Neural Diversity: What We Know from Transcriptomics and Single-Cell RNA Sequencing

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Single-cell RNA sequencing (scRNA-seq) has significantly enhanced our understanding of neurobiology by providing detailed insights into the cellular and molecular diversity of the nervous system. Through the analysis of individual cells, scRNA-seq has identified various cellular phenotypes and regulatory markers essential for neuron differentiation and function. It has also revealed the heterogeneity within neural populations, highlighting the complex networks of transcription factors and signaling pathways that influence neuron development and specialization. Additionally, combining scRNA-seq with connectomics has enriched our understanding of how cellular diversity impacts the nervous system's connectivity and functionality. These advancements point to the potential of scRNA-seq in identifying targets for neurological disorder treatments, marking a significant contribution to neurobiological research.

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Introduction

Single-cell transcriptomics, an emerging set of cellular analytical methods, focuses on understanding the cellular dynamics and their functional implications in multicellular organisms. This field has its roots in the development of single-cell qPCR (Bengtsson et al., 2008), single molecule FISH (smFISH) (Femino et al., 1998), and whole transcriptome analysis (Kamme et al., 2003). With the advancement of RNA sequencing technologies, these transcriptomic methods were refined for analyzing individual cells, leading to the advent of single-cell RNA sequencing (scRNA-seq) (Tang et al., 2009). scRNA-seq has significantly contributed to solving critical biological issues. It has been instrumental in cell classification (Gulati et al., 2020). Additionally, scRNA-seq has played a crucial role in understanding the developmental reduction of stem cell potency (Zakrzewski et al., 2019). Furthermore, it has provided detailed insights into the complex nature of gene regulation within specific cell lineages (Huang & Sanguinetti, 2021).

Neuron development is orchestrated through a sequence of fate-specification processes, well regulated by numerous transcription factors. These transcription factors, which are often highly conserved across species, are crucial for establishing positional identity and promoting cell proliferation (Schuurmans & Guillemot, 2002). In vertebrates, the transcriptome includes at least 1,500 transcription factors (TFs) (Zhou et al., 2017), while in invertebrates, such as *C. elegans*, the count of transcription factors is approximately 620 (Y. Li et al., 2024). scRNA-seq technology has been important in uncovering regulatory and molecular markers that lead to distinct cellular phenotypes. Furthermore, it has been combined with whole-animal connectomics (Michki et al., 2021) and developmental profiling (H. Li et al., 2022) to enhance understanding of the role of cell lineages in regulating complex behavioral and physiological functions (Cook et al., 2019).

Neurogenesis, the creation of neurons from neural stem cells and progenitor cells, is governed by transcription factors and non-autonomous cell signaling (Nguyen & Cheng, 2022). The advent of high-throughput scRNA-seq technologies has deepened our understanding of mRNA expression, cellular subtypes, and their functions (Michki et al., 2021). Additionally, the availability of open-access "cell atlas" datasets has facilitated *in situ* functional analysis and exploration of developmental pathways influencing cell migration, differentiation, and fate (Kiselev et al., 2019). These datasets have been used in elucidating the cellular composition of tissues in various organisms, including zebrafish embryos (Satija et al., 2015), marine annelids (Achim et al., 2015), and adult mice (Grange et al., 2014).

Transcriptomic mapping and classification of neural cell subtypes have seen considerable progress, yet numerous analytical and technological avenues remain to be explored (Keller et al., 2023). Innovations in spatial transcriptomics (Lein et al., 2017), 10X Genomics (Stickels et al., 2021), and sequential fluorescence *in situ* hybridization (seqFISH) (Shah et al., 2016) have been pivotal in profiling transcripts and genes with cellular precision, aiding in the characterization of the spatial distribution of distinct cell sub-types.

The integration of contemporary transcriptomic techniques with connectomic data presents an opportunity to understand the complexities of neurodevelopment and neural diversity. Advances in transcriptomic mapping and classification through atlases has laid the groundwork for identifying neural cell subtypes, but the journey is far from complete. The application of methodologies such as spatial transcriptomics and seqFISH enable the profiling of transcripts and genes with remarkable cellular resolution. Furthermore, the synergy between scRNA-seq's detailed molecular profiles and connectomics' comprehensive mapping of neural connections illustrates the functional implications of cellular diversity in neurogenesis. This confluence of technologies not only deepens our understanding of the nervous system, but also holds the potential to identify novel therapeutic targets for neurological disorders.

Research & Methodology

Overview of Single-Cell RNA Sequencing

Experimental Design and Considerations

Sequencing mRNA from a single cell involves a few challenges that are not typically present in tissue-level sequencing: (1) isolating individual cells and (2) amplifying the small quantities of mRNA present in a single cell. The workflow for scRNA-seq experiments typically adheres to a standard protocol. Initially, an individual cell is isolated and lysed. This is followed by reverse transcription, which targets mRNA using poly[T] priming to generate cDNA. The cDNA, still in minute quantities, is then amplified through PCR and prepared for sequencing (Kolodziejczyk et al., 2015).

Isolating single cells poses a significant challenge in scRNA-seq techniques. To address this, researchers used various methods, with common approaches including sampling from tissues with inherently low cell counts, such as early embryos (Grindberg et al., 2013; Tang et al., 2009). Although this method is slow and has low throughput, it offers a higher consistency in single-cell capture. For tissues with high cell counts, laser capture microdissection (LCM) is used, providing a quicker solution but still maintaining a low throughput (Frumkin et al., 2008; Zhao et al., 2023).

Modern high throughput techniques primarily focus on cells that have been dissociated and suspended in a buffer, often after treatment with trypsin or other hydrolyzing proteins to ensure a single-cell suspension (Kolodziejczyk et al., 2015). Fluorescence-activated cell sorting (FACS) is a widely used method for distributing these cells into microtiter plates. This technique also facilitates the enrichment of specific cells of interest through fluorescent labeling (Olsen & Baryawno, 2018). Moreover, FACS instruments are capable of rapid index sorting, enhancing the efficiency of the process (Hayashi et al., 2010).

After successful cell capture and indexing, the next step is cDNA synthesis via reverse transcription, using poly[T] priming (Hayashi et al., 2010). The challenge of capture efficiency,

which is around 10% for reverse transcribed strands, is highlighted in Islam et al. (2014), contributing to the issue of noisy genes in scRNA-seq. To mitigate amplification bias inherent in PCR or *in vitro* transcription, techniques such as the use of unique molecular identifiers (UMIs) can be introduced. UMIs allow for molecule counting, which corrects PCR-induced artifacts by providing absolute scale measurements, offering a more accurate representation than the relative scale measurements typical of standard RNA-seq methods (Islam et al., 2014).

The UMI method in scRNA-seq uses Tn5 DNA transposase for an adapter transfer to target cDNA through "tagmentation," as described by Islam et al. (2014). These "tagmented" strands act as *in vitro* barcodes, incorporated during reverse transcription (Fu et al., 2011). Reads from PCR-duplicated tags share identical barcode sequences, allowing the quantification of transcript copies in a cell lysate. The transcript count is then based on the count of UMIs linked to tags mapping to that transcript (Kolodziejczyk et al., 2015). This molecule counting approach is crucial for single-cell transcriptome analysis and has been extended to other techniques like CytoSeq (Fan et al., 2015).

Due to the genetic uniqueness of each cell, achieving true technical replication is impossible, necessitating alternative methods for estimating variation. Using external reference component (ERC) molecules as external spike-ins offers a straightforward approach to mitigate technical noise and accurately determine actual gene expression levels (Ding et al., 2015). These spike-ins, introduced into the cell lysate, facilitate understanding the correlation between the quantity of input molecules and the resultant sequencing reads, thus enabling data normalization and technical variation correction (Brennecke et al., 2013; Kolodziejczyk et al., 2015).

scRNA-seq Workflow and Data Analysis

Counts data in scRNA-seq are typically comprised of molecular counts and read counts, depending on whether UMIs are incorporated in the single-cell library construction protocol. The matrices have dimensions of the number of barcodes times the number of transcripts. Raw counts are the whole numbers obtained after trimming, demultiplexing, alingment, and mapping. Normalized counts account for known gene length, sequencing depth, and expression distribution. The read depth in scRNA-seq refers to the number of transcripts detected from each cel and is crucial for determining the confidence in gene expression (Luecken & Theis, 2019).

Protocols using UMIs and those without both yield extensive datasets and require comprehensive computational processing for quality control and gene expression quantification, while also addressing technical noise (Figure 1). The interpretation of biological data is significantly dependent on the various bioinformatic approaches (Stegle et al., 2015). With the prevalent use of bulk RNA-seq, a variety of tools for managing high-throughput transcriptomic data have been developed (Oshlack et al., 2010).

