Opportunities and challenges of single-cell and spatially resolved genomics methods for neuroscience discovery

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ABSTRACT

Over the last decade, single-cell genomics technologies have allowed scalable profiling of cell type-specific features, which substantially increased our ability to study cellular diversity and transcriptional programs in heterogeneous tissues. Yet our understanding of mechanisms of gene regulation or the rules that govern interactions between cell types is still limited. Advent of new computational pipelines and new technologies, such as single-cell epigenomics and spatially resolved transcriptomics, has created opportunities to explore two new axes of biological variation: cell intrinsic regulation of cell states and expression programs and interactions between cells. Here, we summarize the most promising and robust technologies in these areas, discuss their strengths and limitations, and discuss key computational approaches for analysis of these complex datasets. We highlight how data sharing and integration, documentation, visualization, and benchmarking of results contribute to transparency, reproducibility, collaboration, and democratization in neuroscience, and discuss needs and opportunities for future technology development and analysis.

Introduction

Cells in our bodies contain roughly the same genomic information encoded within the DNA, but develop remarkably different properties as a consequence of intrinsic gene expression regulation and inter-cellular communication. Nowhere is this clearer than the mammalian brain, where hundreds of molecularly distinct cell subpopulations have recently been mapped using a combination of single-cell technologies and shown to be organized into neighborhoods and circuits that can be visualized using spatially resolved technologies[1–9]. Intrinsic gene expression regulation and cell-cell interactions represent two orthogonal, and yet interrelated axes of biological variation in complex tissues, that frequently become altered in disease states. Emerging technologies for mapping these modalities create exciting opportunities for uncovering disease changes with fewer *a priori* assumptions than has been possible before. In turn, unbiased profiling of disease tissues has the potential to uncover new disease relevant changes that could be targeted therapeutically.

Here, we strive to provide an overview of the main technologies and approaches currently present in single-cell epigenomics and spatially resolved transcriptomics fields, as well as discuss various strategies for data analysis and considerations in experimental design. In particular, we highlight the proliferation of single-cell epigenomic data collection that has provided exciting opportunities to reveal gene regulatory networks, while highlighting the paucity of methods for functional validation of these predictions. By contrast, spatially resolved transcriptomics approaches vary widely depending on the specific tissue preservation method, size and resolution needed. When coupled with single-cell transcriptomics and rigorous data analysis, such as deconvolution, trajectory analysis and cell-cell interactions prediction, such experiments can provide invaluable insights into tissue biology (**Fig. 1**).

We recommend technology choices as well as computational schemes should be motivated by the biological questions, while balancing discovery, analysis, and validation wherever possible to maximize biological insights. Best practices in computational analysis should guide the experimental design and be considered prior to data generation, taking into consideration the required number of samples, coverage of cells per sample, and design of experimental batches, to facilitate accurate analysis. In turn, the design of the computational scheme for the data analysis should be tailored to the specific features of the dataset as well as to the biological questions, guiding the choices such as *de-novo* vs. reference-based annotations or discrete vs. continuous analysis of cell states.

Technical considerations in study design

High-throughput genomic technologies have created unprecedented opportunities for data-driven discovery of biological processes underlying normal tissue structure, function, and disease changes. Considering the cost of many such studies, responsible experimental design is often required to maximize biological insights, and should start with considerations of best practices in data analysis (**Fig. 2**). We recommend considering the following components for single cell and spatial genomic studies.

Sample size

Evaluation of the necessary sample size for robust analysis is essential, especially when testing changes in cell abundance, differential genes, or trait associations with experimental or clinical conditions. Computational methods should be tailored to the sample size and to the corresponding statistical power of the dataset. There

are tools and resources that can be used to estimate the necessary number of biological replicates and technical replicates ideally required for single-cell/nucleus RNA sequencing (sc/snRNAseq) studies[10–14]. As fewer spatial transcriptomics studies have been conducted so far, recommendations of sample size estimation are driven largely by theoretical and statistical considerations[15,16]. Adhering to the power estimations discussed can greatly increase the confidence in biological findings derived from single-cell RNA sequencing studies, and we predict that the rapid increase in the number of spatial transcriptomic and epigenomic datasets will lead to better understanding of technical variation in the data and inform new methods for quantifying effect sizes that may be specific to the assay or data generation platform. Beyond the number of samples, the number and design of batches should be carefully considered as well. Specifically, ensuring balanced batches across different experimental groups can greatly facilitate batch correction to mitigate technical artifacts. It is important to note that the required sample size is contingent on the intrinsic variation between samples, which may vary depending on the genetic diversity of study population, and could furthermore be influenced by the technical idiosyncrasies of individual platforms or dataset quality.

Underpowered studies may still provide biologically meaningful insights, but require specific considerations. We advise incorporating strategies for orthogonal validation using methods discussed in Companion Piece 3. Alternatively, leveraging large cohorts of bulk datasets can augment the sample size, enabling the correlation of gene and pathway signatures to conditions and traits. For example, different strategies have been suggested to utilize sc/snRNAseq data to deconvolve signatures of cell type abundance hidden within bulk tissue measurements using algorithmic approaches based on deconvolution[17,18] and increasingly deep-learning[19–21]. This strategy significantly reduces the time and cost of experiments, and may be essential in instances where obtaining sufficient biological replicates of tissue specimens is challenging. In essence, acknowledging and accounting for sample size and diversity among samples is crucial for ensuring the reliability and validity of research conclusions.

Cellular coverage

Each profiling experiment involves a decision step to profile a subset of cells present in the tissue, and due to financial and tissue availability constraints, every study will balance the numbers of biological or technical replicates involved with the number of cells that will be profiled. Understandably, heterogeneous tissues such as the brain pose an additional challenge where cell types are not present in equal proportions, and thus effective cell number involved in a study will vary from cell type to cell type.

Thus, consideration for cellular coverage and sequencing depth should guide the computational strategy applied for cell annotations as well as for association analysis depending on the biological question[22,23]. Data downsampling can offer a data driven approach to determining whether a given observation or conclusion is robust, and has been effectively employed to analyze the saturation of cell cluster discovery in mouse brain scRNAseq data[7]. Unfortunately, similar considerations have not been developed yet for spatial transcriptomics or epigenomic studies. Analysis of spatial transcriptomics data in particular will require deep assessment as datasets become more common, and we predict that lessons from stereology[24] may be helpful in interpreting the results of spatial transcriptomics based experiments.

Ideally, when experimental design limitations prohibit from obtaining adequate cellular coverage to comprehensively profile cells in a given tissue, strategies that enrich for a desired cell population can be employed (see companion paper I for methods mining rare cells). Importantly, the computational study design should be guided by the coverage of the dataset, as different clustering algorithms have different sensitivities for detecting rare cell types[25]. Moreover, rare cells can be mis-assigned to transcriptomically similar population if not enough cells are sampled. Mapping cells against a well powered reference atlas dataset can help to overcome this limitation[26], and such atlases are increasing in availability for the brain across various species[1–9], ages, and conditions, offering an important resource that can be leveraged to annotate cell types in smaller studies (see companion paper 1).

