Values of Single-Cell RNA Sequencing in Development of Cerebral Cortex

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Abstract

The single-cell RNA sequencing (scRNA-seq) is a powerful tool for exploring the complexity, clusters, and specific functions of the brain cells. Using scRNA-seq, the heterogeneity and changes in transcriptomic profiles of a single neuron were defined during dynamic development and differentiation of cells in cerebral cortex regions, and in the pathogenesis of neurological diseases. One of the great challenges is that the brain sample is susceptible to interference and confounding. More advanced methodologies of computational systems biology need to be developed to overcome the inherent interference and technical differences in the detection of single-cell signals. It is expected that scRNA-seq will be extended to metabolic profiles of the single neuron cell on basis of transcriptional profiles and regulatory networks. It is also expected if the transcriptional profiles can be integrated with molecular and functional phenomes in a single neuron and with disease-specific phenomes to understand molecular mechanisms of brain development and disease occurrence. scRNA-seq will provide the new emerging neurological disciple of the artificial intelligent single neuron for deep understanding of brain diseases.

Keywords

Single-cell RNA sequencing · Cerebral cortex · Neurons · Brain · Anesthesia

19.1 Introduction

The genome, epigenome, and microenvironment of each single cell in the organism are unique. The gene expression of the single-cell is based on fluctuations in the mechanisms of transcription and translation. The heterogeneity of cells is the basic nature of the homeostasis and development of the living body system to perform specific tasks and functions. It is necessary to define similarities and differences of different cells from morphology, protein level, and even gene level to understand the differences between cells. Single-cell RNA sequencing will become a new approach to monitor gene expression in clinical practice to explore gene expression profiles at the single-cell level using single-cell RNA sequencing (scRNA-seq). The scRNA-seq is a powerful tool to classify and identify cell subtypes [1], characterize rare or small cell populations, and track dynamics of cell-to-cell variations [2].

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The brain is one of the most complex tissues and is intensively investigated on brain cells, and current studies focus on location, morphology, electrophysiological property, target specificity, molecular biomarker, and gene expression pattern [3–6]. Thus, scRNA-seq becomes more important for the understanding of the brain contributions to learning, memory, and other cognitive functions [7]. scRNA-seq can make it possible to understand the heterogeneity and regulatory networks in brain cells at the single-cell level [8]. The present chapter will review recent studies on the use of scRNA-seq in brain cells and summarize the values of analysis method and the significance of results from scRNA-seq in brain cells. We emphasize the importance of clinical application of scRNA-seq in brain cells and potential challenges to be faced in future. We will comprehensively discuss the application of scRNA-seq in the development of the cerebral cortex to better understand the development and function of cerebral nervous system.

19.2 Single-Cell RNA Sequencing Technologies

The hotspot technology of scRNA-seq methods are summarized in Fig. 19.1 to assure the high-resolution analysis of individual cells unbiased and profound. The scRNA-seq contributes to reveal the heterogeneity, dynamics of transcription, and regulatory relationships between genes in a cell [9]. Developed scRNA-seq is applied to investigate the rare cell clusters, which may be omitted by traditional analysis. Specific features of these cells as well as the events of the interactions among cells are unveiled by scRNA-seq, rather than by previous high-throughput analysis [10]. scRNA-seq revealed the dynamic function of individual cell in developmental biology [11–16], neurobiology [17–20], immunology [21–24], and cancer research [25–29]. The landscape of single-cell tumor immune map accelerates the immune treatment on basis of high heterogeneity of immune cells in cancer and identifies molecular characterization of tumors in symptomatic and asymptomatic patients [30].

Using scRNA-seq, the differentiation fate of progenitor cells and the progress of individual cell are defined in the development, during which new cell clusters are distinguished by scRNA-seq [31–33]. The complexity of brain structure enables the delicate regulation in the developmental progress of brain. The nervous system is the most complex organ in mammal, where the cerebral cortex development is the main model system for neural developmental investigations and shares many consistent mechanisms with the developing brain and spinal cord. The Dll1/Notch, Nrg1/ErB, and Fgf10/Fgfr2 pathways were involved in this transformation of neuroepithelial stem cells into radial glial (RG) stem cells. Other transcriptions factors (Ap2γ, Ngn2, Insm1, or Tbr2) are discovered to activate the generation of basal progenitors from RGs, which was inhibited through the Notch and FGF pathways and the epigenetic regulator Ezh2, to differentiate RGs into astrocytes and lead to the termination of neurogenesis. Multiple signaling pathways (Jack/Stat, Notch, BMP, FGF) promote the neurogenic-to-glycogenic switch, although other signalings are still unclear. scRNA-seq is applied to investigate the novel mechanisms implicated in the cerebral cortex development (Fig. 19.2) and to characterize the cellular composition of the mouse cortex at development-embryonic day 14.5, representing a progenitor-driven stage and birth, when neurons corresponding to all six cortical layers were born and gliogenesis has begun. Distinct cortical layer-specific cell types and the spatial and temporal expression patterns of hallmark genes were assigned to 22 cell clusters and described (Fig. 19.3).

