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Lessons from single cell sequencing in CNS cell specification and function

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Abstract

Modern RNA sequencing methods have greatly increased our understanding of the molecular fingerprint of neurons, astrocytes and oligodendrocytes throughout the central nervous system (CNS). Technical approaches with greater sensitivity and throughput have uncovered new connections between gene expression, cell biology, and ultimately CNS function. In recent years,

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No conflict of interest exists.
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single cell RNA-sequencing (scRNAseq) has made a large impact on the neurosciences by enhancing the resolution of types of cells that make up the CNS and shedding light on their developmental trajectories and how their diversity is modified across species. Here we will review the advantages, innovations, and challenges of the single cell genomics era and highlight how it has impacted our understanding of neurodevelopment and neurological function.

Keywords

scRNA-seq; development; cell type; Neurodevelopmental disorders

Current state of single cell RNA-seq methods

ScRNA-seq methods have evolved over the past three decades through technical achievements on three fronts: 1) the preparation of single cell suspensions and subsequent cell capture, 2) amplification and sequencing of single cell transcriptomes, and 3) bioinfomatic analyses to disentangle the large datasets generated through sequencing, ultimately leading to cell classification.

Well- vs droplet based methods

Current scRNA-seq approaches can be generalized into well-based or droplet-based methods to isolate and sequence individual cells from suspension. Well-based approaches require a rigid physical compartment to capture cells (typically by passing them through a microfluidic chamber or flow sorting single cells directly into separate wells of a microtiter plate) and employ a variety of sequencing library preparation techniques. These include STRT-seq (1), CEL-seq (2), SMARTseq (3) and Microwell-seq which produce long complementary DNA (cDNA) fragments (4). While the use of these well based approaches significantly advanced the scRNA-seq field, the greatest innovation came through the development of a droplet-based approach with the Drop-seq protocol in 2015 (5). Drop-seq uses a microfluidics flow plan to capture single cells in oil droplet “reaction chambers”, where their transcripts are barcoded during library production to enable the unmixing of the data to single cell resolution. Droplet-based scRNA-seq methods have quickly become the standard approach (6, 7), especially since the commercialization of droplet-based platforms (e.g. 10X Genomics’ Chromium® and Biorad/Illumina’s TruCell®). Compared with well-based methods, the primary advantage of droplet based methods is the high throughput, enabling the capture and sequencing of thousands of cells per experiment. However, the tradeoff off for this advantage of scale is shorter cDNA lengths which preclude measures of mRNA regulation and alternative splicing.

Bioinformatic analysis

Along with the refinement of sequencing technologies, analytical tools for single cell genomics studies have also come a long way. Previous reviews have extensively described how these approaches make the best use of the rich data arising from scRNA-seq experiments (10, 11). While a consensus guideline or common practice has yet to be established, a general protocol for scRNA-seq analysis has emerged organically over time (12). This process consists of dimension reduction followed by unsupervised clustering and

cell type identification. Once putative cell types are identified, differential gene expression, gene network, or pseudotime analyses can be applied to further characterize differences between cells in the dataset (6). Programs and packages have been developed on both R and python platforms to facilitate the management and analysis of single cell datasets (12, 13). Since most methods rely on accurately determining the relationships between cells, for example using minimum spanning tree or similar techniques (14–16), the choice of bioinformatic methods must be tailored to the structure and topology of the dataset. In particular, pseudotime and other trajectory inference analyses, which evaluate the progression of the transcriptome among single cells and have been used frequently to assess developmental state, especially depend on a carefully constructed topological representation of the dataset. Since only a subset of identified genes are useful or relevant to the biological process of interest, smaller gene groups are used for trajectory inference tests as this hastens the analysis and potentially focuses the results. As such, this process requires researchers to be mindful about the biological validity of the curated list of genes as it can significantly influence the interpretation of the results.