Data Quality and Sequencing depth

The sequencing coverage (3'-end, 5'-end, whole coding region), data quality and sequencing depth (number of unique reads/unique molecular identifiers (UMIs), and number of genes detected per cell) represent important metrics of underlying data quality and should ideally be compared to published studies from the same tissue or cell type, and reported across technical and biological replicates in a study. See **box 1** for an overview of quality control data analysis. For sc/snRNA-seq assays, high-quality data are important to ensure that differences in

expression programs within specific cell types can be robustly detected. Insufficient coverage may overlook biological insights, a factor that must be considered during the differential expression analysis of genes and pathways. While the exact specific number of sequencing reads per cell will depend on both the technology and the nature of the sample, for the most common experimental platform from 10X Genomics, gene expression libraries sequenced at or above 25,000 reads per cell/nucleus would generally be considered as reasonable to identify individual sub types, but may remain too shallow for some cell-type specific responses to disease and infection (*e.g.* microglia and astrocytes) where 50,000 reads per cell/nucleus is more often required to detect subtle gene expression changes. Published reference atlases can provide approximate numbers of genes detected across brain cell types, while data derived from whole dissociated cells typically yield higher numbers of genes detected per cell compared to nuclei.

Epigenetic assays cover a larger sample space (whole genome vs. transcriptome) and thus consequently the sequencing depth per cell should be appropriately higher, with recommended of minimal sequencing depth of 50,000 reads/cell for snATAC-seq libraries. For reliable discovery of gene regulatory elements from snATAC-seq data, the number of cells depends on data quality and the analytical context, yet we advise to have at least 200 cells/nuclei per cell population with biological replicates to ensure reproducibility. These metrics are based on experience and lack systematic and quantitative metanalysis, and therefore should be taken as general guidelines as opposed to prescriptive guidelines. Large scale consortia efforts will likely define these parameters in increasingly greater detail.

These estimates are intended to provide general guidance, and we recommend consulting several published studies prior to embarking on experimental data generation.

While sc/snRNAseq data analysis has reached a point of relative consensus, and broadly applicable recommendations can be proposed, guidelines for analysis and assessing data quality of more recent technologies such as epigenomics and spatial transcriptomics will likely emerge as the number of datasets increase.

Biological considerations in study design

Cellular architecture guiding study design

Apart from technical aspects, the computational study design should also be informed by the distinct characteristics of each cell type and the specific research questions of the study. These elements should guide the choice between a discrete clustering-based analysis of cellular diversity with a case-control differential expression analysis to uncover changes due to conditions and traits, or rather a continuous analysis of gene programs to describe cellular diversity and alignment of cells along continuous trajectories (**Fig.3**). The particular research questions will further shape subsequent downstream analysis steps, which could encompass regulatory networks, cellular interactions, intersection with genetics and other modalities, and more.

Beyond the diversity of cell types, studies have revealed the vast diversity of cell subpopulations and cell states within the brain. Conventional approaches identify cellular diversity by clustering, commonly applying nearest neighbor graphs and community detection algorithms, to subset cells of a specific cell type to sub-clusters capturing transcriptionally distinct cell subsets. Yet, cellular diversity might not be always adequately captured by a discrete model, such as microglia and astrocyte cells that rapidly respond to the changing environment to maintain brain homeostasis, or oligodendrocyte lineage cells that change along the maturation process. Alternative methods that model the continuous variation in gene expression have been developed. For example, inference of gene expression programs enable to model the complexity of cellular functions and response to diverse stimuli, by modeling cells as a combination of expression programs (*e.g.* topic modeling[27], non-negative matrix factorization[28], weighted gene co-expression matrix analysis[29]). Algorithms aligning cells along continuous trajectories of change (e.g. Palantir[30], Monocle[31]), as typically applied in developmental datasets (as described in companion piece 1), can also be applied to study the transitions between cell states in the adult brain, specifically along aging or disease processes. Importantly, as physiological processes involve cooperation of multiple cell types within the brain, new frameworks expand the analysis from the traditional focus on the diversity of individual cell types to multi-cellular environments[4].

Addressing complex cellular microenvironments in study design

The complex spatial arrangement of cells in the central nervous system (CNS) is what enables it to execute its numerous, highly specialized functions, as physiological processes involve multiple cell types working in cooperation. Investigation of changes in cellular communities or microenvironments instead of independent investigation of individual cell types could be a more effective and accurate approach despite the added complexity. Furthermore, future development of effective drugs and therapies will likely require targeting a community of tightly co-regulated cells that together provide the necessary environment for the required healthy function of the brain. In experimental study design, considering the different properties of brain cell types should guide the choice of methods for cell dissociation, nuclei isolation as well as tissue to capture the diversity of cells. In computational study design, beyond the analysis of individual cell types and cellular abundance, communities of cells with coordinated abundance and/or activity can be predicted using single-cell omics. For example, exploring changes in cell state and cell abundance across cell types in aging human brains uncovered Alzheimer's disease associated cellular community that captured coordinated changes in glial, endothelial and neuronal cell types[4,8]. Algorithms for the identification of coordinated cell programs across cell types, co-occurring cellular communities, co-regulated cells, or communities with shared-dynamics have been developed[8,32–34]. To further advance our understanding of the brain's dynamic tissue architecture, we will need to expand these computational methods. In particular, we will need to integrate predictions of the co-regulation and crosstalk between subsets of cells using multiple data modalities.

Spatial transcriptomics (ST) refers to recently developed technologies that enable probing cellular microenvironments *in situ*. ST methods can simultaneously spatially position cells and quantify their transcriptomic profiles, with most methods applicable in histological tissue sections consisting of 1-2 cell layers. There are two broad classes of ST methods that use sequencing or imaging-based readouts. Many ST technologies were first applied to the mouse brain and have been used to spatially map cell types across entire brain regions[35–37], with recent efforts extending this approach to whole mammalian brain[9,38–40]. ST can enrich cell type and state annotations from sc/snRNAseq with spatial information and ascribe meaning to gene expression gradients, and even entire clusters, by identifying their spatial correlates, exemplified in continuous expression gradients in neurons across the medial-lateral and superior-inferior axes of the striatum[41,42]. Broadly, ST methods fall into two categories: *in-situ* RNA-sequencing based technologies aimed at unbiased profiling of whole transcriptomes within tissues, and imaging-based technologies aimed at probing a multiplexed defined set of hundreds of genes. Sequencing or imaging-based ST methods each offer distinct advantages and suffer from different challenges balancing between resolution, sensitivity, and scalability. Thus, their advantages and respective limitations need to be considered carefully when choosing the right method to use. See **Box 2** for detailed description of technical consideration and limitations of imaging- and sequencing- based ST methods.

The computational integration of spatial and single-cell transcriptomics provides a practical approach to construct multi-modal brain atlases. The integration methodologies have matured dramatically in the past few years, now enabling even relatively fine cell type distinctions to be accurately mapped and resolved on high-quality ST datasets[43–46]. For imaging-based ST, integration of spatial and single-cell measurements enables transcriptome-wide imputation unmeasured genes in space[38,47]. There are also emerging computational integration benchmarks for single cell and spatial data, though we lack methods to quantitatively assess the accuracy and robustness of integration between sc/snRNAseq and ST datasets[48,49].

Cohort design and analytical considerations in disease studies

Increasing amounts of brain sc/snRNAseq data from different species, individuals, developmental stages, and pathological states has revealed the diversity of neuronal and non-neuronal cells (see Companion Piece 1). Such studies use a variety of cohort designs and computational schemes to identify transcriptional changes that occur in disease (**Fig. 3**). A carefully balanced case-control cohort is a conventional and powerful approach for identifying gene expression differences. For such a discrete cohort design, applying a statistical test (with an appropriate noise model[50]) with correction for multiple-testing can be used to link cellular changes to the studied trait, correcting for the technical (*e.g.* batch, library quality) and biological (*e.g.* interindividual variability, age, sex) confounders. Alternatively, pseudo-bulk differential gene expression methods (*e.g.* DESeq[51], edgeR[52]) account for variability between biological replicates and thus avoids false discovery and overcomes gene drop-outs noise in sc/sn/RNA-seq[53].