The distinct sensory neuronal types were dissected by scRNA-seq and Notch signaling is indispensable for brain development [17, 34]. Combining the scRNA-seq with electrophysiology, the development of embryonic hippocampal neurons and the neonatal cortical neuron cells were mapped and classified. The

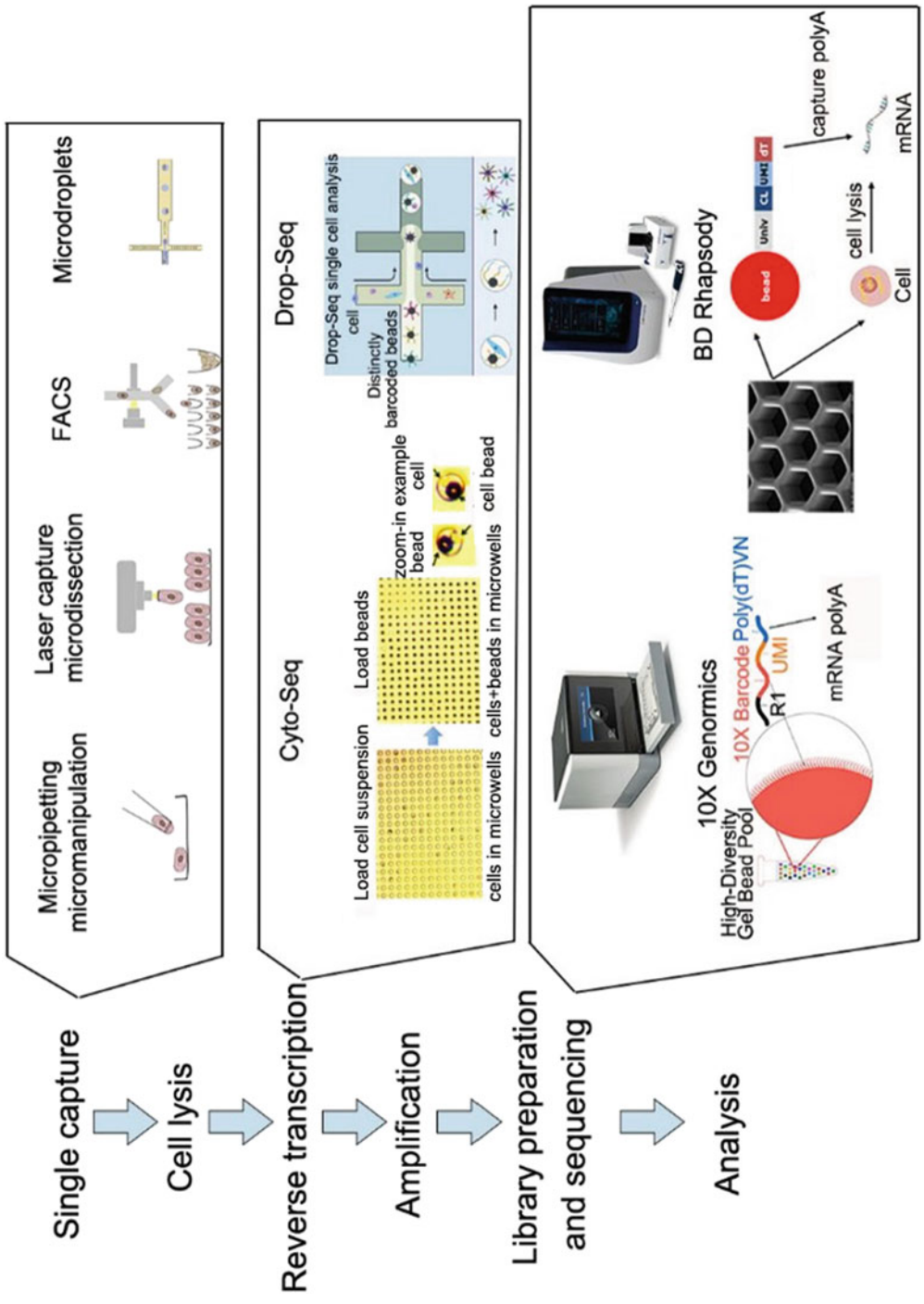

Fig. 19.1 Schematic of single-cell RNA-seq experiment showing each step and different experimental approaches [9]