In computational sequence analysis, standard procedures such as read alignment, generating gene expression counts, quality control, normalization, and downstream modeling are essential.

Stegle et al. (2015) notes that tools designed for bulk cell populations can be adapted for scRNA-seq use, yet there are specific pitfalls to be mindful of when creating experimental protocols. It is important to consider biases such as incomplete knowledge of the target genome or transcriptome annotation (Durruthy-Durruthy et al., 2014). The mapped reads can be interpreted to generate expression levels using the same approaches in general RNA-seq experiments, such as High-throughput sequence analysis (HTSeq), a tool used for analysis of high-throughput sequencing data (Anders et al., 2015). The data obtained from HTSeq could then be passed to DESeq2, software which tests for differential expression of genes, for bulk populations (Michael Love, 2017) or Monocle3 for single cells (Trapnell et al., 2014) when testing and mapping differential expression cascades.

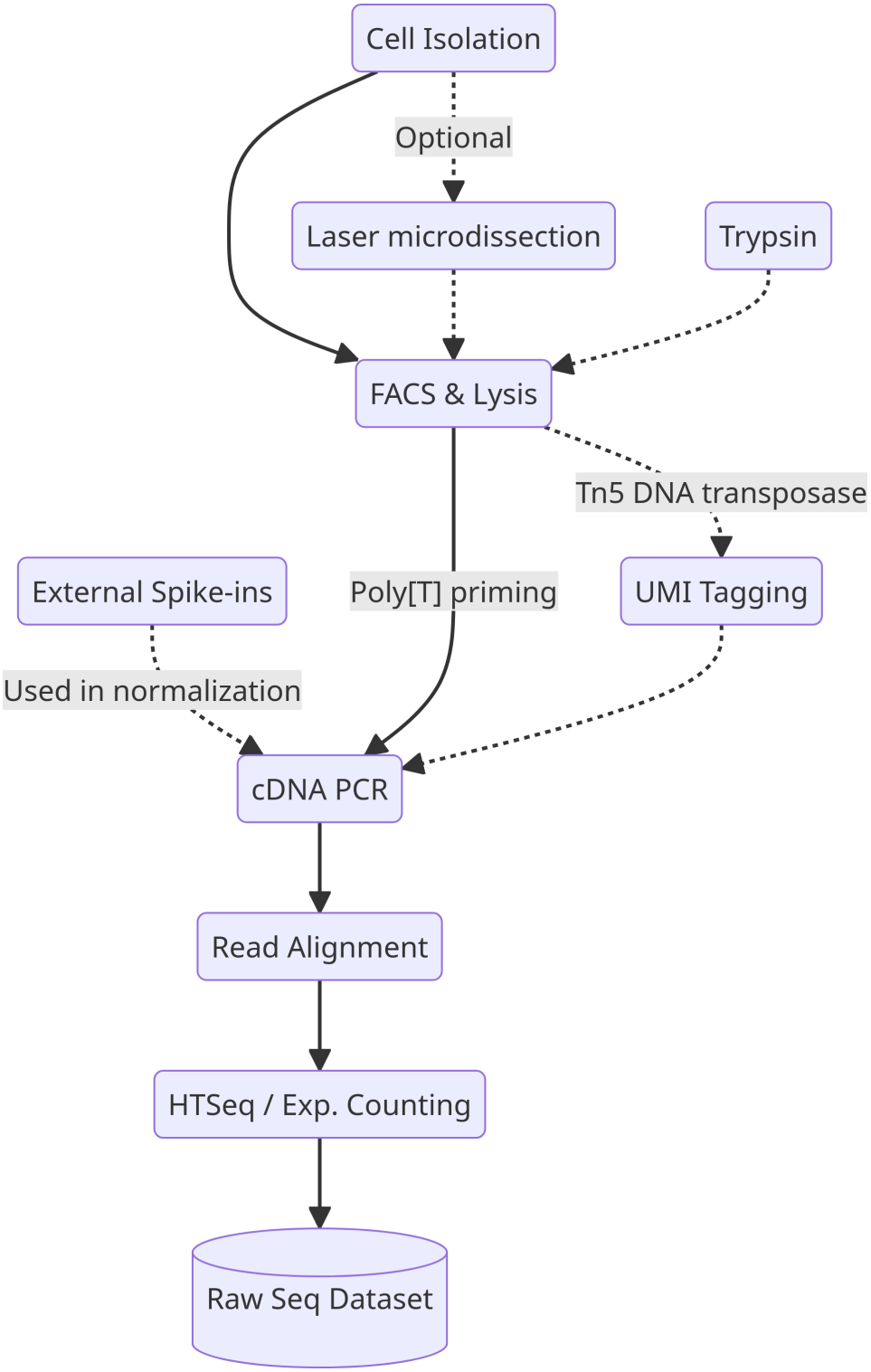
Cell Isolation
Optional
Laser microdissection
Trypsin
FACS & Lysis
Tn5 DNA transposase
External Spike-ins
Poly[T] priming
UMI Tagging
Used in normalization
cDNA PCR
Read Alignment
HTSeq / Exp. Counting
Raw Seq Dataset

Figure 1: Single-cell RNA sequencing sample preparation involves cell isolation, optional treatments, FACS, lysis, spike-in addition, mRNA reverse transcription to tagged cDNA, alignment, and quantification to generate the raw sequencing dataset.

Quality control is a critical step in data analysis. After alignment and obtaining initial read counts, identifying any cell libraries of poor quality is essential (Stegle et al., 2015). Sample mix-ups, external contamination, and sequencing issues can be detected using visualization tools like Kraken2 and fastqc. Assessing RNA capture and amplification is crucial for evaluating sequence integrity. Cells with stressed or degraded RNA should be excluded from downstream analysis (Brennecke et al., 2013).

Principal component analysis (PCA) is commonly applied to the DESeq2 / Monocle3 gene expression matrix for detecting problematic cells, as it clusters good-quality cells together and identifies poor-quality cells as outliers (Lall et al., 2018). These poor-quality cells, possibly undergoing apoptosis during sampling, often show increased mitochondrial gene expression, leading to distinct clustering (Islam et al., 2014). Normalizing raw sequencing data is a standard procedure, particularly in bulk RNA-seq, where library counts are standardized through metrics like fragments per kilobase of exon per million fragments mapped (FPKM). FPKM accounts for both transcript length and library size, ensuring a fair comparison across samples (Anders & Huber, 2010; Robinson & Oshlack, 2010).

The normalization process in scRNA-seq plays a critical role in how data is interpreted (Figure 2). When unique molecular identifiers (UMIs) are utilized during the initial amplification, and assuming each cDNA molecule is sequenced at least once, the count of UMIs per gene accurately reflects the quantity of cDNA molecules for that gene (Stegle et al., 2015). It is recommended to introduce external spike-in molecules into the cell extract prior to reverse transcription and amplification for effective normalization (Ziegenhain et al., 2022). The constant number of mRNA spike-in molecules across cells allows for the identification of variability in UMI counts for spike-in genes, which signals differences in reaction efficiency. Consequently, normalization can adjust for these differences, providing a more accurate estimation of mRNA molecule counts based on cDNA molecule counts (Stegle et al., 2015).

In scRNA-seq, various normalization methods are integrated to address data variability and improve analysis accuracy. The Bayesian Analysis of Single-Cell Sequencing (BASiCS) uses a Bayesian framework to model technical noise and biological variability, offering insights into gene expression levels and technical artifacts. The "Percellome" method, though less commonly referenced, focuses on capturing the per-cell expression profiles to adjust for cell-specific biases (Kanno et al., 2006). Regularized Negative Binomial Regression (RNBR) aims to stabilize variance across different expression levels, making it effective for handling over dispersed count data typical in scRNA-seq (Hafemeister & Satija, 2019). Gamma Regression Model (GRM) applies a gamma distribution to model the continuous nature of gene expression, accommodating the skewness often observed in scRNA-seq data (Ding et al., 2015). Each of these methods, among others, contributes uniquely to the normalization process, addressing specific challenges inherent in single-cell data to enhance downstream analysis (Lytal et al., 2020).