Newer approaches to cohort design and analytical methods consider continuous variation, as well as mixed sources of variation, ranging from sampling along the continuum of disease stages to random sampling of the population in abundant pathologies. For such a continuous cohort design, different analytical methods have been

developed. For instance, linear mixed models can simultaneously take into account orthogonal sources of transcriptional variation and rank genes according to variance explained by specific variables[54,55]. Additionally, manifold learning and trajectory inference enable us to align individuals along a pseudotime of disease progression and infer the intricate cellular dynamics underlying the disease process[8,30,56]. These approaches provide a more nuanced perspective, offering insights into the temporal and spatial aspects of disease progression that might be missed in traditional case-control studies. To provide further confidence in the rigor and robustness of case control datasets, transcriptomic changes predicted to occur in disease states should be validated using orthogonal methodologies. For extensive discussion of validation approaches see Companion Piece 3.

Many neurological diseases are defined by specific histological lesions. ST methods provide a unique opportunity to bridge our historical understanding of these diseases with modern, hypothesis-generating genomics experiments of the same tissue and cells, which will pave the way to linking newly discovered disease-associated states and pathways, to histopathological disease phenotypes, such as aggregates or multiple sclerosis lesions. Such discoveries may be facilitated by imaging-based ST technologies like in situ sequencing, MERFISH or STARmap PLUS[57], but the limited genes detected by these technologies may limit the discovery of novel cell states uniquely associated with histopathology, without the accompanying transcriptome-wide profiling by sc/snRNA-seq. Efforts to further expand the multiplexing (*i.e.* to increase the number of genes and isoforms that can be measured in imaging ST) is an important area of future technology development. Sequencing-based ST approaches, including the original Spatial Transcriptomics or dBIT-Seq[58,59] largely lack the resolution needed to precisely pair these histological lesions with gene expression in single cells, yet neighborhood-based analyses are feasible. New sequencing-based approaches to capture individual cells with high spatial resolution and sufficient gene coverage, such as Stereo-Seq, Slide-Seq, Slide-Tags, or the recently Visium HD could help overcome this challenge[60,61].

Emerging multi-omics spatial technologies as a bridge between modalities

The benefits of recent ST technological advancements provide an opportunity to combine ST and sc/snRNAseq to create a standardized cell atlas of the nervous system across diverse organisms by bridging anatomical, functional and molecular analysis of neural cell types. A key opportunity associated with ST is its ability to serve as a bridge between the fields of cellular-molecular genomics and systems neuroscience. Specifically, technological approaches are increasingly enabling genomic measurements of cells to be directly paired with measurements of connectivity and neural activity. For example, BARseq combines sequencing based-barcoding of neuronal projections with ISS-based spatial mapping of gene expression and neuronal cell typing[62]. Other studies have combined two-photon calcium imaging and cFOS-staining with ST to probe molecular identities of neurons activated in different behaviours[36,63]. Furthermore, electrophysiological recordings have been coupled to STARmap *in vitro* in other systems[64]. These developments provide exciting avenues to marry spatial transcriptomics with functional studies of neural circuits.

Epigenomic technologies such as CUT&Tag, discussed below, have recently been successfully applied at spatial level using the dBIT-Seq technology, allowing the genome-wide mapping of histone modifications in the mouse and human brain at different stages of development, at a resolution approaching single-cell (20-50μm) (spatial CUT&Tag)[65] or at hundreds of loci at subcellular resolution[66]. In addition, the recent developments of spatial ATAC-seq[67,68] and spatially resolved single-cell translatomics (RIBOmap)[69] potentiate spatial multi-omics mapping of epigenome, transcriptome, and translatome from the same brain samples to understand gene regulation mechanisms at both transcriptional and post-transcriptional level. Moreover, multiomic approaches combining RNA and CUT&Tag (and ATAC) have also been developed at a spatial level[70], while combined ST-lipidomics[71] and ST-metabolomics[72] are emerging, indicating that the simultaneous probing of several modalities might become standard in the spatial omics area, as it has in the single-cell/nucleus arena.

The Allen Brain Atlas Common Coordinate Framework[73] is a three dimensional average map of the adult mouse brain, provides an anatomical reference to standardize spatial measurements of neural activity and connectivity. The integration of ST-based cell maps with such Common Coordinate Frameworks provides an opportunity for charting brain atlases. This is an active area of computational development[74], where a major challenge is the accurate mapping of two-dimensional ST datasets to three-dimensional coordinates at cellular resolution. Furthermore, it is likely ST data will itself challenge some traditionally defined neuroanatomical boundaries [38,75]. New computational approaches will be needed to learn cytoarchitectural features from the spatial data itself and

use it to improve our understanding of regional boundaries. Ultimately, functional perturbations of cells may also be required to refine these regional definitions.

The ability to map ST data to common coordinate frameworks, as discussed above, will likely open opportunities to correspond molecular histopathology measurements to *in vivo* phenotypes. For example, functional MRI can measure correlates of neural activity of a given brain region in the context of a particular behavioral task, and alterations of such activity have been observed in a wide range of neurological and psychiatric disorders[76,77]. For example, 7T MRI can resolve disease structures such as iron-positive lesion rims in multiple sclerosis[78], while PET imaging can resolve metabolic tissue states, with ST measurements.

With the rapid proliferation of ST, systematic benchmarking of different methods is needed. These efforts should formally evaluate the consequences of spatial resolution, sensitivity and multiplexing levels for cell typing and cell-cell interactions analysis of brain tissue. An emerging computational challenge is the integration of ST datasets across experimental batches, studies, and technologies[79,80]. While sc/snRNAseq methods are applicable to a certain extent, new methods to integrate imaging- and sequencing-based ST as well as formal benchmarks to evaluate such methods will be necessary.

Finally, the current cost and throughput of both sequencing- and imaging-based ST are prohibitive to map the whole human brain at the coverage of the mouse brain atlases. Technological or platform investments to increase the feasibility of large-scale ST and apply to 3D brain volumes, prioritizing human brain regions relevant to disease and disorders such as Autism or Schizophrenia, and developing computational approaches to approximate full brain maps from incomplete 3D volumes or to predict ST profiles from MR and metabolic PET imaging provide future avenues for exploration. For example, myeloid cell subtypes - visualized by cell type specific molecular RNA and protein tagging - can be mapped to inflamed tissue areas based on iron-sensitive MR imaging[81] or metabolic tracers as used in PET imaging[82].

Considering cell regulation in the study design

Inference of cell-cell interactions

Cell-cell interactions (CCIs) play key roles in the specification and function of the nervous system. Myriads of neuronal cell types interact to form synaptic connections and neural circuits across multiple scales, whereas glial and vascular cell derived signals are important regulators of neuronal synapses and brain development and homeostasis. Furthermore, neuro-immune interactions are prominent in many neurological disorders. Cell-cell interactions can be predicted using sc/snRNAseq by coupled expression of known ligand-receptor pairs. As RNA abundance only plays a stoichiometric role in mediating signaling activity, there is significant risk of false positive results, and multiple algorithms have been developed with diverse computational strategies and statistical frameworks to limit the sources of noise, such as CellPhoneDB[83], CellChat[84], and NicheNet[85].