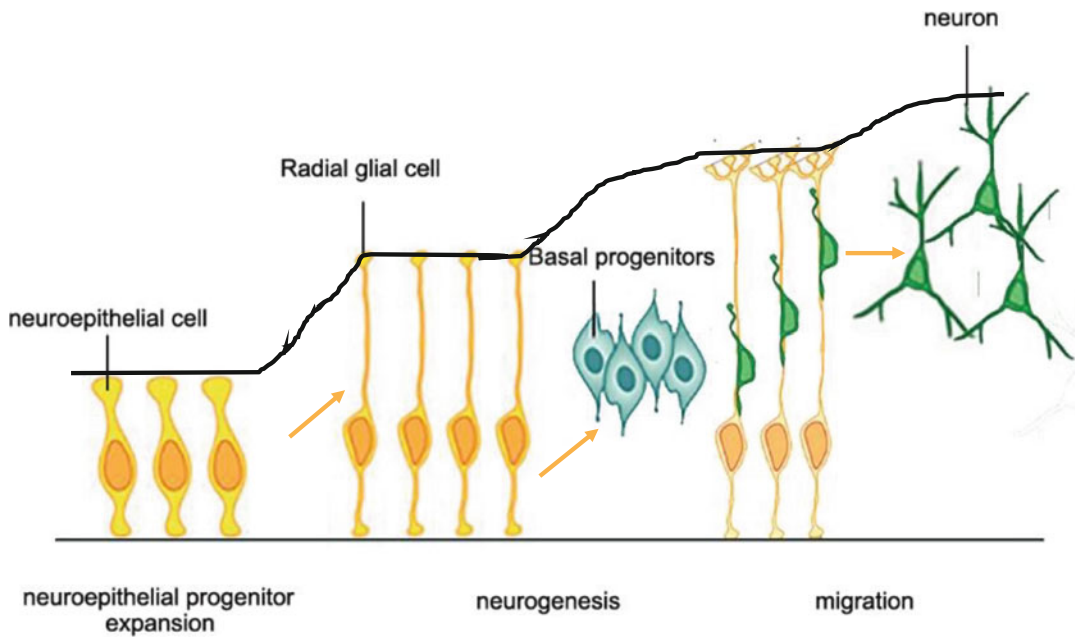

Fig. 19.2 Development of mouse cerebral cortex. Cerebral cortex develops via a complex sequence of cell proliferation, differentiation, and migration events [93]

new marker genes of the cerebral cortex during human development and the specific developmental characteristics, as well as the developmental timeline of excitatory neurons were found using scRNA-seq [19]. By the scRNA-Seq in situ, the neurons location and the difference between the neighbor neurons spatially were defined [35].

The single-cell transcriptomic profiles developed along with the qPCR development and single-molecule fluorescence in situ hybridization are the primary method of the transcripts analysis [36–39]. The whole-transcriptome analysis and the subsequent RNA sequencing are adapted for analyzing single cells [40–43]. The scRNA-Seq was applied to investigate the early embryonic development and global patterns of gene expression variations [44, 45], although the amount of biological materials were a limiting factor [2]. The unbiased analyzing of scRNA-Seq is adapted for the hundreds of thousands of cells endowed of heterogeneity [46].

The methods for capturing single cells from enormous cells include mouth pipetting, serial dilution, robotic micromanipulation, and flow-assisted cell sorting; while the methods for isolating rare single cells still lag behind, including Nanofilters [47], MagSweeper [17], Laser-capture microdissection, CellSearch [48], CellCelector [49], and DEP-Array [50]. It is necessary to amplify the RNA sequence for scRNA-seq due to that the total RNA in a mammalian cell is only 10 pg and the mRNA is only 0.1 pg. SMART-Seq is a whole-transcriptome amplification (WTA) method performed using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase to protect the full-length amplification from the strong plague of the 3′ mRNA bias. MMLV has both template-switching and terminal transferase activity, which leads to the addition of contemplated cytosine residues to the 5′ end of the cDNA [1]. The templates can be switched by MMLV and transcribe the other strand to amplify the full-length cDNA transcripts by adding a poly