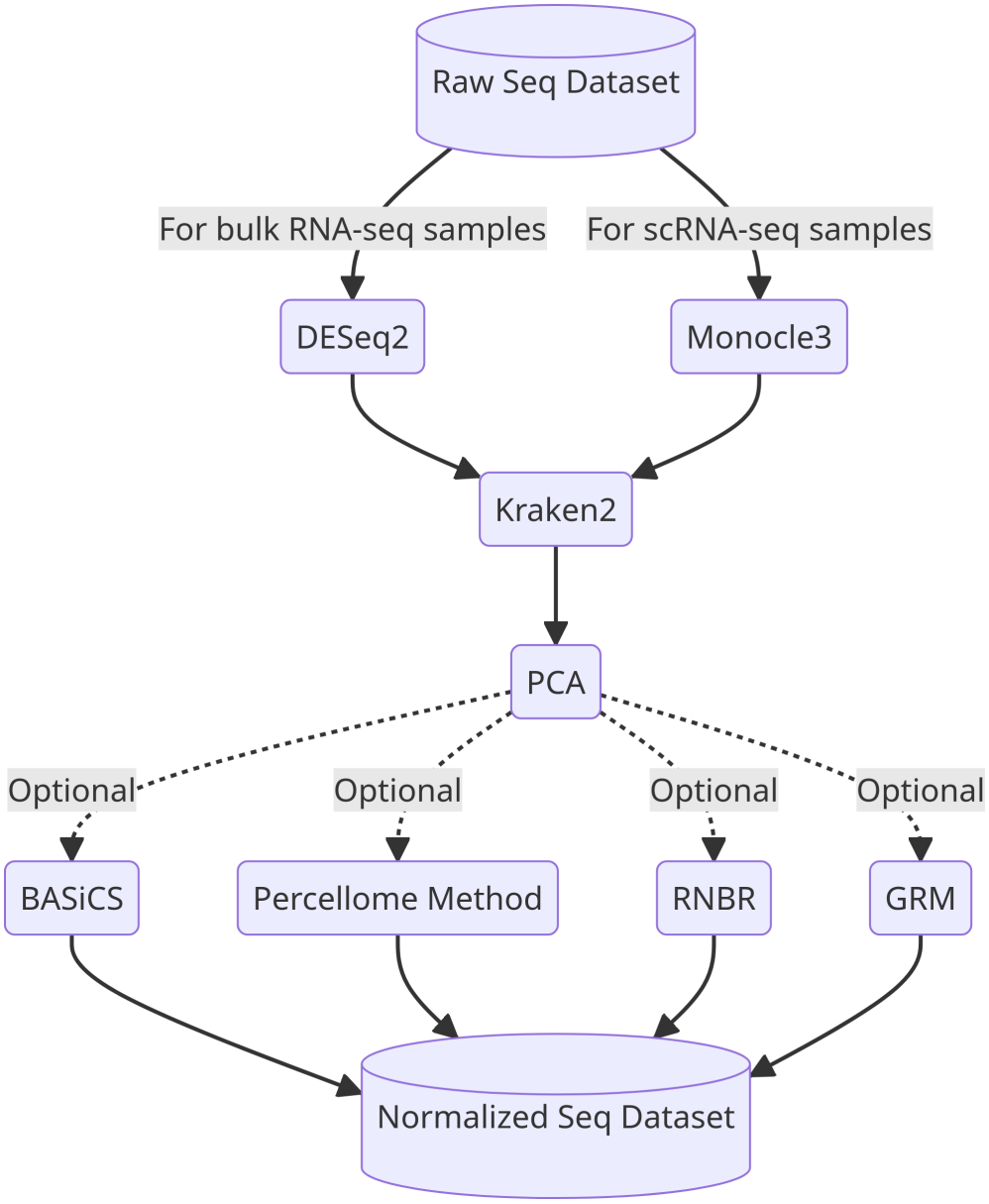

Figure 2: Raw sequencing data is analyzed using DESeq2 for bulk RNA-seq or Monocle3 for scRNA-seq, followed by Kraken2. PCA is then performed, with optional advanced normalization techniques, to generate a normalized sequencing dataset.

By constructing a normalized dataset, it becomes feasible to generate data-specific profiles from the processed data. Single-cell transcriptomics facilitates the identification of both known and novel cell types within a sample (Jaitin et al., 2014; Michki et al., 2021; Tang et al., 2010). Furthermore, scRNA-seq is instrumental in uncovering the underlying heterogeneity of tissues

by clustering cells based on their expression profiles. The approach to cell clustering is bifurcated into two categories, contingent on the presence or absence of pre-existing knowledge or hypotheses about the cells' relationships. In the absence of prior hypotheses, unbiased clustering techniques are used to categorize cells according to their differentiation stages, utilizing the Monocle3 for pseudotime differentiation profiles, alongside regression analytics and graph-autocorrelation analysis (Sharon et al., 2019; Trapnell et al., 2014).

Single-cell transcriptomics is widely used to identify both known and novel cell types within a sample (Jaitin et al., 2014). Earlier research demonstrated that clustering tissues based on their overall expression profiles is feasible (Blekhman et al., 2008). scRNA-seq is particularly effective for uncovering hidden heterogeneity within tissues, allowing for the identification of distinct subsets that may represent previously unrecognized cell types (Stegle et al., 2015). Furthermore, these methods offer insights into cellular differentiation processes. By analyzing a population of cells at various stages of differentiation towards a particular cell type simultaneously, it becomes feasible to position these cells at specific points within the differentiation sequence. This can be achieved through unsupervised clustering techniques that do not depend on pre-identified marker genes (Treutlein et al., 2014).

When the spatial location of cells is known, it is often plausible to assume that cells in closer proximity are more likely to be of the same type compared to those that are farther apart. Consequently, clustering cells by integrating both spatial and quantitative data using a Markov random field (MRF)-based approach has shown potential (Pettit et al., 2014). The MRF approach is a statistical modeling technique used to analyze spatial data by considering the dependency of a cell's characteristics on its neighboring cells, thereby enabling more contextually informed clustering and analysis.

The nervous system's cellular composition is not only vast but also organized in a complex hierarchical structure, influenced by cell-type-specific gene-regulatory programs and alternative splicing mechanisms (Feng et al., 2021). This molecular diversity, particularly evident in over 100 transcriptomically defined neuronal types of the adult mouse cortex, underscores the importance of integrating both spatial and molecular data for a comprehensive analysis (Feng et al., 2021). Such integration can enhance our understanding of neuronal identity and function, which are crucial for investigating the roles neurons play in various biological processes and diseases (Zhong et al., 2023). This approach aligns with the use of MRF models to analyze spatial data, emphasizing the dependency of a cell's characteristics on its neighboring cells, thereby enabling more contextually informed clustering and analysis (Yang et al., 2022).

Applications in Developmental Neurobiology

Neurons are foundational in sensation, movement, information processing, and behavior (Peng et al., 2021). Their genetic diversity and specific distribution across the nervous system endow different neuron types with distinct roles (Beine et al., 2022). Advances in connectomics, the study of the spatial dynamics of complex synaptic connection networks, for species such as

C. elegans (Cook et al., 2019), *D. melanogaster* (Scheffer et al., 2020), *M. musculus* (Yao et al., 2023), and *D. rerio* (Svara et al., 2022) have revealed the involvement of numerous neural circuits in the development and progression of neurological disorders and neurodegenerative diseases. These conditions are often linked to changes in axon guidance proteins (Van Battum et al., 2015), neuronal morphological alterations, demyelination (Schäffner et al., 2023), cellular apoptosis (Lo et al., 1995), and disruptive cell signaling events leading to neuron activity pathology (Calvo-Rodriguez & Bacskai, 2021; Hara & Snyder, 2007; Mishina & Snider, 2014).

Single-cell transcriptomics serves as a modern technique that both complements and contrasts with established neural research methods like optogenetics, protein assays, membrane biophysics, and calcium imaging (Ahmad & Budnik, 2023). Traditional techniques, despite their significance, overlook the extensive spatial diversity among neurons (Russell et al., 2022). The recent use of single-cell transcriptomic approaches, such as scRNA-seq, snRNA-seq, and other techniques (Figure 3), enables the detection of molecular signatures at the individual cell level, offering detailed genomic insights and aiding in the classification of neuronal subpopulations (Cebrian-Silla et al., 2021).

A common variant of scRNA-seq involves the seuquencing of neuron nuclei known as single nuclei RNA sequencing (snRNA-seq) (Smajić et al., 2022). The use of scRNA-seq and snRNA-seq techniques also makes it possible to perform DNA and transcriptional cataloging of neural cells, including neurons and astrocytes (Armand et al., 2021; Xing et al., 2023). Comprehensively characterizing neural diversity, especially through precise identification of neuron-specific transcriptional features, can enhance our understanding of neural circuits and predict the developmental changes in spatially distinct neurons (Xing et al., 2023). Integrating single nucleus/single-cell RNA sequencing (sn/scRNA-seq) with spatial transcriptomics techniques like seqFISH (Eng et al., 2019) holds significant promise for creating more detailed molecular maps.