ST can identify neuroglial tissue microenvironments that consist of spatially co-localized cell types that are specialized to support specific neural circuit and underlie neural pathologies. As ST jointly resolves spatial positions and transcriptomes of cell types in tissues, it is uniquely suited for inferring CCIs by identifying cells that are in close proximity as well as mapping complementary expression of receptor-ligand pairs in neighboring cells. This can resolve short range interactions (autocrine, juxtracrine, paracrine) such as neuronal-glial interactions at a cellular and molecular level[86,87]. As with cell type mapping, integration with sc/snRNAseq can enhance CCI analysis in ST by leveraging whole transcriptome information from the former data[88]. Current limitations of ST based mapping of CCIs should be considered, as detailed in **Box 3**. Finally, CCIs can be linked to predicted downstream cellular phenotypes by inferring biological pathway activities or gene regulatory networks from single cell or spatial data[85]. However, no existing approaches can yet trace CCIs from spatial information to gene expression to epigenomic profiles and other modalities.

Considering cell intrinsic regulation in the experimental study design

Uncovering the major regulators driving distinct transcriptional programs is a critical step towards understanding of brain function and dysfunction and can inform therapeutic strategies and drug discovery efforts. New analytical approaches and emerging technologies that allow researchers to profile epigenetic states of single cells, provide pivotal new information related to the intrinsic mechanism driving expression programs, and should be

considered in the study design.

The most mature technology for identifying regulatory DNA measures chromatin accessibility- a state in which genomic DNA is actively accessed by macromolecules. Chromatin accessibility thus reflects whether proteins, typically transcription factors (TFs) and high-density of nucleosomes, are bound at a particular genomic locus[89]. Chromatin accessibility often correlates with gene expression, but it is not a direct proxy for it. As the chromatin accessibility landscape is highly cell type-specific, it provides a robust epigenomic measurement to identify cell types and states. It can be measured by commercially available assays such as snATACseq. Analysis of chromatin accessibility has been mainly used for identifying putative gene regulatory elements (enhancers, promoters, silencers, insulators, etc.), though it cannot necessarily distinguish among classes of gene regulatory elements. It also retains signals related to nucleosome occupancy, though less tailored to infer nucleosome positioning as compared with techniques such as MNase-seq.

Moreover, available multiomic assays enable joint profiling of RNA abundance (snRNAseq) and chromatin accessibility (snATACseq) within a single nucleus, linking changes in gene regulatory element accessibility to changes in gene expression. One of the largest impacts of locating cell type- and context-specific gene regulatory elements is the identification of disease-associated noncoding variants predicted to impact gene regulation, allowing for fine-mapping of thousands of genetic risk loci. Further, chromatin accessibility-based mapping of quantitative trait loci (QTLs) provides more direct observation of the effect of a variant compared to the more indirect effects observed in expression QTL mapping due to linkage disequilibrium[90,91]. The variants predicted to be functional based on residence in a gene regulatory element or existence of a chromatin accessibility QTL can be further prioritized in validation experiments and linked to nearby genes via co-accessibility or 3D chromatin contacts. Of note, regulatory elements predicted by chromatin accessibility are putative and necessitate downstream functional validation. For information on approaches that can validate the functional role of a specific element please refer to Companion Piece 3.

Each cell has only two copies of each genomic locus (alleles) leading to unique challenges for single nucleus epigenomics in: (i) sparsity, (ii) scale, and (iii) cell-type specificity[92–94]. To partially address the challenges of data sparsity and specificity, snATAC-seq data is typically converted post-clustering to pseudo-bulk to increase reliability by summing the information over hundreds-to-thousands of cells, each with a few thousands of fragments captured. Unlike sc/snRNAseq which often fails to capture the lowest expressed genes, the dropout in snATACseq is likely stochastic across the genome[95]. Moreover, snATACseq has a larger feature space compared to sc/snRNAseq, as a typical multi-cell type dataset could have >1 million regulatory elements, in comparison to sc/snRNAseq which has <30,000 unique transcripts. Because of this, snATAC-seq often requires a higher sequencing depth per cell and the larger size of the cell x feature space presents unique challenges the analysis.

The epigenetic landscape of a cell is altered along differentiation, maturation, ages and disease states, thus it is interesting to compare chromatin accessibility and other epigenetic features between cases and controls or along biological processes (**Fig. 3**). Such comparisons from snATACseq data should be performed at the pseudobulk level[53] to reduce noise, while maintaining biological and cellular diversity by aggregating all cells from a single cell type from a given individual into a single pseudobulk profile. The use of pseudobulk profiles circumvents issues with sparsity by combining signal across many individual cells, and thus we recommend a minimum of 100 cells per profile based on current studies. Once pseudobulk objects are created, differential testing should be performed using one of many tools that have been carefully benchmarked previously[96], for example DESeq2[51] or edgeR[97]. As tools vary in the degrees of false positives and false negatives, the analyses should be tailored to the specific application and the tolerance for false positives versus false negatives, as discussed previously[96,98].

Additional technologies in the field of epigenomics are available, enabling the measurement of histone modifications, DNA methylation and chromatin contacts (as described in more details below). Integration of these methods will provide a more complete view of the intrinsic mechanisms underlying gene expression regulation within and across cells types.

Analytical approaches to infer gene regulatory networks

Gene Regulatory Networks (GRN), the interconnected set of molecular regulators and their targets, together orchestrate the biological programs responsible for specific gene functions and ultimately govern all cellular and

biological activities, by controlling the activation and deactivation of individual genes. GRN analysis has become an increasingly important tool in neuroscience research. The complexity of the nervous system, characterized by its diverse cell types and intricate interconnections, makes it especially suitable for the GRN analysis. GRN analysis has uncovered the pivotal role of GRNs in multiple brain processes, such as differentiation of neuron and glial cells.

Regulators of gene expression are not restricted to regulatory proteins, and include various non-coding RNAs such as long, short, and antisense ncRNAs. Regulators can act as activators or repressors via different regulatory elements (enhancer, promoter). Each stage of gene expression is regulated, including post-transcriptional regulation (splicing, translation, transport, degradation) and post-translational regulation (modifications, transport, localization, degradation). As the targets of each regulator include additional regulators, a network of interconnected regulators is formed, represented as a graph, where nodes capture regulators and their relationships are represented as directed and weighted edges.

As many of these regulatory modalities are not measured with single-cell resolution due to the lack of scalable technologies, GRN inference is mainly focused on TF regulation, inferred from available single-cell omics measurements[99,100]. The inference of TF regulation is highly challenging due to several reasons: (1) TFs can regulate expression of target genes at large genomic distances by binding to remote enhancer regions, often by folding to form close contacts proximal to their targets, limiting our ability to link a DNA element to its target gene; (2) We lack reliable species-specific mapping of TFs to the DNA sequence motifs they can bind; (3) Physical binding of a TF to a DNA element can lead to activation, repression, or no modulation, under different cellular contexts; (4) TF binding often depends on the chromatin state. Thus, beyond relying on sc/snRNAseq data to infer GRNs[101], the use of single-cell multiomic data, which includes both epigenomic (DNA contact, accessibility and methylation, or histone modification) and transcriptomic information, can enhance the accuracy and precision of identifying these regulatory relationships. This is achieved through integrative analysis that examines the correlations and colocalizations of open regions, methylation sites, or regulatory motifs with gene expression.