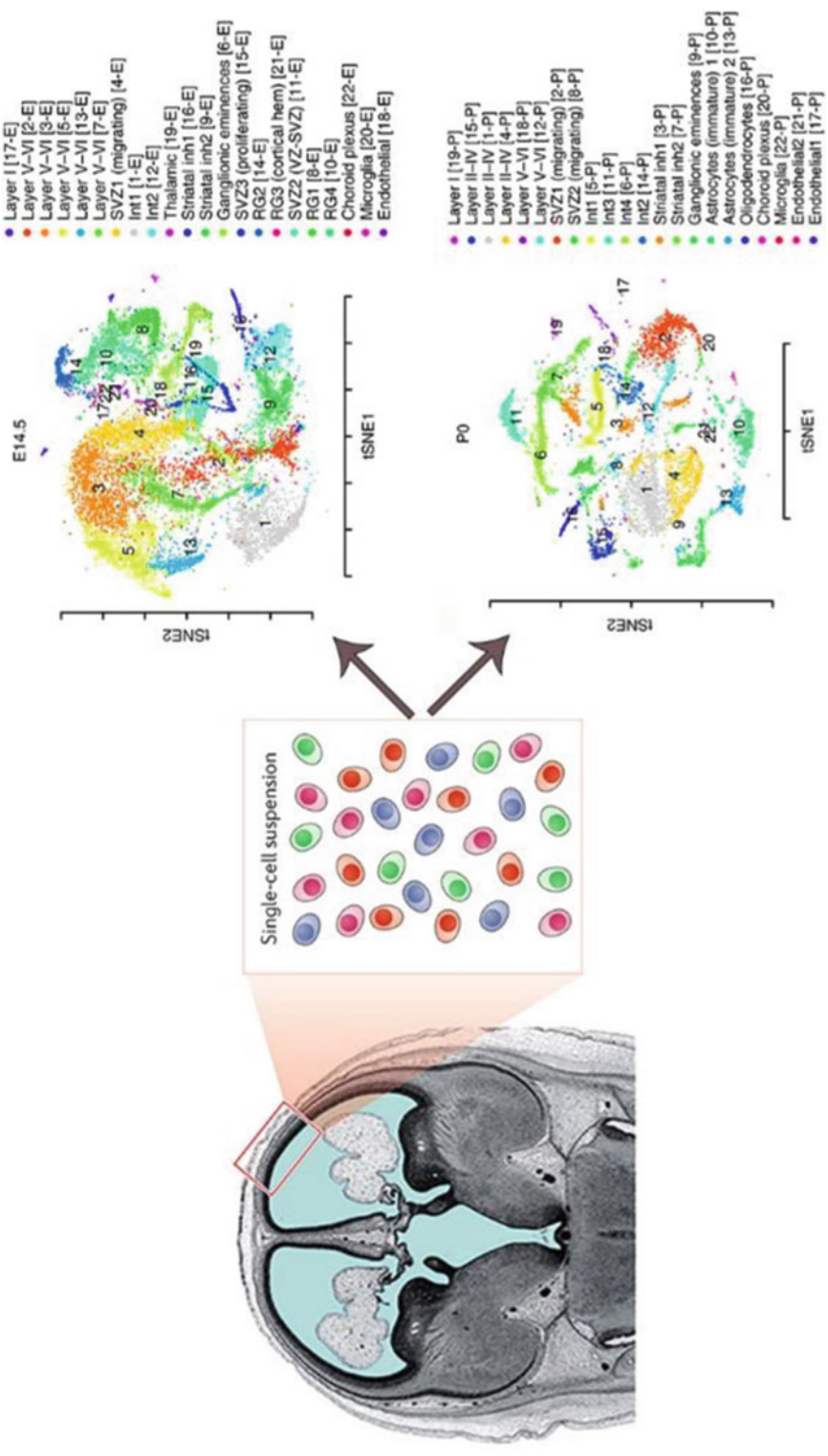

Fig. 19.3 Cell clusters analysis of developing cerebral cortex by scRNA-seq. t-SNE visualization of the 22 clusters identified in the E14.5 and P0 cortex [94, 95]

(G) template with an adaptor sequence. To overcome the distortion of the mRNA strand during the amplification progress, cDNA is labeled with barcodes thus specific cDNA sequence is assigned to the specific cells [51–54]. With the revolutionary development of technologies, MARS-Seq, Cyto-Seq, Drop-Seq, inDrop, and the scRNA-Seq are promoted extensively.

19.3 scRNA-seq and Developing Cerebral Cortex

The diversity, function and the range of transcriptional regulation are studied on brain cells from the cerebral cortex with RNA-seq. As an emerging tool, scRNA-seq is gradually applied to study the complexity of brain cells, new cell populations, specific genetic characteristics, and potential regulatory networks [55, 56]. Brain cells include highly complex nerve cell types/subtypes with special morphology, excitability, connectivity, and cell location [57]. Different neuronal cell types and subtypes, and new cell-specific markers were found using scRNA-seq. For example, Amit et al. extracted 3005 single cells from the cerebral cortex and hippocampus from mice, performed scRNA-seq analysis, and found nine major cell populations, including S1 and CA1 vertebral neurons, transfer neurons, oligodendrocytes, astrocytes, microglia, vascular endothelial cells, parietal cells, and ependymal cells. Novel and specific molecular markers of different cell types were also discovered, e.g., Gmll549 for S1 vertebral neurons, Pnoc for transferred neurons.

The individual adult neurons are freshly isolated from a limited regional sample of neurosurgical tissue [58]. Freshly separated neurosurgical tissue is better for analyzing individual neurons, while more samples of postmortem tissues are available in clinical practice. Lake et al. developed a new method of the neuron nucleus and RNA sequencing for the brain, separated the 3227 individual neurons from six different regions of the cerebral cortex for RNA sequencing, and found 16 subtypes of neurons in the cortex cells with molecular biomarkers. The cerebral cortical neurons are evaluated and developed originally from the subependymal neural progenitors, and neural progenitor cells complete the development process of the cerebral cortex through the proliferation, differentiation, and migration. The temporal and spatial characteristics of cerebral cortex development from rodents to primates include differentiation characteristics from progenitors to various types of neurons, with a clear relationship between mental disorders and target gene expression.