Table 1: Figure 3: sn/scRNA-seq and other single-cell transcriptomic profiling techniques compared based on their resolution, commonly used protocols, and capture efficiency.

	Resolution	Protocol	Capture Efficiency
Single-cell RNA sequencing	Single cell (cytoplasm)	10X Genomics, SMART-seq2	~ 20,000 genes
Single-nuclei RNA sequencing	Single nucleus (nucleoplasm)	10X Genomics, SMART-seq2	500 - 20,000 genes

Other single-cell transcriptomic profiling	Single cell (cytoplasm)	seqFISH(+)	~ 20,000 genes

These three techniques are commonly used in uncovering and detailing the transcriptomic characteristics of neural populations. Comparative studies across different species have shown that analyzing various neuron subtypes in analogous regions via single-cell transcriptomics serves as an effective initial approach (Kebschull et al., 2020; Tosches et al., 2018). Furthermore, Smart-seq2 and 10X Genomics, as leading sequencing protocols, have been widely used to explore the gene-expression diversity among neural cells in humans (Maynard et al., 2021), *C. elegans* (Taylor et al., 2019), and other species.

The article by Davie et al. (2018) presents a detailed exploration of the *D. melanogaster* brain using scRNA-seq, focusing on the identification and characterization of cell types and their lineage across the lifespan of the organism. This approach is particularly notable for its read depth, as it captures a totalizing transcriptional snapshot of the cellular diversity within the adult *Drosophila* brain, identifying 87 distinct cell clusters. This granularity is further enhanced by the application of stringent filtering and validation through targeted cell-sorting using FAC-sorting, which ensures the accuracy and specificity of the cell type identification (H. Li et al., 2017).

A critical aspect of the scRNA-seq methodology highlighted in the study is the use of droplet microfluidics technology, which facilitates the high-throughput sequencing of thousands of individual cells. This technology is instrumental in achieving the sequencing depth, allowing for a detailed analysis of both protein-coding genes and non-coding RNAs across different neuronal cell types (Wang et al., 2021). The study documents the changes in gene expression profiles at various ages, providing insights into the stability of neuronal identity despite the overall decline and changes in RNA content with age (Davie et al., 2018).

The integration of Single-Cell rEgulatory Network Inference and Clustering (SCENIC) for gene network analysis is an added element of the scRNA-seq workflow. SCENIC is a computational method used to infer gene regulatory networks from scRNA-seq data. It identifies potential regulators and their target genes by analyzing the co-expression patterns across the single-cell transcriptomes (Aibar et al., 2017). SCENIC then uses these networks to define the regulatory states of individual cells, providing insights into the transcriptional control mechanisms that govern cell identity and function (Bravo González-Blas et al., 2023). By identifying regulatory networks and linking them to specific cell types and states, the study not only categorizes cells based on their transcriptomic profiles but also provides a functional context to these profiles (Davie et al., 2018). This approach is important for understanding not only how different cell types contribute to overall brain function, but also how these contributions might change as the brain ages.

The article leverages scRNA-seq technology not just to catalog the cell types within the *Drosophila* brain but also to identify the regulatory mechanisms that define these cell types at a molecular level. The detailed analysis of cell lineage and typing, enriched by high-throughput

sequencing and advanced bioinformatics tools, sets a high standard for future studies in the field of cellular and developmental neurobiology and offers a model for similar analyses in other organisms.

A study by Feng et al. (2021) details the application of scRNA-seq in neurobiology, particularly by detailing the complexity of alternative splicing across different neuronal types. The research demonstrates the potential of scRNA-seq to provide insights into the cellular and molecular mechanisms that define neuronal identity and function, both of which are critical for understanding normal brain and nervous system function, as well as the pathogenesis of neuronal disoders (Feng et al., 2021).

Feng et al. (2021) utilized scRNA-seq to explore the diversity of neuronal cell types in the adult mouse cortex, focusing on how alternative splicing contributes to this diversity. The study identified distinct splicing programs between major neuronal classes such as glutamatergic and GABAergic neruons (Feng et al., 2021). This level of detail demonstrates the role of alternative splicing in fine-tuning neuronal functions and interactions (Tasic et al., 2018). The findings suggest that even subtle changes in splicing patterns can significantly alter neuronal behavior and interactions. Understanding these patterns aids in mapping the complex neural circuits and provides insights into how disruptions in these patterns could lead to neurological disease.

The research also demonstrated the importance of RNA-binding proteins (RBPs) in regulating alternative splicing across different neuronal types. The differential expression of RBPs serves as a gateway to understanding the developmental trajectories of neurons (Zeisel et al., 2015). For instance, during brain development, neurons undergo complex changes in gene expression that dictte their mature functions (Dillman & Cookson, 2014). RBPs play a role in this process by selecting enhancing or reprssing the inclusion of exons in mature mRNA, thereby influencing the protein's function after synthesis (Schneider-Lunitz et al., 2021). This mechanism allows neurons to respond adaptively to developmental clues and environmental stimuli (Zeisel et al., 2015).

Moreover, the study's findings on the activity of these RBPs during neuronal development provides insights into the temporal dynamics of splicing decisions (Feng et al., 2021). For example, certain RBPs might be highly active during early developmental stages, guiding the initial splicing decisions that establish the foundational attributes of the neuron. As development progresses, the expression or activity of these RBPs may decrease, and other RBPs may take over to refine the neuron's properties to suit its specific role within the neural circuitry (Thompson et al., 2019).

Feng et al. (2021) also demonstrated the integration of scRNA-seq with advanced computational and bioinformatic techniques such as *de novo* motif analysis and position-dependent RNA mapping. In *de novo* motif analysis, this technique is useful in identifying novel sequence motifs within RNA that are potential binding sites for RBPs (Kazan & Morris, 2013). Feng et al. (2021) utilized this approach to analyze the datasets generated by scRNA-seq, identifying unique motifs that are differentially expressed across various cell types. By identifying these

motifs, the researchers could infer the presence and activity of specific RBPs that interact with these sequences to regulate splicing events (Feng et al., 2021). This analysis involves algorithms that scan the RNA sequences to detect recurring patterns that are statistically significant compared to an established background model.

Furthermore, position-dependent RNA mapping is involved in determining where along the RNA molecule the RBPs bind (Jensen & Darnell, 2008). These maps visually represent the binding sites of RBPs along the RNA sequence and correlate these positoins with the effects on splicing. This spatial information is important because the impacts of RBP binding can vary dramatically depending on its location relative to the exon-intron boundaries. For instance, binding close to the splice site might block splicing machinery access, leading to exon skipping, where as binding further away might enhance the inclusion of exons by stabilizing or recruiting spliceosomal factors (Feng et al., 2021).

The findings from the studies presented offer an avenue for future studies to explore the dynamics changes in alternative splicing and scRNA-seq during neuronal development, aging, or in response to neurological disease. Such studies could utilize scRNA-seq to track the changes in splicing over time or in response to treatments, providing a dynamic view of neuronal plasticity. This could particularly inmpact developmental neurobiology, where the understanding of sensitive temporal changes in gene expression is significant for identifying mechanisms of cellular development.

Challenges and Limitations

Despite the significant advancements in scRNA-seq and its applications in neurobiology, several challenges and limitations persist. One major challenge is the technical variability inherent in scRNA-seq data, which can arise from differences in cell capture efficiency, mRNA isolation, and amplification biases. These technical variations can obscure true biological differences, making it difficult to distinguish between noise and meaningful biological signals (Michki et al., 2021).

Another limitation is the cost and complexity of scRNA-seq experiments, which can be prohibitive for many research institutions. The requirement for specialized equipment and high-throughput technologies means that only well-funded laboratories can afford to conduct these studies at a large scale. While scRNA-seq provides a high-resolution view of cell-to-cell variability, it still faces challenges in capturing the full transcriptomic complexity due to incomplete mRNA capture and the degradation of sensitive RNA molecules during processing. This can lead to an under-representation of certain transcripts, potentially skewing the understanding of cell states and functions.