Multiple computational methods have been developed to infer and to analyze GRNs at single-cell resolution from sc/snRNAseq data, including Correlation-, Regression-, Information-theory-, Bayesian- Boolean- and Deep-learning- based methods. An overview of such methods is provided in **Table 1**. Of course, different methods may produce divergent results, posing challenges in determining the most accurate representation of the network.

Identifying differentially accessible regions (DARs) between biological conditions is fundamental for pinpointing differentially active regulatory elements and regulators. Many methods for differential expression have been repurposed for finding DARs (*e.g.,* DESeq2[51], edgeR[97], and limma voom[102]). Pairing DARs with differential expression analysis can enable the inference of context-associated GRNs using packages such as cisTopic[103], Signac[104], DORCs[105], FigR[106], SCENIC+[107], ArchR[108], MIRA[109], scBPGRN[110], and Symphony[111].

Additionally, CRISPR/Cas9-based screens, assessed at the single-cell level via Perturb-seq or Genome and Transcriptome sequencing (G&T-seq)[112], can also inform the inference of GRNs or experimentally validate them.

Computational models of GRNs are valuable for modeling complex data, generating hypotheses, and directing future research efforts. However, the field is still evolving, and several challenges lie ahead. First, efficient analysis of GRNs requires large scale high-quality data, often constrained by technical limitations and high cost, and the computational complexity. Next, biological variation and technical noise might hinder the distinction between true regulatory interactions and fluctuations in gene expression. Thus, there is a need for further development of specialized statistical methods with heightened sensitivity for accurate DAR identification in future research efforts. Furthermore, gene expression regulation encompasses multiple layers of molecular interactions, making it difficult to accurately model and analyze GRNs. Finally, gene expression is a dynamic process, but experimental measurements only provide a static snapshot, greatly complicating the temporal dynamic analysis of GRNs. As a consequence of these shortcomings, our understanding of GRNs in the brain and the available tools to analyze them are lacking, yet future research in this field, empowered by new technologies, improved methodologies, and the accumulation of large-scale datasets, are expected to yield important insights into brain function and dysfunction.

Emerging technologies for single-cell epigenomics

Various types of genetic and environmental perturbations can lead to changes in cell state driven by modulation of epigenetic states. To uncover underlying regulatory mechanisms of such perturbations, requires large scale single-cell epigenetic data. Emerging technologies now allow researchers to profile epigenetic states of single-cells, and in this section, we highlight emerging data modalities beyond DNA accessibility, discuss main technologies, and summarize their strengths and weaknesses (**Fig. 4**).

Histone modifications

Histones undergo multiple post-translational modifications involved in transcription regulation by affecting transcription factor binding and RNA polymerase activity. Different histone modifications patterns define specific genomic features, such as enhancers, promoters, or coding regions, and some can be associated with transcriptional states (e.g. activation, repression, elongation, poised[113]). Therefore, profiling histone modifications at single-cell resolution represents an emerging area of technological innovation that could facilitate the profiling of *cis*-regulatory elements (CREs) that are likely to be functional, as opposed to all accessible DNA loci.

Profiling histone modifications was traditionally done by immunoprecipitation followed by sequencing (ChIP-Seq) that required large amounts of input material, hampering single cell applications. Novel CUT&Run[114] and CUT&Tag[115] methods, related to the ChiC[116] method and based on a fusion of MNase/Tn5 transposase with protein A, have been recently developed and successfully applied to single-cells, allowing the probing of individual[115,117–121] or combination of histone modifications[122–125]. Methods to investigate chromatin dynamics during cell state transitions have been recently developed, taking advantage of multiomic single-cell chromatin accessibility and transcriptomic data[105,126], or single-cell histone modification data, such as chromatin velocity[122,124].

Chromatin Contact

Genome organization and DNA methylation represent additional layers of gene expression regulation[127,128]. Genome topology can modulate enhancer-promoter communication[129] and there is considerable evidence linking disease relevant structural variants or epigenetic changes to changes in 3D genome organization[130].

Genome organization has been traditionally studied using chromatin conformation capture assays such as Hi-C or micro-C in bulk or in FAC-sorted populations, yet recent advances in technologies have enabled profiling in single cells/nuclei[131–133]. Moreover, recent technologies allow co-profiling of chromatin architecture and transcriptome at a single cell level[134]. However, current methods require significant sequencing depth compared with ATAC-seq, and lack robust experimental and computational benchmarking and validations.

Methylation

The most common DNA base modification in mammalian species is cytosine 5-methylation (5mC) and its oxidated derivatives including 5'-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fc) and 5-carboxylcytosine (5caC)[135]. The mammalian CNS is associated with highly unique patterns of cytosine modifications including unusually high levels of non-CpG methylation (5mCH) and 5hmC[135–137]. For the majority of somatic tissues in mammalian species, 5mC is almost exclusively found in the CpG dinucleotide context (5mCG). However, in mature human cortical neurons, 5mCH could account for more than half of cytosine methylation with between 2%-8% of CH sites methylated depending on neuronal types[138].

The single-nucleus profiling of cytosine modifications reveals the epigenetic states at different genomic scales[137]. While the depletion of 5mCG and/or enrichment of 5hmCG at regulatory elements indicates a local epigenomically permissive state, the cell-type and developmental specificity of 5mCH can inform the epigenomic states of mega-scale regions such as topological associated domains, or intermediate -scale regions such as gene bodies. Existing single-cell methylome techniques provide sparse measurements at individual cell level typically covering 5% to 10% of the genome[138]. The aggregation of single-cell methylome profiles for identified cell types (pseudobulk) has been applied to reconstruct cell-type-specific profiles that are reminiscent of traditional bulk methylome profiles. The pseudobulk approach provides a reasonable approximation for steady-state cell populations in the adult brain but inevitably leads to the under-appreciation of ongoing cellular dynamics in developing brains. We anticipate that data imputation tools and trajectory inference algorithms that can effectively use sparse single-cell methylome profiles will expand the knowledge of methylation dynamics during brain development[139].

The development of single-cell profiling techniques for 5mC and 5hmC has revealed the cell-type specific

patterns of cytosine modifications in mammalian brains. The snmC-seq family assays were developed based upon earlier methods such as scBS-seq and provided significantly enhanced throughput allowing the generation of hundreds of thousands of single-cell methylome profiles[138,140,141]. SnmC-seq has also been extended to multiomic approaches including the joint profiling of chromatin conformation and DNA methylation by snm3C-seq and the simultaneous profiling of transcriptome, DNA methylation, chromatin accessibility by snmCAT-seq[142,143]. A method for single-cell profiling of 5hmC snhmC-seq was recently developed by integrating chemical protection of 5hmC by bisulfite conversion and selective deamination by APOBEC3A[144]. The absence of easy-to-use and commercially available assays has severely impeded the adoption of single-cell methylome methods. A recently developed combinatorial-indexing based approach sciMETv2 provides a feasible route towards commercialization[145]. Lastly, methods that can generate high-coverage methylome from a single cell, but likely from a smaller number of cells, could be useful for analyzing highly specific cell populations such as ones associated with a neural circuit in adult brains or daughter cells derived from asymmetric divisions during neural development.