Lessons from single cell RNA-seq in CNS specification and function

Early single cell neuroscience studies focused on developing tissues due to their accessibility and ease in generating single cell solutions. These first cells were collected primarily by direct microscopic visualization and manual picking followed by gene expression analysis with DNA microarrays. Known classifier genes were used to identify cell types and demonstrated unexpected diversity in populations previously thought to be homogeneous (17–20). The heterogeneous nature of cell types was extended in later studies that used well-based capture techniques and RNA sequencing library production procedures (21–23). One common limitation of these early studies was the small number of cells collected, leaving open questions about whether the entire population had been adequately mapped. Nevertheless, these early studies pushed the field to recognize that commonly held lineage trees were probably overly simplistic and that datasets showed the developing brain holds a greater capacity to generate cellular diversity than previously appreciated. More recent scRNA-seq studies have classified cells from adult tissue and a range of neurological disorders to test the premise that disease etiology may progress through specific cell types. These studies have taken advantage of new techniques to isolate and sequence the transcriptomes from individual nuclei using an extension of scRNA-seq - single nuclei RNA-seq (snRNAseq) (22, 24–28). These modern scRNA-seq and snRNA-seq datasets have supported and extended the findings of earlier studies, leading to a finer-grained understanding of cell complexity in the mammalian CNS.

One of the most exciting recent impacts of scRNA-seq is a newfound understanding of brain differences among species, in particular studies that have identified novel gene expression profiles and cell types in the human brain. It is now clear that many developmental mechanisms, including gene expression and the generation of cellular diversity, are shared between mammalian species - especially between primates. However, genes encoding axon guidance molecules, retinoic acid and PDGF signaling pathways, cationic membrane channels and neurotransmitter-synthesizing enzymes have been found to be uniquely expressed in some human progenitors and neurons (29–32). In addition, human-specific

neural stem cells and inhibitory neurons have also been identified (22, 33–36), as have genes differentially expressed in human neural stem and progenitor cells (36–38). As more species-specific differences are uncovered, it will be crucial to continue testing the roles of these genes and cells to decode how they participate in brain development and function.

For the most part, all single cell studies rely on unbiased clustering followed by the use of cardinal marker genes to guide cell classification. For example in scRNA-seq studies of the developing neocortex, cells expressing a list including *SOX2*, *PAX6* and *SLC1A3* are identified as radial glia while cells expressing *EOMES* and other genes are classified as intermediate progenitor cells. Similarly, inhibitory neurons are classified by the expression of *LHX6* and *DLX5* while excitatory neurons express *TIAM2* and *PRDM8*. These marker genes, which may vary across studies and are mined from prior "non-genomic" studies, have been validated to ensure they mark the appropriate cells in vivo. One interesting observation from many scRNA-seq studies to date is the fact that multiple clusters have been identified that express the same cardinal marker genes and many clusters also exhibit heterogeneous gene expression. These findings have led to important discussions on "what is and what defines a cell type," and whether these molecular distinctions have biological relevance.

Cellular diversity: cell types, subtypes, and states

A major challenge in matching gene expression data with cell identity and biological function is that there is currently no consensus on how single cell transcriptional profiles should be grouped during scRNA-seq analyses. This is further complicated by the vast differences obtained with even small changes to the analytical algorithms. Accordingly, changes in these protocols have a direct impact on distinctions between possible "cell types" identified in each experiment. The most popular clustering methods (Louvain-Jaccard, k-means e.g.) typically start with dimensional reduction (PCA, tSNE or UMAP) of the original single cell transcriptome datasets. The low dimensional space is then interpreted to establish a neighborhood graph based on correlation or distance between data points. The choice of dimensional reduction method and the parameters of the neighborhood graph (i.e. size of neighbors) can each influence the final clustering outcomes in subtle to substantial ways (39). Currently, the choices of these intermediate analysis steps are completely subjective, and this can have an overstated influence on the interpretation of results and comparisons between experiments since each scRNA-seq dataset is unique due to sampling differences and batch effects across runs. Therefore, while it is easy to assume that cells in different clusters are distinct once the analysis is complete, it remains unclear whether the observed inter-cluster and intra-cluster gene expression variabilities signify important biological events or are due to subjective database management and technical noise. One logical approach to minimize technical artifacts is to ensure that the clustering algorithm is adjusted to capture at a minimum the known complexity of cell types in the system (i.e. all known cell types can be annotated), and then to determine whether novel or additional clusters concurrently appear.