Additionally, the interpretation of scRNA-seq data requires sophisticated computational tools and expertise in bioinformatics, which are not universally available. The complexity of data analysis and the need for advanced statistical models to correct for technical artifacts add another layer of difficulty in fully exploiting the potential of scRNA-seq. Moreover, while ‘cell

atlas' style scRNA-seq datasets effectively characterize the transcriptomes of a majority of cells from a region of interest, they may fail to identify cell populations that are traditionally grouped together through in situ and/or functional analyses. This limitation arises because cluster analysis may not always align with known biological groupings, potentially overlooking subtle yet significant cellular distinctions (Kiselev et al., 2019). Additionally, broad scRNA-seq studies often do not leverage the extensive collection of genetic labeling tools available, which can highlight traditionally clustered cell populations for more detailed study.

For instance, a targeted approach to scRNA-seq is essential for accurately describing nuanced developmental systems, such as the specification of unique neural subtypes derived from the type II neuroblast (NB) lineages in *Drosophila*. Including cells not derived from type II lineages, which constitute the majority of the fly brain, would introduce significant noise and confound the analysis, underscoring the need for precision in sample selection and data interpretation (Michki et al., 2021). This highlights a critical challenge in scRNA-seq applications: the need for methodological adaptations to address specific biological questions and contexts, which broad, untargeted approaches may not sufficiently meet.

In terms of assessing neural cells, another limitation of single-cell sequencing is the vulnerability of certain neuron subtypes during the cell isolation process. For example, cortical layer five pyramidal tract neurons may not easily survive the isolation, leading to potential underrepresentation or absence in the scRNA-seq data (Tasic et al., 2018). This issue underscores the challenge of obtaining a complete and accurate representation of neural diversity, as the survival rates of different neuron types can significantly influence the results and interpretations of scRNA-seq studies (Xing et al., 2023). Such limitations necessitate the development of gentler and more refined cell isolation techniques that can preserve the integrity and viability of sensitive neuron subtypes.

Conclusions

The study of neural diversity through transcriptomics and scRNA-seq has enhanced understanding of the nervous system's cellular and molecular complexity. By analyzing individual cells, scRNA-seq aided in the uncovering of a variety of cellular phenotypes and regulatory markers that are essential for neuron differentiation and function. This approach has revealed the extensive heterogeneity within neural populations, highlighting the intricate networks of transcription factors and signaling pathways that govern neuron development and specialization. Each cell type, from progenitors to fully differentiated neurons, exhibits unique transcriptional signatures that scRNA-seq can identify and decode, providing insights into their functional roles and developmental trajectories.

Furthermore, the integration of scRNA-seq with connectomics has enriched our comprehension of how cellular diversity influences the nervous system's connectivity and functionality. Connectomics, combined with the detailed cellular insights provided by scRNA-seq, allows researchers to map these connections at unprecedented resolution. This synergy enhances our

understanding of the structural and functional organization of the brain, offering new perspectives on how neural circuits support cognitive and behavioral outcomes. The data generated through these studies are crucial for constructing more accurate models of brain function, which are essential for developing targeted interventions for neurological disorders.

The insights gained from this technology not only deepen our understanding of the cellular basis of neural function but also open up new avenues for therapeutic intervention. For instance, identifying specific cell types that are disproportionately affected in various neurodegenerative diseases could lead to more precise treatments. Continued innovations in scRNA-seq technology and its applications can continue to further elucidate the complexities of neurodevelopment and neural diversity. Furthermore, advancements in computational and bioinformatics tooling are being developed to handle the massive datasets generated by scRNA-seq, enabling the identification of subtle but critical changes in gene expression that may influence disease states or therapeutic responses.

References

Achim, K., Pettit, J.-B., Saraiva, L. R., Gavriouchkina, D., Larsson, T., Arendt, D., & Marioni, J. C. (2015). High-throughput spatial mapping of single-cell RNA-seq data to tissue of origin. *Nature Biotechnology*, *33*(5), 503–509. https://doi.org/10.1038/nbt.3209

Ahmad, R., & Budnik, B. (2023). A review of the current state of single-cell proteomics and future perspective. *Analytical and Bioanalytical Chemistry*, *415*(28), 6889–6899. https://doi.org/10.1007/s00216-023-04759-8

Aibar, S., González-Blas, C. B., Moerman, T., Huynh-Thu, V. A., Imrichova, H., Hulselmans, G., Rambow, F., Marine, J.-C., Geurts, P., Aerts, J., Van Den Oord, J., Atak, Z. K., Wouters, J., & Aerts, S. (2017). SCENIC: Single-cell regulatory network inference and clustering. *Nature Methods*, *14*(11), 1083–1086. https://doi.org/10.1038/nmeth.4463

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology*, *11*(10), R106. https://doi.org/10.1186/gb-2010-11-10-r106

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2), 166–169. https://doi.org/10.1093/bioinformatics/btu638

Armand, E. J., Li, J., Xie, F., Luo, C., & Mukamel, E. A. (2021). Single-Cell Sequencing of Brain Cell Transcriptomes and Epigenomes. *Neuron*, *109*(1), 11–26. https://doi.org/10.1016/j.neuron.2020.12.010

Beine, Z., Wang, Z., Tsoulfas, P., & Blackmore, M. G. (2022). Single Nuclei Analyses Reveal Transcriptional Profiles and Marker Genes for Diverse Supraspinal Populations. *The Journal of Neuroscience*, *42*(47), 8780–8794. https://doi.org/10.1523/JNEUROSCI.1197-22.2022

Bengtsson, M., Hemberg, M., Rorsman, P., & Ståhlberg, A. (2008). Quantification of mRNA in single cells and modelling of RT-qPCR induced noise. *BMC Molecular Biology*, *9*(1), 63. https://doi.org/10.1186/1471-2199-9-63

Blekhman, R., Oshlack, A., Chabot, A. E., Smyth, G. K., & Gilad, Y. (2008). Gene Regulation in Primates Evolves under Tissue-Specific Selection Pressures. *PLoS Genetics*, *4*(11), e1000271. https://doi.org/10.1371/journal.pgen.1000271

Bravo González-Blas, C., De Winter, S., Hulselmans, G., Hecker, N., Matetovici, I., Christiaens, V., Poovathingal, S., Wouters, J., Aibar, S., & Aerts, S. (2023). SCENIC+: Single-cell multiomic inference of enhancers and gene regulatory networks. *Nature Methods*, *20*(9), 1355–1367. https://doi.org/10.1038/s41592-023-01938-4

Brennecke, P., Anders, S., Kim, J. K., Kołodziejczyk, A. A., Zhang, X., Proserpio, V., Baying, B., Benes, V., Teichmann, S. A., Marioni, J. C., & Heisler, M. G. (2013). Accounting for technical noise in single-cell RNA-seq experiments. *Nature Methods*, *10*(11), 1093–1095. https://doi.org/10.1038/nmeth.2645

Calvo-Rodriguez, M., & Bacskai, B. J. (2021). Mitochondria and Calcium in Alzheimer's Disease: From Cell Signaling to Neuronal Cell Death. *Trends in Neurosciences*, *44*(2), 136–151. https://doi.org/10.1016/j.tins.2020.10.004

Cebrian-Silla, A., Nascimento, M. A., Redmond, S. A., Mansky, B., Wu, D., Obernier,

K., Romero Rodriguez, R., Gonzalez-Granero, S., García-Verdugo, J. M., Lim, D. A., & Álvarez-Buylla, A. (2021). Single-cell analysis of the ventricular-subventricular zone reveals signatures of dorsal and ventral adult neurogenesis. *eLife*, *10*, e67436. https://doi.org/10.7554/eLife.67436

Cook, S. J., Jarrell, T. A., Brittin, C. A., Wang, Y., Bloniarz, A. E., Yakovlev, M. A., Nguyen, K. C. Q., Tang, L. T.-H., Bayer, E. A., Duerr, J. S., Bülow, H. E., Hobert, O., Hall, D. H., & Emmons, S. W. (2019). Whole-animal connectomes of both Caenorhabditis elegans sexes. *Nature*, *571*(7763), 63–71. https://doi.org/10.1038/s41586-019-1352-7

Davie, K., Janssens, J., Koldere, D., De Waegeneer, M., Pech, U., Kreft, Ł., Aibar, S., Makhzami, S., Christiaens, V., Bravo González-Blas, C., Poovathingal, S., Hulselmans, G., Spanier, K. I., Moerman, T., Vanspauwen, B., Geurs, S., Voet, T., Lammertyn, J., Thienpont, B., … Aerts, S. (2018). A Single-Cell Transcriptome Atlas of the Aging Drosophila Brain. *Cell*, *174*(4), 982–998.e20. https://doi.org/10.1016/j.cell.2018.05.057