Single molecule epigenomic assays

Finally, single-molecule epigenomic assays use high-throughput long-read sequencing technologies (e.g. Pacific Biosciences, or Oxford Nanopore) to make high-throughput single-molecule genomic measurements of chromatin accessibility[146–150], as well as single-molecule sequencing of intact RNA isoforms[151], at the resolution of single-cells[152]. Single-molecule chromatin accessibility profiling approaches allow one to 'deconvolute' the population averages provided by approaches like DNase-seq and ATAC-seq – that is, one can explicitly map the presence of nucleosomes, transcription factors, and their respective co-occupancy patterns on individual DNA molecules.

For instance, it is now possible to identify heterogeneity of nucleosome positioning and transcription factor binding[146,147], and has been informative in dissecting complex epigenome / regulatory pathways[153,154]. A key unmet challenge is currently the throughput of long-read sequencing and its cost, which are expected to be reduced in the coming years.

Data sharing, dissemination and visualization

Data sharing, dissemination, and effective visualization are crucial aspects of modern research, especially in the realm of single-cell data analysis, given the speed of the data generation, the need for large scale datasets for capturing the full complexity and diversity of cells in the brain, to ensure transparency and reproducibility.

One significant step towards enhancing transparency is the open sharing of code. Utilizing platforms like Jupyter notebooks allows researchers to share not only their results but also the entire analysis pipeline. This practice is particularly valuable for highly customized data analysis that extends beyond standard packages. It enables others to reproduce and validate complex analyses, fostering trust and collaboration within the scientific community. It is essential to follow best practices in documenting data analysis methods and parameters when using complex analysis packages. Ideally, there is a convergence to shared data formats and structures such as AnnData in single cell analysis, and first efforts for extensions via the Open Microscopy Environment (OME) Standard are recommendable[155]. Benchmarking algorithms is another vital aspect of advancing single-cell data analysis. By rigorously evaluating the performance of analysis methods, researchers can identify the most reliable and efficient tools for their specific research questions. This process contributes to the continuous improvement of analytical techniques and ensures the validity of scientific findings.

To further enhance the accessibility and usability of data, adhering to the principles of FAIR data (Findable, Accessible, Interoperable, and Reusable) is essential. Proper curation of patient and experimental metadata within datasets ensures that critical context accompanies the data, making it more valuable for researchers and promoting data reuse.

Data visualization plays a pivotal role in translating complex datasets into understandable insights. While shiny apps have initially been used extensively to allow exploration of single-cell data, various single-cell data portals, such as UCSC Cell Browser, Cell Annotation Platform and CELLxGENE, have made significant strides in democratizing data access and navigation. However, for these efforts to thrive and expand, there is a pressing need for broader community engagement and sustained financial support. Visualizing spatial genomic data, particularly datasets integrated with single-cell omics data, presents unique challenges. These datasets offer critical insights into the spatial organization of cells within tissues. To make this information more accessible to

the research community, concerted efforts are required to develop online resources that facilitate data exploration and visualization.

Overall, we see effective data sharing, documentation, visualization, and benchmarking as integral to the progress of single-cell data analysis. These practices promote transparency, reproducibility, and collaborations, and ultimately will lead to better understanding of complex biological systems.

Limitations of this perspective

Emerging genomic technologies have the potential to transform our understanding of nervous system development, structure, and function. This Review aims to discuss the challenges of designing studies that are rigorous, well-powered, and informative, and outlined several key applications including building atlases, uncovering disease processes, and predicting gene regulatory relationships. Clearly, applications of these technologies extend beyond those specific use cases, and space limitations required us to omit some details, particularly related to development and cross-species comparisons. These applications involve their own sets of opportunities and challenges that we regretfully could not cover in this article.

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Declaration of conflicts of interest

S.A.L. declares a financial interest in AstronauTx Ltd. and Synapticure. All other authors declare no competing

interests.

Main Figures

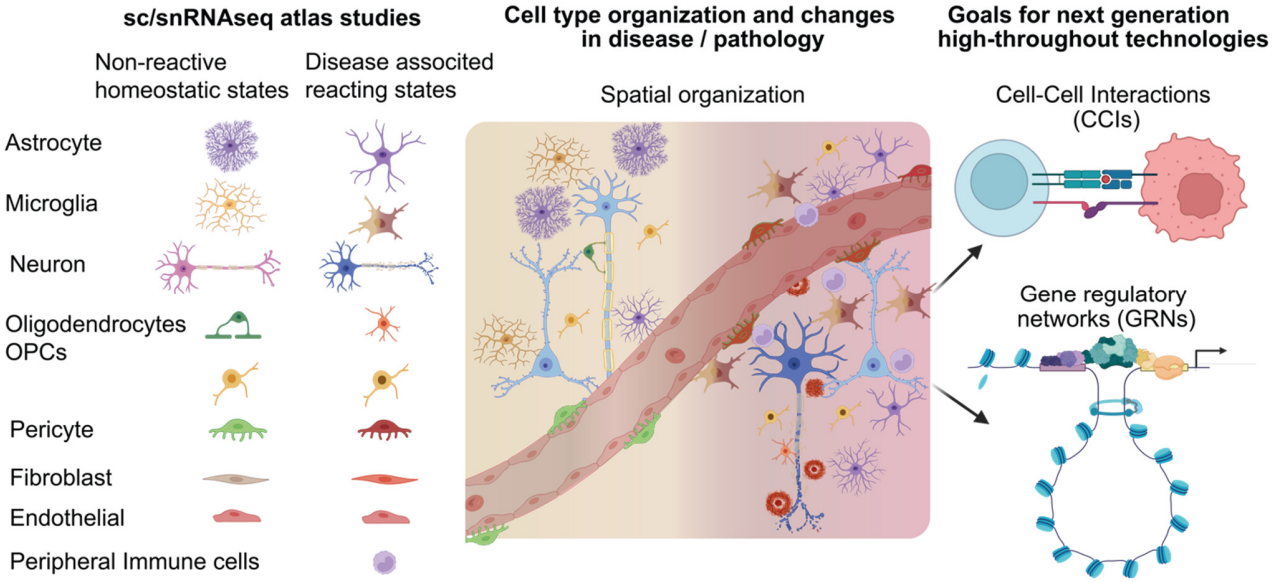


Figure 1. Biological considerations: cellular architecture and research questions. Emerging single cell atlas studies have created reference resources for defining cell types in normal and pathological brain tissue. Cell types and states defined by these studies exist in complex and dynamic communities *in vivo* and more disease associated states and types may emerge in the future. Advent of spatial transcriptomics technologies helps to define cellular neighborhoods and identify candidate networks of molecular interactions, while advanced single cell genomic technologies can provide insights into dynamic intracellular pathways underlying cellular transitions.

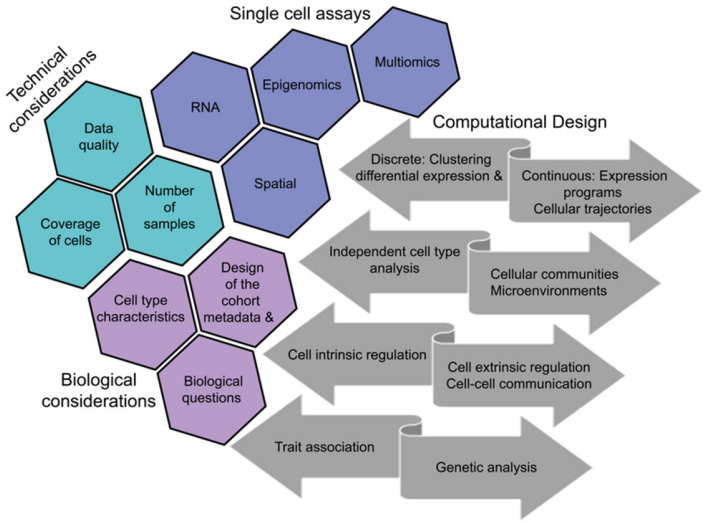


Figure 2. Outline of key considerations involved in designing high-throughput single-cell and spatial transcriptomics studies.