The developmental mechanisms of the cortex with different functional divisions are involved in intrinsic and extrinsic biological mechanisms. For example, Gbx2 contributes to the development of normal cortical regions, rather than thalamic cortical projections. Providing the first clear evidence that thalamic innervation is not necessary for the basic generation of cortical area maps, and the formation of cortical regions depends primarily on the mechanisms within the telencephalon [59]. The intrinsic mapping center of the telencephalon controls the size and location of the cortical region. The morphine and fibroblast growth factor 8 (FGF8) released from the commissural plate at the distal prefrontal tip of the brain periphery can be adjusted along the frontal tail. Other factors play complementary roles, such as Fgf17 [60], BMP [61], and Wnt [62]. The thalamic input is required to establish genetic and functional divisions between the primary and adjacent high-grade sensory cortexes [63]. In addition, in-cortical self-generating activities also contribute to the formation of cortical columns [64] (Fig. 19.4).

The precise gene spatiotemporal expression profile of the cerebral cortex is important for the evolution, development, and function of the nervous system [65–67]. The temporal and spatial characteristics of gene expression during cortical development are different from genetic characteristics of different brain regions in the same period, or developmental stages of the same brain region. Using immunofluorescence technology, the diversity of Drosophila neurons was found to be dependent upon the integration of time and space patterns [68]. The temporal and

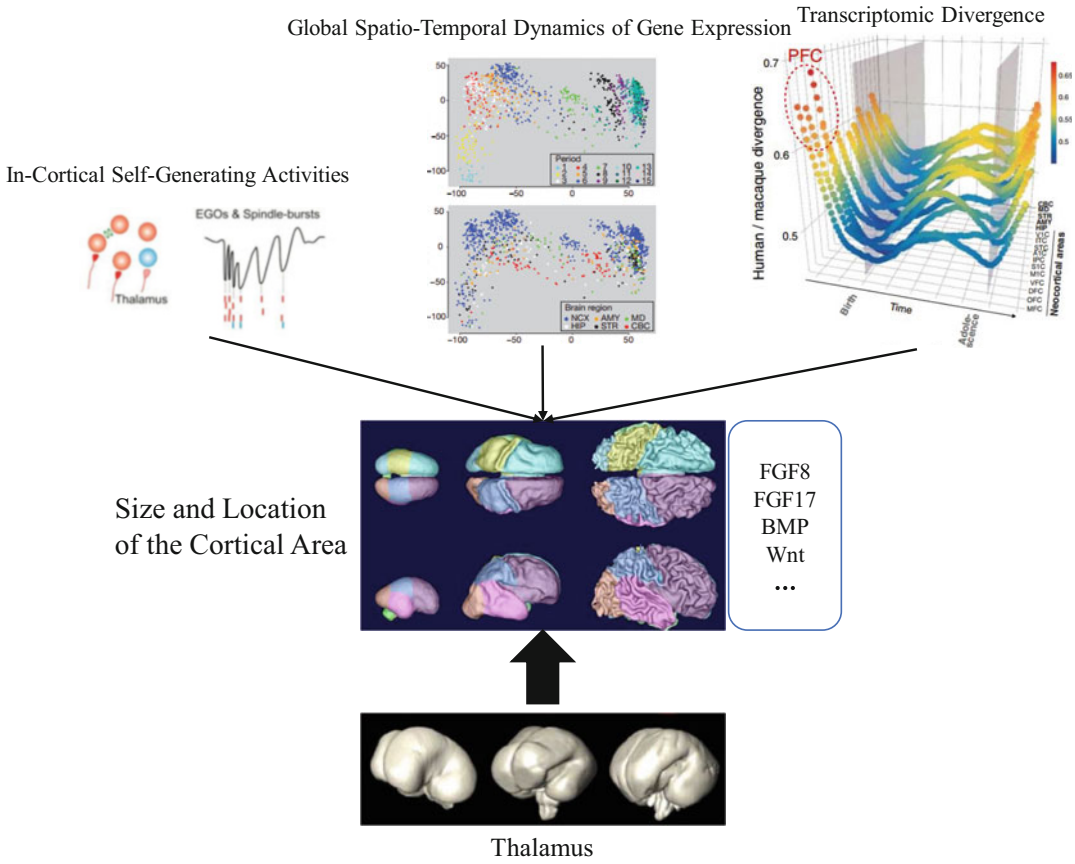

Fig. 19.4 Factors involved in cerebral cortex development. The formation of cortical regions depends mainly on the mechanisms within the cortex, self-generating activities, temporal-spatial differences in gene expression, and the involvement of the thalamus in cortical development [18, 60–64, 96, 97] (Credit: Studholme Lab/UWMC)

spatial characteristics of mouse brain development were defined with a new algorithm by detecting repetitive patterns in spatiotemporal gene expression data of developing mouse brains. Expression patterns can reveal regional differences in brain development [69]. Previous studies analyzed exon-level transcriptome data from multiple brain regions and neocortex regions of developmental brain and adult brain by transcriptome sequencing. About 90% of expressed genes were in the whole-transcriptome or exogenous sublevels are different before birth, and then the similarity of transcriptomes in the same region increases, forming different co-expression networks [70].

