the current status of each cells. By comparing the “topic histograms”, the similarity between cells can be evaluated and used to build hierarchical structures. This new technique is based on R and thus friendly to who is not very familiar with coding. But the model selection can be difficult, since finding a balance between denser models/better distance matrix and the risk of overfitting is difficult.

Weighted correlation network analysis—Weighted correlation network analysis (WGCNA) [26] was developed for analyzing correlation patterns among genes across samples in bulk transcriptome. It has recently been applied to single-cell sequencing data and provides useful hints in precisely dissecting the developmental path [54] or identifying new cell populations of adult NSCs in brain regions that do not reside in traditional stem cell niches [32]. WGCNA is powerful in identifying modules that are consistent with highly inter-connected genes from each individual cells, and further pin-points the critical hub genes. However, since single-cell sequencing data are obtained from each individual cells, analyzing data from one cell by WGCNA may be less meaningful since this method focuses on the co-expression of genes [26].

Applications of single-cell sequencing in NSC studies

The proliferation and differentiation of stem cells are broadly studied in the field of developmental biology. Previous studies using traditional bulk RNA-seq would pool a group of stem cells/intermediate cells base on cell morphology, location, or cell-type specific marker. During such procedure, information regarding the heterogeneous nature and the intermediate states of stem cells is lost. This problem can be elegantly resolved by employing the aforementioned single-cell sequencing and transcriptome analysis. Single-cell sequencing technology allows researchers to identify and analyze the unique molecular signatures of individual cells that drive NSC activation and lineage development.

Identifying molecular signatures and cascades of NSCs underlying adult neurogenesis

Conventional methods for gene profiling are not sufficient to identify heterogeneous subtypes and developmental dynamics of adult NSCs. Thus, scRNA-seq has been used for the identification of characteristic molecular signals driving stem cell development and neurogenesis which include a unique transcription factors and surface proteins. In one study, analysis of scRNA-seq with dimension-reduction algorithm (Waterfall) by reconstructed somatic stem cell dynamics with temporal resolution [43]. With Waterfall algorithm, the molecular transitions identified by scRNA-seq revealed common features among different adult NSCs during hippocampal neurogenesis. The authors also identified two new genes, Aldoc and Stmn1, which are specifically expressed in adult NSCs. The authors further verified the expression of these two genes by immunohistology.

The SVZ of the postnatal forebrain contains several cell types that possess NSC characteristics [4, 13]. However, the molecular signatures and the differentiation potential for each of the cell types have not been well-characterized. Using scRNA-seq and WGCNA, Luo et al. interrogated CD133+/GFAP– ependymal cells in the adult mouse forebrain [32]. The authors identified a subset of quiescent NSCs that could be activated and differentiated into neurons and glia upon stimulation such as alleviated VEGF level. To confirm their findings, CD133+ cells in the fourth ventricle were stimulated by injecting bFGF and VEGF. As a result, these cells were triggered to proliferate, migrate, and differentiate into Map2+ and GFAP+ cells.

It is still controversial as to whether new neurons are generated in the postnatal cerebral cortex. Bifari et al. hypothesized that nascent neurons might be generated by progenitors in the meninges and meningeal substructures in the postnatal brain [6]. Using lineage tracing, the authors identified a population of neural progenitor cells in the meninges of postnatal mice. These cells migrate through the caudal ventricular zone and differentiate into functional and integrated neurons of the posterior cortex. In this study, scRNA-seq analysis revealed that transcriptomes of neurospheres of PDGFR β + meningeal cells overlap with those of Prom1+ cells from the VZ/SVZ. This result confirms that the identified neural progenitors in the meninges possess a radial glia-like molecular signature, e.g., the PDGFR β [23].

Determining NSC response to injury and disease

Regeneration by endogenous NSCs in response to injury or disease has become one of the most focused topics in the field. scRNA-seq technology has allowed researchers to identify genes expressed in the activated stem cells to better understand this processes, identify responsible pathways activated or interrupted by the disease, and identify potential new targets for therapeutic development.