Dillman, A. A., & Cookson, M. R. (2014). Transcriptomic Changes in Brain Development. In *International Review of Neurobiology* (Vol. 116, pp. 233–250). Elsevier. https://doi.org/10.1016/B978-0-12-801105-8.00009-6

Ding, B., Zheng, L., Zhu, Y., Li, N., Jia, H., Ai, R., Wildberg, A., & Wang, W. (2015). Normalization and noise reduction for single cell RNA-seq experiments. *Bioinformatics*, *31*(13), 2225–2227. https://doi.org/10.1093/bioinformatics/btv122

Durruthy-Durruthy, R., Gottlieb, A., Hartman, B. H., Waldhaus, J., Laske, R. D., Altman, R., & Heller, S. (2014). Reconstruction of the Mouse Otocyst and Early Neuroblast Lineage at Single-Cell Resolution. *Cell*, *157*(4), 964–978. https://doi.org/10.1016/j.cell.2014.03.036

Eng, C.-H. L., Lawson, M., Zhu, Q., Dries, R., Koulena, N., Takei, Y., Yun, J., Cronin, C., Karp, C., Yuan, G.-C., & Cai, L. (2019). Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH+. *Nature*, *568*(7751), 235–239. https://doi.org/10.1038/s41586-019-1049-y

Fan, H. C., Fu, G. K., & Fodor, S. P. A. (2015). Combinatorial labeling of single cells for gene expression cytometry. *Science*, *347*(6222), 1258367. https://doi.org/10.1126/science.1258367

Femino, A. M., Fay, F. S., Fogarty, K., & Singer, R. H. (1998). Visualization of Single RNA Transcripts in Situ. *Science*, *280*(5363), 585–590. https://doi.org/10.1126/science.280.5363.585

Feng, H., Moakley, D. F., Chen, S., McKenzie, M. G., Menon, V., & Zhang, C. (2021). Complexity and graded regulation of neuronal cell-type–specific alternative splicing revealed by single-cell RNA sequencing. *Proceedings of the National Academy of Sciences*, *118*(10), e2013056118. https://doi.org/10.1073/pnas.2013056118

Frumkin, D., Wasserstrom, A., Itzkovitz, S., Harmelin, A., Rechavi, G., & Shapiro, E. (2008). Amplification of multiple genomic loci from single cells isolated by laser micro-dissection of tissues. *BMC Biotechnology*, *8*(1), 17. https://doi.org/10.1186/1472-6750-8-17

Fu, G. K., Hu, J., Wang, P.-H., & Fodor, S. P. A. (2011). Counting individual DNA molecules by the stochastic attachment of diverse labels. *Proceedings of the National Academy of Sciences*, *108*(22), 9026–9031. https://doi.org/10.1073/pnas.1017621108

Grange, P., Bohland, J. W., Okaty, B. W., Sugino, K., Bokil, H., Nelson, S. B., Ng, L.,

Hawrylycz, M., & Mitra, P. P. (2014). Cell-type–based model explaining coexpression patterns of genes in the brain. *Proceedings of the National Academy of Sciences*, *111*(14), 5397–5402. https://doi.org/10.1073/pnas.1312098111

Grindberg, R. V., Yee-Greenbaum, J. L., McConnell, M. J., Novotny, M., O'Shaughnessy, A. L., Lambert, G. M., Araúzo-Bravo, M. J., Lee, J., Fishman, M., Robbins, G. E., Lin, X., Venepally, P., Badger, J. H., Galbraith, D. W., Gage, F. H., & Lasken, R. S. (2013). RNA-sequencing from single nuclei. *Proceedings of the National Academy of Sciences*, *110*(49), 19802–19807. https://doi.org/10.1073/pnas.1319700110

Gulati, G. S., Sikandar, S. S., Wesche, D. J., Manjunath, A., Bharadwaj, A., Berger, M. J., Ilagan, F., Kuo, A. H., Hsieh, R. W., Cai, S., Zabala, M., Scheeren, F. A., Lobo, N. A., Qian, D., Yu, F. B., Dirbas, F. M., Clarke, M. F., & Newman, A. M. (2020). Single-cell transcriptional diversity is a hallmark of developmental potential. *Science*, *367*(6476), 405–411. https://doi.org/10.1126/science.aax0249

Hafemeister, C., & Satija, R. (2019). Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biology*, *20*(1), 296. https://doi.org/10.1186/s13059-019-1874-1

Hara, M. R., & Snyder, S. H. (2007). Cell Signaling and Neuronal Death. *Annual Review of Pharmacology and Toxicology*, *47*(1), 117–141. https://doi.org/10.1146/annurev.pharmtox.47.120505.105311

Hayashi, T., Shibata, N., Okumura, R., Kudome, T., Nishimura, O., Tarui, H., & Agata, K. (2010). Single-cell gene profiling of planarian stem cells using fluorescent activated cell sorting and its "index sorting" function for stem cell research. *Development, Growth & Differentiation*, *52*(1), 131–144. https://doi.org/10.1111/j.1440-169X.2009.01157.x

Huang, Y., & Sanguinetti, G. (2021). Uncertainty versus variability: Bayesian methods for analysis of scRNA-seq data. *Current Opinion in Systems Biology*, *28*, 100375. https://doi.org/10.1016/j.coisb.2021.100375

Islam, S., Zeisel, A., Joost, S., La Manno, G., Zajac, P., Kasper, M., Lönnerberg, P., & Linnarsson, S. (2014). Quantitative single-cell RNA-seq with unique molecular identifiers. *Nature Methods*, *11*(2), 163–166. https://doi.org/10.1038/nmeth.2772

Jaitin, D. A., Kenigsberg, E., Keren-Shaul, H., Elefant, N., Paul, F., Zaretsky, I., Mildner, A., Cohen, N., Jung, S., Tanay, A., & Amit, I. (2014). Massively Parallel Single-Cell RNA-Seq for Marker-Free Decomposition of Tissues into Cell Types. *Science*, *343*(6172), 776–779. https://doi.org/10.1126/science.1247651

Jensen, K. B., & Darnell, R. B. (2008). CLIP: Crosslinking and immunoprecipitation of in vivo RNA targets of RNA-binding proteins. *Methods in Molecular Biology (Clifton, N.J.)*, *488*, 85–98. https://doi.org/10.1007/978-1-60327-475-3_6

Kamme, F., Salunga, R., Yu, J., Tran, D.-T., Zhu, J., Luo, L., Bittner, A., Guo, H.-Q., Miller, N., Wan, J., & Erlander, M. (2003). Single-Cell Microarray Analysis in Hippocampus CA1: Demonstration and Validation of Cellular Heterogeneity. *The Journal of Neuroscience*, *23*(9), 3607–3615. https://doi.org/10.1523/JNEUROSCI.23-09-03607.2003

Kanno, J., Aisaki, K., Igarashi, K., Nakatsu, N., Ono, A., Kodama, Y., & Nagao, T. (2006). "Per cell" normalization method for mRNA measurement by quantitative PCR and microarrays. *BMC Genomics*, *7*(1), 64. https://doi.org/10.1186/1471-2164-7-64

Kazan, H., & Morris, Q. (2013). RBPmotif: A web server for the discovery of sequence and structure preferences of RNA-binding proteins. *Nucleic Acids Research*, *41*(Web Server issue), W180–W186. https://doi.org/10.1093/nar/gkt463

Kebschull, J. M., Richman, E. B., Ringach, N., Friedmann, D., Albarran, E., Kolluru, S. S., Jones, R. C., Allen, W. E., Wang, Y., Cho, S. W., Zhou, H., Ding, J. B., Chang, H. Y., Deisseroth, K., Quake, S. R., & Luo, L. (2020). Cerebellar nuclei evolved by repeatedly duplicating a conserved cell-type set. *Science*, *370*(6523), eabd5059. https://doi.org/10.1126/science.abd5059

Keller, D., Verasztó, C., & Markram, H. (2023). Cell-type-specific densities in mouse somatosensory cortex derived from scRNA-seq and in situ RNA hybridization. *Frontiers in Neuroanatomy*, *17*, 1118170. https://doi.org/10.3389/fnana.2023.1118170

Kiselev, V. Y., Andrews, T. S., & Hemberg, M. (2019). Challenges in unsupervised clustering of single-cell RNA-seq data. *Nature Reviews Genetics*, *20*(5), 273–282. https://doi.org/10.1038/s41576-018-0088-9

Kolodziejczyk, A. A., Kim, J. K., Svensson, V., Marioni, J. C., & Teichmann, S. A. (2015). The Technology and Biology of Single-Cell RNA Sequencing. *Molecular Cell*, *58*(4), 610–620. https://doi.org/10.1016/j.molcel.2015.04.005