Systematic analysis of temporal and spatial gene expression trajectories during cerebral cortical development because of the coexistence of multiple cell types in emerging tissues at different stages of maturation and differentiation. The scRNA-seq of primary and medial ganglionic eminence (MGE) micro-dissected from germinal zone and cortical plate samples was performed at various stages of peak neuronal firing from progenitor cells to neuronal differentiation during post-mitotic neuronal differentiation [71]. Those cortex areas mainly include DFC, HIP, AMY, STR, MD, and CBC from the 60 days post-conception to the 11-year-old macaque. Human-specific transcriptomics and

spatiotemporal transcription specificity were associated with neurological diseases such as autism and schizophrenia, synapse formation, and neuronal development [72].

The classification of neurons is based on morphological, chemical, and electrophysiological differences [73, 74, 75], as well as the different molecular features that researchers use to study. The large-scale characterization of approximately 1000 neurologically important gene expressions was defined with in situ hybridization in the visual and adult temporal cortex of the adult brain prior to the application of scRNA-seq. Changes in gene expression profiles distinguish cortical functions between species [74]. Compared with scRNA-seq, those methods have significantly lower flux and higher workload. For example, the isolation 49 cells from mouse cerebral cortex and hippocampus, including 23 GABAergic neurons, 19 glutamatergic neurons, and 7 non-neuronal cells, took much longer time in the previous study [76], as compared with clarification of mouse somatosensory cortex and hippocampal CA1 region cells using scRNA-seq. About 47 different subclasses of molecules were identified and corresponded to cell types, morphologies, and locations. The authors found a layer I neuron expressing Pax6 and a unique mitotic oligodendrocyte subclass labeled by Itpr2. The diversity of cortical cell types on basis of transcription factors forms a complex hierarchical surveillance to demonstrate mechanisms for maintaining adult cell type identity [73]. Three thousand two hundred and twenty-seven data sets of single nerves from six different regions of the cerebral cortex were generated using a scalable approach to sequencing and quantifying RNA molecules in neuronal nuclei isolated from postmortem brains. Using iterative clustering and classification methods, 16 neuronal subtypes were identified by annotating biomarkers and cortical cell structures [77]. The cortex of different brain regions of human embryos was analyzed using scRNA-seq at 22 and 23 weeks. The distribution characteristics of local gene expression and neuronal maturation were evaluated using the modified STRT-seq method in human cerebral cortex [18]. With the advancement of scRNA-seq, more than 20,000 cells in the primary visual cortex and motor cortex of adult mice were sequenced and 133 cell types were identified. Excitatory neurons are regionally specific, and one subtype is only distributed in a certain cortex, where different subtypes also show different long-range projection modes by retrograde labeling [78].

Cortical development is experienced from progenitors to complex neural networks. The mechanisms by which neuronal diversity and the connections form complex neural networks can improve the discovery of pathogenic targets of neurodevelopmental disorders [79]. As the major components of a complex neural network, excitatory neurons migrate to the cortical plate, form six cortical layers with a stereotyped connection pattern, and contribute to the configurations of the functional circuit [71]. Those neurons account for approximately 80% of neurons in the cortex and interact with a small number of inhibitory cortical neurons in important ways [79]. In the cerebral cortex, GABAergic interneurons are evolved into high heterogeneity of cell types with unique temporal and spatial capabilities to affect neuronal circuits. Up to 50 different types of GABAergic neurons are distributed in the cerebral cortex and derived from the subcutaneous progenitors in the ventral surface of the lower cerebral ventricle. Internal neuronal diversity occurs through the implementation of intracellular genetic processes in progenitor cells over a longer period of time until the internal neurons acquire mature features [80]. A few precursor cells of inhibitory neurons are present in the early prefrontal cortex using scRNA-seq, of which the most are in the cell cycle, except for during the rest period [19]. Glial cells, including astrocytes, oligodendrocyte Schwann cells, and microglia, do not transmit transmitters like neurons, but form the environment where neurons survive and form neural networks [81]. Their dysfunction is associated with neurological diseases [82]. Human glial cells wrap more than single astrocytes in mice which can wrap more than 100,000 synaptic structures [82], support the role of neurons, and participate in the

development of the nervous system, plasticity, and disease. Glial cells are also involved in synapse formation, regulating synaptic function, and blood flow [83].