Response of adult neural stem cells to injury—Adult NSCs are activated upon injury and have the ability to proliferate and differentiate to support the natural healing mechanism [7, 17, 41]. To better understand the process of NSC activation, Llorens-Bobadilla et al. analyzed NSCs from the SVZ by scRNA-seq to identify molecular signatures of quiescent and activated NSCs [31]. Single-cell analysis allowed identification of genes that driving stem cell activation/proliferation after ischemic brain injury. In this study, the unique transcriptomes of quiescent and activated NSCs from the SVZ of the mouse brain in response to ischemic injury were determined by scRNA-seq analysis. Single cells were isolated by their expression of GLAST and Prominin1 (CD133). It was found that ischemic brain injury activates dormant NSCs via the interferon gamma signaling pathway accompanied by down-regulation of glycolytic metabolism, Notch, and BMP signaling. An increase in lineage-specific transcription factors was also observed before activation of NSCs. Heterogeneous response of dormant NSCs and their associated pathways were identified. Different states of NSCs from quiescence to activation were characterized, which could not be revealed with pooled or population-based studies. Similarly, scRNA-seq analysis identified distinct injury responses in different types of dorsal root ganglion neurons as well as regeneration genes after nerve transection injury [21].

Identify pivot genes responsible for NSC-related developmental disorders—

The recent outbreak of Zika virus (ZIKV) infection and associated microcephaly has created a worldwide health concern [37]. ZIKV infection leads to dysregulation of cell cycle and gene transcription, and cell death in human NSCs [46]. These studies confirm that NSCs are a direct ZIKV target and provides mechanistic understanding of ZIKV infection and microcephaly. In an attempt to identify ZIKV receptor, Nowakowski et al. employed scRNA-seq analysis and immunohistochemistry to determine ZIKV targeted cell populations and molecular mechanism that lead to microcephaly [36]. A highly conserved gene AXL was identified as a candidate receptor for the entry of ZIKV into NSCs. AXL is strongly expressed in human radial glia, brain, capillaries, microglia, and in retinal progenitors. Since these selectively expressed proteins in radial glial cells (embryonic NSCs) promote ZIKV entry during neurogenesis, they could play a role in the microcephaly cases. However, a more recently published study by Eggan group at Broad Institute of MIT and Harvard showed that deletion of AXL receptor has no effect on ZIKV entry or ZIKV-mediated cell death in human induced pluripotent stem cell (iPSC)-derived neural progenitors or cerebral organoids [53]. Although scRNA-seq analysis identified many candidate genes, the ZIKV receptor still remains to be determined.

Understanding iPSCs

The iPSC-derived organoids created a greater potential for developmental, regenerative, and artificial organ research. Camp et al. used scRNA-seq technique in combination with bioinformatic algorithms (e.g., hierarchical clustering, principle component analysis, and covariation network analysis) to determine cell composition and progenitor-to-neuron lineage relationships in human cerebral organoids and fetal neocortex [9]. The study revealed the similarity and differences in the transcriptomes among these organoids. These authors showed that cells in organoid cortex-like regions have gene profiles highly similar to tissues in fetal development, indicating that organoid culture systems are a good model for investigating certain genetic features in cortical development. Cells from two human neocortex specimens 12–13 weeks post conception exhibited cell markers highly similar to organoid cortical cells in active genes and signaling pathways involved in cortical processes, e.g., cell proliferation, self-renewal, production of ECM, migration, adherence, delamination, and differentiation. Results identified that 90% of the genes involved in transcription regulation between fetal and organoid cells types were similar between the two groups. Seventy percent of the genes involved in Notch/Delta signaling were also similar between the two groups, and 96% of the genes involved in neurite outgrowth. However, beyond the 80% similarity, it is also noted that findings obtained in organoid research may not be translatable to fetal development since there is still 20% diversity. The study also identified underdeveloped SVZ in organoid model, indicating that organoids may not be a good model system for studying SVZ.