Thus far, the evidence for transcriptionally separate cell types and subtypes is based largely on comparisons to cell lines and transcriptionally consistent cells such as embryonic stem cells. However, the degree of transcriptional uniformity varies across cell types. Cells

serving complex functions, such as cortical neurons, may be more transcriptionally dynamic than stem cells. Indeed, many studies have identified a wide diversity of excitatory and inhibitory neuron lineages (22, 23, 28, 35, 36, 40–42). A necessary step, therefore, is to validate the appearance of any putative cell types or subtypes directly in vivo. Spatial transcriptomic analysis, including a new effort termed Visium® by 10X Genomics, is an important new method to validate single cell identity with in vivo location and has already been used to query gene expression changes in pathological samples (43–46).

While common features (shared marker gene expression, similar electrophysiology or morphology etc.) may generally characterize a cell type, discrete transcriptional differences between subtypes may be biologically important and influence the development and function of their resident tissues. However, the inability to mark and query these subtypes in vivo has been a longstanding barrier in validating scRNA-seq identified heterogeneity. This is primarily because cell type/subtype distinctions are most often based on the combinatorial expression of multiple genes or co-expressed gene networks rather than individual marker genes (22, 28, 36, 37, 41). While this characteristic can be easily visualized using bioinformatics tools, it has hampered the development of specific labeling tools to identify and study these types/subtypes in vivo. Future advances in this area are dependent on new labeling strategies, or perhaps creative modifications of existing methods such as intersectional fate mapping.

Cell state and continuum

The challenge of querying scRNA-seq findings in vivo is amplified when interpreting temporal transitions in cells, including cell state and cell continuum (47, 48). Unlike cell types, which are mutually exclusive and non-interchangeable, both cell states and cell continuum incorporate the concept that transcriptome features can vary developmentally and can thereby temporally shift within a cell type. From the perspective of transcriptomics, cell states have definable boundaries (e.g. early/developmental vs. late/mature), whereas cell continuum is thought to represent gradually shifting transcriptome characteristics that may blur the boundaries between cell types. Thus, although this still remains to be empirically determined, cell states may parcellate developmental progression (48–50), even within individual subtypes, and in some circumstances may be misclassified as cell subtypes themselves. In contrast, cell continuum trajectories may mask the fact that groups of cells are fundamentally different cell types. Developing tools to identify, confirm and track these properties in vivo, will be critical for determining how changes to state and continuum impact neural development, neurological disorders, and contribute to the mechanisms underlying species divergence.

Epigenomics and splicing

Concurrent with the advances in transcriptomic analyses has been the understanding that the expression of the genome is significantly controlled by chromatin conformation, non-coding RNA molecules, and RNA splicing. Measuring these additional factors in parallel to mRNA levels yields a more complete gene expression profile of the cell. Accordingly, many functional genomics studies have incorporated these processes into scRNA-seq approaches, providing layers of information through which gene expression results can be better

understood. For example, with the addition of ATAC-seq, gene expression profiles can now be combined with an index of chromatin accessibility of sites neighboring active or inhibited loci (51, 52). This procedure enables a temporal and physical connection between histone modifiers and transcription factor activity. The activity of microRNAs (miRNA) can also influence post transcriptional expression, and methods like CLIP-seq (53) can now map miRNA expression in single cells. In addition, splitting single cell libraries into two concurrently analyzed aliquots has recently enabled miRNA and mRNA co-measurements on the same cells (54). Adding yet more information are recent advances in using scRNA-seq to examine exon usage/mRNA splicing. These studies indicate that measurements of RNA processing and isoform expression may be important for a full characterization of cell type, interspecies differences, and as drivers for neurological disorders (55, 56).