Lall, S., Sinha, D., Bandyopadhyay, S., & Sengupta, D. (2018). Structure-Aware Principal Component Analysis for Single-Cell RNA-seq Data. *Journal of Computational Biology*, *25*(12), 1365–1373. https://doi.org/10.1089/cmb.2018.0027

Lein, E., Borm, L. E., & Linnarsson, S. (2017). The promise of spatial transcriptomics for neuroscience in the era of molecular cell typing. *Science*, *358*(6359), 64–69. https://doi.org/10.1126/science.aan6827

Li, H., Horns, F., Wu, B., Xie, Q., Li, J., Li, T., Luginbuhl, D. J., Quake, S. R., & Luo, L. (2017). Classifying Drosophila Olfactory Projection Neuron Subtypes by Single-Cell RNA Sequencing. *Cell*, *171*(5), 1206–1220.e22. https://doi.org/10.1016/j.cell.2017.10.019

Li, H., Janssens, J., De Waegeneer, M., Kolluru, S. S., Davie, K., Gardeux, V., Saelens, W., David, F. P. A., Brbić, M., Spanier, K., Leskovec, J., McLaughlin, C. N., Xie, Q., Jones, R. C., Brueckner, K., Shim, J., Tattikota, S. G., Schnorrer, F., Rust, K., … Zinzen, R. P. (2022). Fly Cell Atlas: A single-nucleus transcriptomic atlas of the adult fruit fly. *Science*, *375*(6584), eabk2432. https://doi.org/10.1126/science.abk2432

Li, Y., Chen, S., Liu, W., Zhao, D., Gao, Y., Hu, S., Liu, H., Li, Y., Qu, L., & Liu, X. (2024). A full-body transcription factor expression atlas with completely resolved cell identities in C. elegans. *Nature Communications*, *15*(1), 358. https://doi.org/10.1038/s41467-023-42677-6

Lo, A. C., Houenou, L. J., & Oppenheim, R. W. (1995). Apoptosis in the Nervous System: Morphological Features, Methods, Pathology, and Prevention. *Archives of Histology and Cytology*, *58*(2), 139–149. https://doi.org/10.1679/aohc.58.139

Luecken, M. D., & Theis, F. J. (2019). Current best practices in single-cell RNA-seq analysis: A tutorial. *Molecular Systems Biology*, *15*(6), e8746. https://doi.org/10.15252/msb.20188746

Lytal, N., Ran, D., & An, L. (2020). Normalization Methods on Single-Cell RNA-seq Data: An Empirical Survey. *Frontiers in Genetics*, *11*, 41. https://doi.org/10.3389/fgene.2020.00041

Maynard, K. R., Collado-Torres, L., Weber, L. M., Uytingco, C., Barry, B. K., Williams,

S. R., Catallini, J. L., Tran, M. N., Besich, Z., Tippani, M., Chew, J., Yin, Y., Kleinman, J. E., Hyde, T. M., Rao, N., Hicks, S. C., Martinowich, K., & Jaffe, A. E. (2021). Transcriptome-scale spatial gene expression in the human dorsolateral prefrontal cortex. *Nature Neuroscience*, *24*(3), 425–436. https://doi.org/10.1038/s41593-020-00787-0

Michael Love, S. A. (2017). *DESeq2*. [object Object]. https://doi.org/10.18129/B9.BIOC.DESEQ2

Michki, N. S., Li, Y., Sanjasaz, K., Zhao, Y., Shen, F. Y., Walker, L. A., Cao, W., Lee, C.-Y., & Cai, D. (2021). The molecular landscape of neural differentiation in the developing Drosophila brain revealed by targeted scRNA-seq and multi-informatic analysis. *Cell Reports*, *35*(4), 109039. https://doi.org/10.1016/j.celrep.2021.109039

Mishina, Y., & Snider, T. N. (2014). Neural crest cell signaling pathways critical to cranial bone development and pathology. *Experimental Cell Research*, *325*(2), 138–147. https://doi.org/10.1016/j.yexcr.2014.01.019

Nguyen, P.-K., & Cheng, L. Y. (2022). Non-autonomous regulation of neurogenesis by extrinsic cues: A *Drosophila* perspective. *Oxford Open Neuroscience*, *1*, kvac004. https://doi.org/10.1093/oons/kvac004

Olsen, T. K., & Baryawno, N. (2018). Introduction to Single-Cell RNA Sequencing. *Current Protocols in Molecular Biology*, *122*(1), e57. https://doi.org/10.1002/cpmb.57

Oshlack, A., Robinson, M. D., & Young, M. D. (2010). From RNA-seq reads to differential expression results. *Genome Biology*, *11*(12), 220. https://doi.org/10.1186/gb-2010-11-12-220

Peng, H., Xie, P., Liu, L., Kuang, X., Wang, Y., Qu, L., Gong, H., Jiang, S., Li, A., Ruan, Z., Ding, L., Yao, Z., Chen, C., Chen, M., Daigle, T. L., Dalley, R., Ding, Z., Duan, Y., Feiner, A., … Zeng, H. (2021). Morphological diversity of single neurons in molecularly defined cell types. *Nature*, *598*(7879), 174–181. https://doi.org/10.1038/s41586-021-03941-1

Pettit, J.-B., Tomer, R., Achim, K., Richardson, S., Azizi, L., & Marioni, J. (2014). Identifying Cell Types from Spatially Referenced Single-Cell Expression Datasets. *PLoS Computational Biology*, *10*(9), e1003824. https://doi.org/10.1371/journal.pcbi.1003824

Robinson, M. D., & Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology*, *11*(3), R25. https://doi.org/10.1186/gb-2010-11-3-r25

Russell, L. E., Dalgleish, H. W. P., Nutbrown, R., Gauld, O. M., Herrmann, D., Fişek, M., Packer, A. M., & Häusser, M. (2022). All-optical interrogation of neural circuits in behaving mice. *Nature Protocols*, *17*(7), 1579–1620. https://doi.org/10.1038/s41596-022-00691-w

Satija, R., Farrell, J. A., Gennert, D., Schier, A. F., & Regev, A. (2015). Spatial reconstruction of single-cell gene expression data. *Nature Biotechnology*, *33*(5), 495–502. https://doi.org/10.1038/nbt.3192

Schäffner, E., Bosch-Queralt, M., Edgar, J. M., Lehning, M., Strauß, J., Fleischer, N., Kungl, T., Wieghofer, P., Berghoff, S. A., Reinert, T., Krueger, M., Morawski, M., Möbius, W., Barrantes-Freer, A., Stieler, J., Sun, T., Saher, G., Schwab, M. H., Wrede, C., … Stassart, R. M. (2023). Myelin insulation as a risk factor for axonal degeneration in autoimmune demyelinating disease. *Nature Neuroscience*, *26*(7), 1218–1228. https://doi.org/10.1038/s41593-023-01366-9

Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S., Hayworth, K. J., Huang, G. B., Shinomiya, K., Maitlin-Shepard, J., Berg, S., Clements, J., Hubbard, P. M., Katz, W. T., Umayam, L., Zhao, T., Ackerman, D., Blakely, T., Bogovic, J., Dolafi, T., … Plaza, S. M. (2020). A connectome and analysis of the adult Drosophila central brain. *eLife*, *9*, e57443. https://doi.org/10.7554/eLife.57443

Schneider-Lunitz, V., Ruiz-Orera, J., Hubner, N., & van Heesch, S. (2021). Multifunctional RNA-binding proteins influence mRNA abundance and translational efficiency of distinct sets of target genes. *PLoS Computational Biology*, *17*(12), e1009658. https://doi.org/10.1371/journal.pcbi.1009658

Schuurmans, C., & Guillemot, F. (2002). Molecular mechanisms underlying cell fate specification in the developing telencephalon. *Current Opinion in Neurobiology*, *12*(1), 26–34. https://doi.org/10.1016/S0959-4388(02)00286-6

Shah, S., Lubeck, E., Zhou, W., & Cai, L. (2016). In Situ Transcription Profiling of Single Cells Reveals Spatial Organization of Cells in the Mouse Hippocampus. *Neuron*, *92*(2), 342–357. https://doi.org/10.1016/j.neuron.2016.10.001

Sharon, N., Chawla, R., Mueller, J., Vanderhooft, J., Whitehorn, L. J., Rosenthal, B., Gürtler, M., Estanboulieh, R. R., Shvartsman, D., Gifford, D. K., Trapnell, C., & Melton, D. (2019). A Peninsular Structure Coordinates Asynchronous Differentiation with Morphogenesis to Generate Pancreatic Islets. *Cell*, *176*(4), 790–804.e13. https://doi.org/10.1016/j.cell.2018.12.003