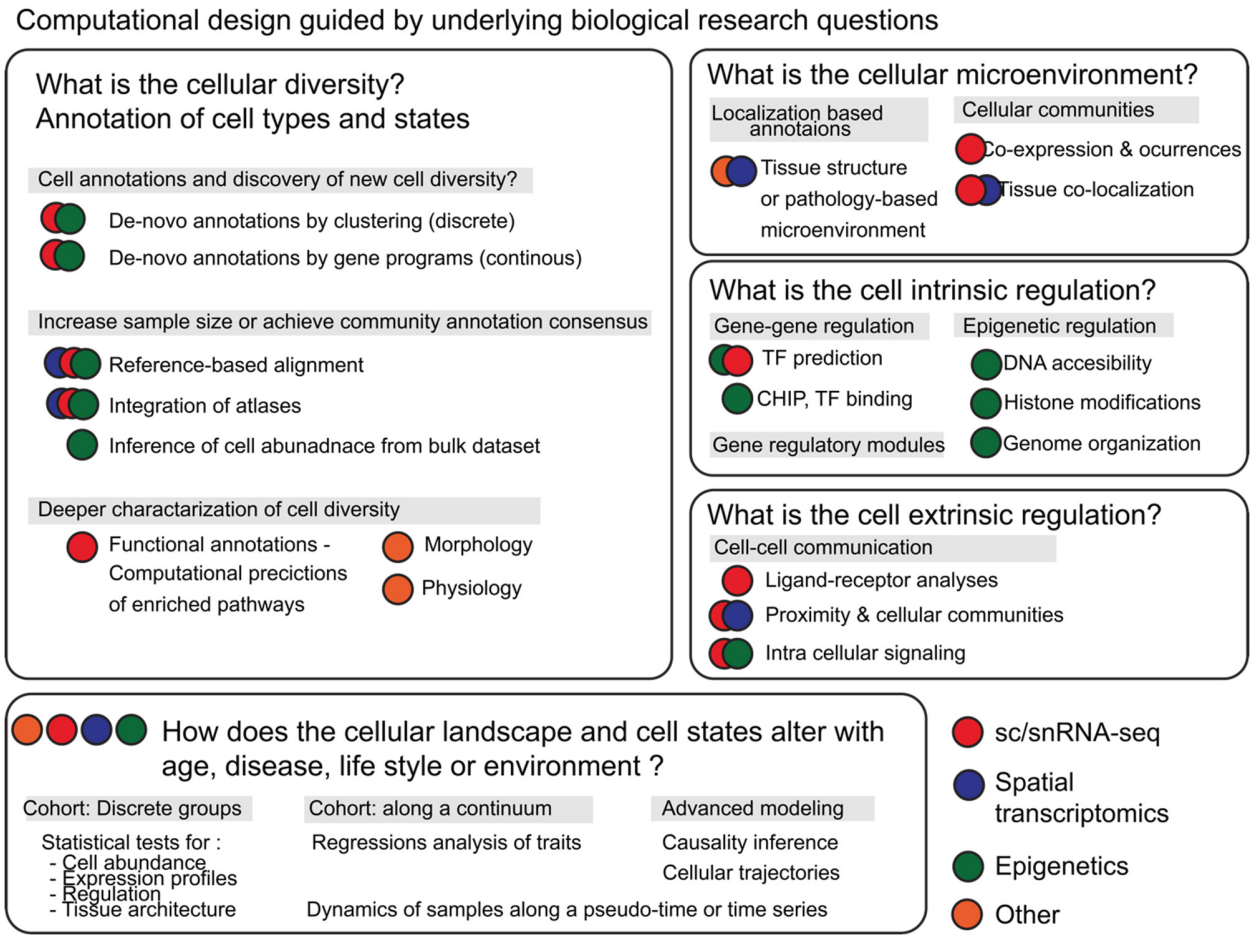


Figure 3. Outline of computational design of high-throughput single-cell/nucleus and spatial omics studies. TF - Transcription factor; CHIP - chromatin immunoprecipitation.

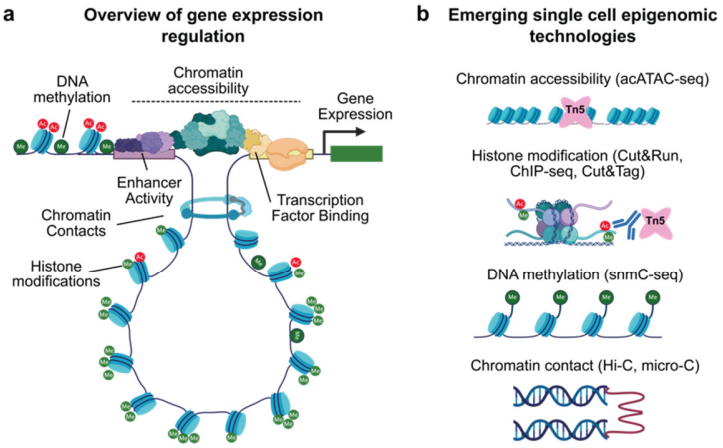


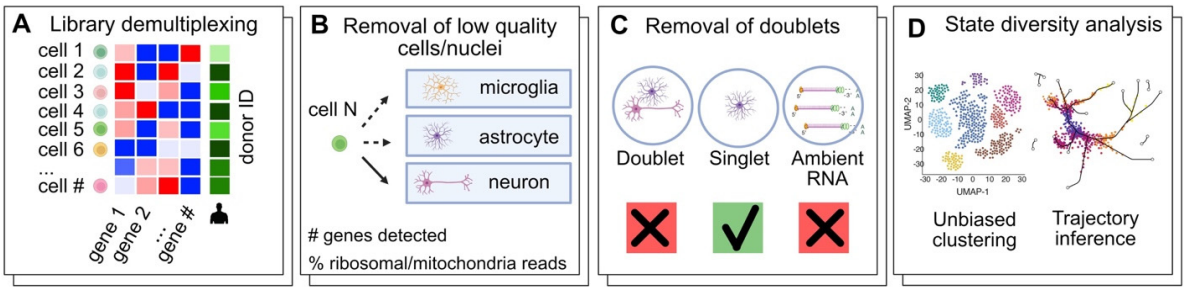
Figure 4. Epigenomic technologies. **a**) Overview of the major modalities regulating gene expression currently studied using high-throughput single cell technologies. **b**) Summary of key single-cell epigenomics technologies. Tn5 – hyperactive Tn5 transposase, Me – methyl group, Ac- acetyl group.