Beyond cell classification

It is now clear that scRNA-seq analysis provides a powerful methodology for identifying and characterizing the cellular constituents of the CNS. However, due to caveats and questions about how the transcriptome ultimately defines cellular identity, scRNA-seq analyses should be viewed as a starting point from which additional methods must be employed to fully elucidate the functional characteristics of cell types. In recent years, several applications of scRNA-seq have emerged to help fill this divide in the neurosciences. For example, Patch-seq enables direct insight into the relationship between the electrophysiology and transcriptional profile of neurons isolated following whole-cell patch clamp recordings (57, 58). This approach offers unprecedented resolution in identifying the molecular underpinnings of neuronal subtypes and their roles in the neural circuitry. Similarly, Act-seq (activated cell population sequencing) has been developed to detect the acute transcriptional changes associated with immediate early gene expression in response to neuronal activity (59). These two approaches have the potential to reveal the cellular components of diverse neural circuits that respond during cognitive and behavioral functions in an unbiased manner. In the optimal scenario, single cell transcriptome profiling can lead to the development of genetic tools to trace the location, physiology and connectivity of neural cell types and to study their roles in behavior (60). Identifying the connections between molecular state and mechanism will undoubtedly enhance our understanding of the relationship between CNS structure and function in both healthy and diseased states.

Cell specific changes in disease/neurodevelopmental disorders

While many of these technical approaches have been used to provide a detailed examination of normal CNS development, they have also been used to interrogate the cell-type specific etiology of several neurodevelopmental disorders (NDD) and neuropathologies. In many cases, cell subtypes (rather than the broader cell types) are specifically affected. For example, it has long been appreciated that the loss of myelin produced by oligodendrocytes leads to neurodegeneration in multiple sclerosis (MS). Recently however, scRNA-seq analysis demonstrated that the depletion of specific oligodendrocyte subtypes is associated with disease progression and clinical outcome (61). In addition, distinct subpopulations of neurons may be more susceptible to insult in MS (62). In Autism Spectrum Disorder, studies have shown that upper layer excitatory neurons, inhibitory neurons and microglia may

independently affect disease progression (26, 63, 64). Similarly, an association between genes and multiple cell types has been discovered through genome-wide association studies in schizophrenia (65). These recent studies demonstrate that the ability to identify and measure fine-grained differences between types and subtypes of cells may lead to direct clinical impacts on care and prevention of these disorders. Nevertheless, the ultimate success of uncovering these disease mechanisms relies on understanding the cellular landscape of normal brains and continued advances in establishing connections between gene expression and CNS function.

Concluding remarks

It is undeniable that scRNAseq has made a large impact on many biomedical fields. As with many innovative scientific techniques, it has swiftly changed from a rarified method to one that is widely used throughout neuroscience. With its wider adoption and extraordinary resolution, scRNA-seq has greatly accelerated the pace at which neurodevelopmental processes and disease mechanisms of the CNS are understood. At the same time, new questions have emerged as we have ventured into the complex domains of single cells. Answers to these questions will require both continued technological development, and perhaps more importantly, a reexamination of former theories of neurodevelopment. One of the most important future steps will be the discovery and refinement of techniques to directly validate scRNA-seq models in vivo.

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Box 1:

M-MLV reverse transcriptase.

Perhaps the most significant and revolutionary technological development came with the application of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) to RNA sequencing efforts due to its thermostability, lack of RNAse H activity, and longer read lengths. During reverse transcription, M-MLV RT adds additional dCTP to the 3' end of the newly synthesized (cDNA) strand. The overhanging nucleotides can be used as anchors for additional oligonucleotides, enabling a mechanism termed template switching (8). Template switching allows the addition of primer binding sites at the end of RT procedure; combined with poly(d)T capturing, the process enables amplification of cDNA covering the full length of RNA transcripts. This powerful system became the foundation and the central mechanism of high through-put scRNA-seq (1, 9).