Challenges and future directions

scRNA-seq is a powerful tool for investigating cellular and molecular diversity within heterogeneous cell populations. It also provides a method to resolve dynamic changes during differentiation and clarify the genes that play a role in NSC homeostasis and regeneration

after injury. Over the past 7 years, major progress in both technological developments and research applications in single-cell sequencing analysis has been achieved. The technique in combination with bioinformatic approaches has begun to unravel key questions in stem cell biology that have been difficult to address with bulk transcriptome profiling. It has already revolutionized our understanding in stem cell biology. However, since it is still a relatively new and complicated technique, it lacks a “standard” or step-wise detailed protocol to guide beginners from choosing the appropriate cell isolation methods, sequencing, and analysis methods to properly interpret biological results. Other drawbacks include the high cost and low speed of transcriptome sequencing of individual cells. Ideally, scRNA-seq could be performed rapidly at a reasonable cost to profile hundreds and thousands of single cells.

In traditional studies, cells are characterized into different populations using a handful of markers, which represent only a small portion of their transcriptome. Instead, cells should be analyzed by their entire gene expression profile to reduce the risk of misinterpretation due to the lack of specificity of markers [29]. In addition, the combination of scRNA-seq with other modalities, such as epigenomics and proteomics, are expected to yield a greater insight into the capture of the biological state of cell or tissue [15, 40]. Furthermore, if the location of each cell in the tissue of interest could be recorded and correlated to the profile, such a “3D” biological model should further advance our knowledge of cell biology and pathology. Although scRNA-seq allows mapping the transcriptome profile of each individual cells, a high-speed analyzing method to read and understand the entire dataset generated from high-throughput single-cell sequencing is still lacking. Further improvements in this technique and development of analysis tools will lead to more breakthroughs in biomedical research. As methods continue to mature, the applications of single-cell sequencing are expected to have a major impact on developmental study of NSCs and future development of therapeutics for CNS disease and injury.

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Fig. 1.
Methods and applications of scRNA-seq analysis

Table 1

Summary of several recently published works using single-cell sequencing

Citation	Cell types	Isolation methods	WTA methods	# of cells per sequencing	Sequencing depth	Alignment rate	Sequencing methods	Population control	Noise control	Analyzing algorithm
[31]	Adult NSC in SVZ (uninjured/-ischemia); CD133+/Prom1+ and PSA- and PSA- ⁺ NCAM ⁺	Flow cytometry		104	8.3 M read pairs	59%	Smart-seq2 [38]	Two replicates of 1000 cells	RNA spike-in standards	PCA, unsupervised hierarchical clustering, pseudotemporal ordering [48]
[43]	Adult hippocampal quiescent neural stem cells	Nestin-CFP labeling	SMART-seq protocol (Ramsköld et al., 2012)+ DNase I treatment to remove genomic DNA	142 Nestin-CFP ^{pos} and 26 CF ^{pos} single cells	2~10 M reads	87%	Smart-seq (Ramsköld et al. 2012)	Total RNA from wild-type adult mouse dentate gyri serially diluted to 3 pg		Waterfall: unsupervised hierarchical clustering analysis, PCA, k-mean clustering, pseudotime
[32]	Adult mouse forebrain CD133+ and CD133- cells	FACS plus manual picking under the microscope		28 (including different CD133+ and CD133- population)	15~20 M reads		SOLID-3 and Illumina HiSeq 2500		Sequencing reproducibility controls and control for batch effect	WGCNA
[36]	SVZ and SVZ tissue samples	Cl single-cell auto prep integrated fluidic circuit (fluidigm)	SMARTer ultra-low RNA Kit (Clontech),	715 single cells						PCA, t-SNE, K-means clustering
[49]	Multiple time points during the transition from embryonic fibroblast to induced neurons	Captured on medium-sized microfluidic RNA-seq chip	SMARTer ultra-low RNA kit for illumina	405 single cells	1~7 M of 100 or 75 bp paired-end		Illumina Nextera XT DNA sample preparation kit according to the protocol supplied by fluidigm			PCA, tSNE
[9]	Fetal samples 12~13 weeks post conception Cerebral organoids derived from iPSCs (iPSC line PS409b2)	Laser-micro-dissected zones and FACS purified cells	cDNA sequencing libraries constructed using illumina Nextera XT DNA sample preparation kit	226 single-cell 100 bp paired-end	2~5 M 100 bp paired-end reads		Illumina HiSeq 2500	12~13 wpc vs organoids		t-stochastics neighboring embedding, monocle