Smajić, S., Prada-Medina, C. A., Landoulsi, Z., Ghelfi, J., Delcambre, S., Dietrich, C., Jarazo, J., Henck, J., Balachandran, S., Pachchek, S., Morris, C. M., Antony, P., Timmermann, B., Sauer, S., Pereira, S. L., Schwamborn, J. C., May, P., Grünewald, A., & Spielmann, M. (2022). Single-cell sequencing of human midbrain reveals glial activation and a Parkinson-specific neuronal state. *Brain*, *145*(3), 964–978. https://doi.org/10.1093/brain/awab446

Stegle, O., Teichmann, S. A., & Marioni, J. C. (2015). Computational and analytical challenges in single-cell transcriptomics. *Nature Reviews Genetics*, *16*(3), 133–145. https://doi.org/10.1038/nrg3833

Stickels, R. R., Murray, E., Kumar, P., Li, J., Marshall, J. L., Di Bella, D. J., Arlotta, P., Macosko, E. Z., & Chen, F. (2021). Highly sensitive spatial transcriptomics at near-cellular resolution with Slide-seqV2. *Nature Biotechnology*, *39*(3), 313–319. https://doi.org/10.1038/s41587-020-0739-1

Svara, F., Förster, D., Kubo, F., Januszewski, M., Dal Maschio, M., Schubert, P. J., Kornfeld, J., Wanner, A. A., Laurell, E., Denk, W., & Baier, H. (2022). Automated synapse-level reconstruction of neural circuits in the larval zebrafish brain. *Nature Methods*, *19*(11), 1357–1366. https://doi.org/10.1038/s41592-022-01621-0

Tang, F., Barbacioru, C., Bao, S., Lee, C., Nordman, E., Wang, X., Lao, K., & Surani, M. A. (2010). Tracing the Derivation of Embryonic Stem Cells from the Inner Cell Mass by Single-Cell RNA-Seq Analysis. *Cell Stem Cell*, *6*(5), 468–478. https://doi.org/10.1016/j.stem.2010.03.015

Tang, F., Barbacioru, C., Wang, Y., Nordman, E., Lee, C., Xu, N., Wang, X., Bodeau, J., Tuch, B. B., Siddiqui, A., Lao, K., & Surani, M. A. (2009). mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods*, *6*(5), 377–382. https://doi.org/10.1038/nmeth.

1315

Tasic, B., Yao, Z., Graybuck, L. T., Smith, K. A., Nguyen, T. N., Bertagnolli, D., Goldy, J., Garren, E., Economo, M. N., Viswanathan, S., Penn, O., Bakken, T., Menon, V., Miller, J., Fong, O., Hirokawa, K. E., Lathia, K., Rimorin, C., Tieu, M., … Zeng, H. (2018). Shared and distinct transcriptomic cell types across neocortical areas. *Nature*, *563*(7729), 72–78. https://doi.org/10.1038/s41586-018-0654-5

Taylor, S. R., Santpere, G., Reilly, M., Glenwinkel, L., Poff, A., McWhirter, R., Xu, C., Weinreb, A., Basavaraju, M., Cook, S. J., Barrett, A., Abrams, A., Vidal, B., Cros, C., Rafi, I., Sestan, N., Hammarlund, M., Hobert, O., & Miller, D. M. (2019). *Expression profiling of the mature* C. Elegans *nervous system by single-cell RNA-Sequencing*. https://doi.org/10.1101/737577

Thompson, M., Bixby, R., Dalton, R., Vandenburg, A., Calarco, J. A., & Norris, A. D. (2019). Splicing in a single neuron is coordinately controlled by RNA binding proteins and transcription factors. *eLife*, *8*, e46726. https://doi.org/10.7554/eLife.46726

Tosches, M. A., Yamawaki, T. M., Naumann, R. K., Jacobi, A. A., Tushev, G., & Laurent, G. (2018). Evolution of pallium, hippocampus, and cortical cell types revealed by single-cell transcriptomics in reptiles. *Science*, *360*(6391), 881–888. https://doi.org/10.1126/science.aar4237

Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S., Morse, M., Lennon, N. J., Livak, K. J., Mikkelsen, T. S., & Rinn, J. L. (2014). The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nature Biotechnology*, *32*(4), 381–386. https://doi.org/10.1038/nbt.2859

Treutlein, B., Brownfield, D. G., Wu, A. R., Neff, N. F., Mantalas, G. L., Espinoza, F. H., Desai, T. J., Krasnow, M. A., & Quake, S. R. (2014). Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. *Nature*, *509*(7500), 371–375. https://doi.org/10.1038/nature13173

Van Battum, E. Y., Brignani, S., & Pasterkamp, R. J. (2015). Axon guidance proteins in neurological disorders. *The Lancet Neurology*, *14*(5), 532–546. https://doi.org/10.1016/S1474-4422(14)70257-1

Wang, X., He, Y., Zhang, Q., Ren, X., & Zhang, Z. (2021). Direct Comparative Analyses of 10X Genomics Chromium and Smart-seq2. *Genomics, Proteomics & Bioinformatics*, *19*(2), 253–266. https://doi.org/10.1016/j.gpb.2020.02.005

Xing, Y., Zan, C., & Liu, L. (2023). Recent advances in understanding neuronal diversity and neural circuit complexity across different brain regions using single-cell sequencing. *Frontiers in Neural Circuits*, *17*, 1007755. https://doi.org/10.3389/fncir.2023.1007755

Yang, Y., Shi, X., Liu, W., Zhou, Q., Chan Lau, M., Chun Tatt Lim, J., Sun, L., Ng, C. C. Y., Yeong, J., & Liu, J. (2022). SC-MEB: Spatial clustering with hidden Markov random field using empirical Bayes. *Briefings in Bioinformatics*, *23*(1), bbab466. https://doi.org/10.1093/bib/bbab466

Yao, S., Wang, Q., Hirokawa, K. E., Ouellette, B., Ahmed, R., Bomben, J., Brouner, K., Casal, L., Caldejon, S., Cho, A., Dotson, N. I., Daigle, T. L., Egdorf, T., Enstrom, R., Gary, A., Gelfand, E., Gorham, M., Griffin, F., Gu, H., … Zeng, H. (2023). A whole-brain monosynaptic input connectome to neuron classes in mouse visual cortex. *Nature*

Neuroscience, *26*(2), 350–364. https://doi.org/10.1038/s41593-022-01219-x

Zakrzewski, W., Dobrzyński, M., Szymonowicz, M., & Rybak, Z. (2019). Stem cells: Past, present, and future. *Stem Cell Research & Therapy*, *10*(1), 68. https://doi.org/10.1186/s13287-019-1165-5

Zeisel, A., Muñoz-Manchado, A. B., Codeluppi, S., Lönnerberg, P., La Manno, G., Juréus, A., Marques, S., Munguba, H., He, L., Betsholtz, C., Rolny, C., Castelo-Branco, G., Hjerling-Leffler, J., & Linnarsson, S. (2015). Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science*, *347*(6226), 1138–1142. https://doi.org/10.1126/science.aaa1934

Zhao, P., Mondal, S., Martin, C., DuPlissis, A., Chizari, S., Ma, K.-Y., Maiya, R., Messing, R. O., Jiang, N., & Ben-Yakar, A. (2023). Femtosecond laser microdissection for isolation of regenerating C. Elegans neurons for single-cell RNA sequencing. *Nature Methods*, *20*(4), 590–599. https://doi.org/10.1038/s41592-023-01804-3

Zhong, C., Tian, T., & Wei, Z. (2023). Hidden Markov random field models for cell-type assignment of spatially resolved transcriptomics. *Bioinformatics*, *39*(11), btad641. https://doi.org/10.1093/bioinformatics/btad641

Zhou, Q., Liu, M., Xia, X., Gong, T., Feng, J., Liu, W., Liu, Y., Zhen, B., Wang, Y., Ding, C., & Qin, J. (2017). A mouse tissue transcription factor atlas. *Nature Communications*, *8*(1), 15089. https://doi.org/10.1038/ncomms15089

Ziegenhain, C., Hendriks, G.-J., Hagemann-Jensen, M., & Sandberg, R. (2022). Molecular spikes: A gold standard for single-cell RNA counting. *Nature Methods*, *19*(5), 560–566. https://doi.org/10.1038/s41592-022-01446-x