Many long-chain noncoding RNAs (lncRNA) express in brain tissue and regulate neuronal function, responsible for the development of diseases. lncRNA exhibits stronger in the tissue and cell specificity, as compared to mRNA [84]. The subtype-dependent enrichment of lncRNA was noticed in cortical progenitors isolated and sequenced from in human fetal brain cerebral cortex within 4 h after autopsy [85]. Liu et al. deeply dissected lncRNA from polyadenylated and total RNA obtained at different developmental stages in human neocortex using strand-specific RNA-seq and analyzed the transcriptome of individual cells. Single-cell transcriptomics of hundreds of neocortical cells revealed that many lncRNAs abundantly expressed in a single cell and are cell type specific. Among those, LOC646329 is a lncRNA rich in single radial glial cell and regulates cell proliferation [86]. A variety of lncRNAs are involved in the cellular processes of brain development and the spatiotemporal expression of lncRNA in a cohort of 13 lncRNA null mutant mouse models showed different between developing and adult brains, between transcriptomes and phenomes, between temporal and spatial brain development, and between selected and non-selected brain regions. Among those, a variety of cellular pathways and processes changed after deletion of the lncRNA locus, and four of the lncRNAs affected the expression of adjacent protein-coding genes in a cis-like manner [87]. In addition to lncRNA, microRNAs play an important role in posttranscriptional regulation and complexity during brain development and are considered as important triggers of brain development and neurological or psychiatric diseases [88]. The limited understanding of in vivo miRNA targets and their intensity in single cells makes it difficult to define miRNA-mRNA networks. Single-cell analysis using binary and co-expression networks is carried out by combining high-throughput sequencing of RNA and immunoprecipitation-cross-linked immunoprecipitation with AGO2 antibody (AGO2-HITS-CLIP) The miRNA-mRNA interaction as a functional module undergoes dynamic transformation during brain development and shows cell-specific and highly dynamic during development and throughout the evolution process. For example, the interaction between ORC4 and miR-2115 abundant in radial glial cells, can control the proliferation rate of radial glial cells during human brain development [89] (Fig. 19.5). Those studies on brain cells based on scRNA-seq enable us to better understand the similarities and differences between brain cells.

19.4 Application of scRNA-seq in Neurologic Diseases

The scRNA-seq as a powerful tool can provide new insights for understanding molecular mechanisms of the functional and dysfunctional regulations in a single neuron. It is the time to deeply understand the occurrence and development of neuro-related diseases, e.g., degenerative diseases and brain tumors, at the level of single-cell transcriptome, find disease-specific cell populations, and discover target genes for therapy.

Alzheimer's disease (AD) is a harmful neurodegenerative disease without effective treatments, due to the heterogeneity among neurocytes and among immune cells associated with the development and progression of AD. A microglial cell type with molecular markers, spatial locations, and signaling pathways was identified to contribute to neurodegenerative disease, accompanied with AD-associated brain immune cell populations. Such specific microglia specifically present in neurodegenerative diseases, with great potential for the future treatment of AD and other neurological diseases.

Glial cells and stem cells undergo genetic mutations during development and may develop human gliomas, due to the composition of different apparent states and cell types. In recent years, more and more single-cell RNA sequencing has

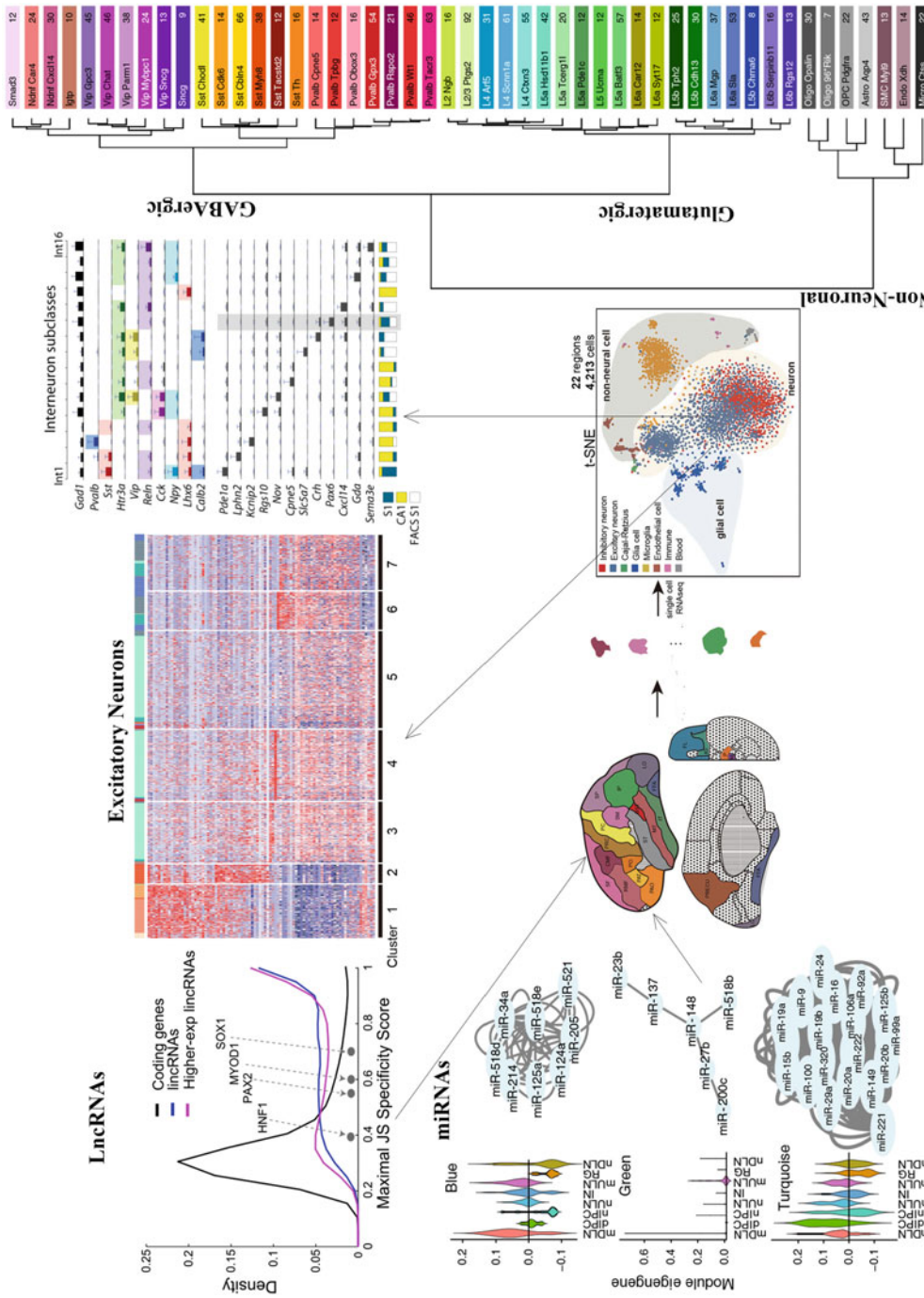

Fig. 19.5 Noncoding RNA (LncRNA and miRNA) characteristics and differentiation of cerebral cortical cells excitatory neurons and interneurons [19, 73, 76, 84, 89]

been applied in the study of glioma. Patel et al. [90] found that 430 cells from 5 primary glioma samples were associated with oncogene signaling, proliferation, immune response, and hypoxic signaling using scRNA-seq. The clear heterogeneity among intra-glioma cell subtypes was identified, as an important breakthrough in understanding the heterogeneity, biology, prognosis, and treatment of glioma.

Tumor stem cells can drive the growth of tumor cells, but there is no particularly good evidence for the existence of tumor stem cells in human solid tumors. Tirosh et al. analyzed 4347 individual oligodendrocytes using scRNA-seq, and found that the majority of tumor cells differentiated into two specific glia oligodendrocytes and astrocytes as well as a small number of cells in the undifferentiated state ranged from genome expression level to the development process and associated with cancer stem cell signaling pathways. Proliferating characteristics of gene defined were consistent with the tumor stem cells promoting tumor growth in human oligodendroglioma. This scRNA-seq provides insight into the developmental structure of oligodendroglioma at the single-cell level and strong support for disease treatment.

19.5 Limitation

Transcriptome features of glutamatergic neurons vary widely among cortical regions. It is questioned whether each of those transcriptome features represents a unique cell type or reflects a heterogeneous transcriptional state in a single projection neuron that may be affected by a series of nerve activity and other factors [91, 92]. By combining the transcriptome profile to other phenotypes, e.g., morphology, electrophysiological properties, and function, more molecular subtypes and phenomes of neurons are characterized using scRNA-seq. The gene expression in the cortical cellular and molecular networks at a single cell will generate important information to determine the molecular interactions between the connected genome and transcriptome within a cell. There are urgent needs to furthermore explore intercellular and intermolecular heterogeneity, the degree of selectivity and differentiation of cortical projections, and disease-specific biomarkers and mechanisms of circuit development and maturity.

19.6 Summary and Prospect

scRNA-seq is a powerful tool for exploring the complexity, clusters, and specific functions of the brain cells. Using scRNA-seq, the heterogeneity and changes in transcriptomic profiles of a single neuron were defined during dynamic development and differentiation of cells in cerebral cortex regions, and in the pathogenesis of neurological diseases. One of the great challenges is that the brain sample is susceptible to interference and confounding. More advanced methodologies of computational systems biology need to be developed to overcome the inherent interference and technical differences in the detection of single-cell signals. It is expected that scRNA-seq will be extended to metabolic profiles of the single neuron cell on basis of transcriptional profiles and regulatory networks. It is also expected if the transcriptional profiles can be integrated with molecular and functional phenomes in a single neuron and with disease-specific phenomes to understand molecular mechanisms of brain development and disease occurrence. scRNA-seq will provide the new emerging neurological disciple f of the artificial intelligent single neuron for deep understanding of brain diseases.

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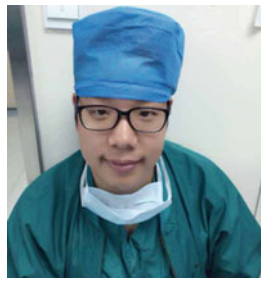

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