Tables and Boxes

Table 1. Common Gene Regulatory Network (GRN) Tools

	Method Description	Advantages	Pitfalls	Available tools
Correlation-based	These methods, are based on calculating the correlation coefficient (*e.g.* Pearson correlation or Spearman rank correlation) between pairs of genes across multiple samples	Simple and computationally efficient	Cannot capture complex regulatory relationships and cannot differentiate between direct and indirect interactions	SCENIC[33]/SCENIC+[107], GENIE3[156], PPCOR[157], LEAP[158]
Regression-based	These methods model the expression level of a gene as a function of the expression levels of other genes. Techniques include linear regression, LASSO, and ridge regression.	Can capture direct interactions	They may not handle non-linear relationships	GRNBoost2[159], SINGE[160]
Information theory-based	Use measures such as mutual information to infer relationships between genes	Can handle non-linear relationships and can differentiate direct from indirect interactions	May have difficulty with high-dimensional data	ARACNE[161], PIDC[162], SCRIBE[163], and CLR[164]
Bayesian network	Probabilistic graphical models that represent the dependencies among a set of variables	Can model complex relationships and differentiate direct from indirect interactions	May have difficulty with large networks as they are computationally intensive	GRNVBEM[165], BANJO[166] and BNFinder[167,168]
Boolean network	Model gene expression as on/off states and gene interactions as logical functions	Computationally efficient and can handle large networks	Oversimplify gene expression and cannot capture graded changes in expression levels	SCNS[169]
Traditional Deep learning	Interpret gene regulatory networks from sc/snRNAseq data and deduce causal relationships between genes	Can handle complex, non-linear relationships and high-dimensional data	Require large amounts of data and can be computationally intensive	CNNC (Convolutional Neural Networks for coexpression)[170], Foundation models[171]
Emerging Deep learning	Based on foundation models, *i.e.* deep learning models trained on vast amounts of data in an self-supervised fashion [172]. Emerging in single cell transcriptomics as well [171], based on transfer learning from reference atlas and expand beyond.	The attention mechanisms in the employed transformer architecture reflect the underlying GRN structure.	Only in early stages. Require large amounts of data and can be computationally intensive	A single cell transcriptomics transfer learning based GRN model[171]
Differential equations	Model gene interactions as a system of differential equations	Can model time-dependent changes in gene expression	Require time-series data and can be computationally intensive	SCODE[173], GRISLI[174]

Box1 Guidelines for data processing and quality controls in single-cell / nucleus RNA seq studies.



Box 1 Fig. *Quality control (QC) in sc/snRNAseq involves evaluating both cells and genes.*

QC is an essential step in the analysis of sc/snRNASeq. Nevertheless, while too permissive thresholds might lead to technical artefacts such as false positives in differential gene expression (DGE) analysis and misclassification of cell types, too stringent parameters can lead to false negatives and failure to detect relevant biology. Thus, it is crucial to implement robust QC workflows before further downstream analysis. For cell QC, begin by excluding cell barcodes likely representing dead cell debris or free-floating RNAs (ambient RNA), as they don't correspond to intact individual cells. A straightforward approach for assessing cell quality is calculating metrics such as the number of transcripts (unique molecular identifiers, UMIs), or detected genes. In practice, the number of UMIs and genes detected notably varies between brain cell types like microglia and neurons, and between datasets based on quality and sequencing depth. Hence, assigning a cell type- and dataset- specific threshold for filtering low-quality cells is important. This can be done by initial classification of cells to broad cell classes based on trained classifiers on existing datasets, and assessing the appropriate thresholds based on the distribution of number of detected genes within each cell class.

Another frequently used cell quality measure is the proportion of mitochondrial RNA, with high proportions possibly indicating damaged cells. Nonetheless, such high mitochondrial content cells should not be automatically excluded as they might signify metabolic changes such as increased mitochondrial activities or be informative in the context of neurodegenerative diseases. We recommend that mitochondrial content should be taken into account, but not used as the only exclusion criteria for low quality cells.

Conversely, cells with unexpectedly high counts may indicate doublets (or multiplets), where cell barcodes correspond to multiple cells. It is essential to remove doublets as they can constitute a significant portion of cell barcodes in high-throughput sc/snRNAseq methods. Yet we caution against frequently used filtration methods solely based on the number of detected transcripts, especially in complex tissues like the brain, as they are not accurate enough, while specialized algorithms that model doublet cells are much more robust (e.g. Scrublet[175], DoubletFinder[176], and scds[177]). Also, transitory cell states, which might present cell signatures of different cell populations and that are frequent in development and disease (companion paper I), might also be mistaken as doublets.

Ambient RNA transcripts, which are free-floating and barcoded with the cell/nuclei, can impact the cellular expression profile and potentially bias cell annotations and functional interpretations. Given the varying extent of ambient RNA, dependent on tissue quality and cell or nuclei isolation protocols, it is essential to evaluate each dataset individually. The ambient RNA can be corrected as necessary using methods like CellBender[178], SoupX[179] and DecounX[180]. We also recommend extracting signatures of ambient RNA directly from the data by compiling abundant transcripts in empty droplets, to assess the contamination within each dataset and to ensure that the corrected expression profiles remain undistorted.

Furthermore, QC can be applied at the gene level although recommended only if the computational resources are constrained or the noise in the dataset is high. One can filter out genes with limited expression (*i.e.* detected in a small number of cells), by choosing an appropriate filtration threshold considering the number of cells expected from the smallest cell population. In addition, methods to identify informative genes and focus the analysis on this gene subset can be applied (such as variance stabilizing transformation).

Finally, data normalizing and corrections for technical features such as batch and sample quality are critical to exclude technical artifacts from the downstream analysis, yet over-correction and normalization could result in the loss of the biological signal. Thus, carefully examination of the data after correction is necessary, and comparison to reference atlases can be used as a benchmark.

It is important to note the evolving guidelines, especially for newer data types like single-cell epigenomics and spatial transcriptomics methods that have their unique pre-processing and QC challenges that vary between platforms. As technologies mature, these challenges are expected to diminish. For example, in multiplexed in-situ methods, (e.g. Stomics, MERFISH, Xenium) the main challenge is image analysis, specifically cell segmentations and data to noise ratio. While, for in-situ sequencing-based methods, the main challenge lies in data resolution and coverage, which require more advanced computational techniques to deconvolute the expression signal to cell types and states, or find expression patterns based on communities of cells or based on the spatial gradient to the center of pathology.

Box 2. Technical considerations and current limitations of Special Transcriptomics methods

Sequencing-based spatial transcriptomics (ST) technologies utilize RNA-sequencing to enable unbiased profiling of whole transcriptomes in tissues. Various methods differ across their cell/transcript capture approach, spatial resolution, throughput (*i.e*. sample size, number) and sensitivity. For example, whereas some methods directly capture transcripts from tissues (e.g. Visium[181], Slide-Seq[35], others spatially barcode cells or nuclei (e.g. Slide-Tags[60]) or tissue areas (e.g. DBiT-seq[58], Nanostring CosMX/GeoMX[182]) prior to sequencing. The major benefit of sequencing-based ST is the discovery-based analysis of cellular transcriptomes *in situ*. This can be applied to healthy or diseased neural tissue samples with little prior information about tissue architecture and without target gene selection or differential gene analysis from prior sc/snRNAseq. Many methods are readily scalable as they require minimal specialized equipment (*e.g.* Visium) and rely on standard histological methods and commercially available kits and sequencing reagents. The major limitations of these methods are summarized below:

Spatial resolution: Most techniques do not offer true single cellular resolution as they profile multiple cells (*e.g.* Visium with 55 micron resolution) or transcripts from neighboring cells (*e.g.* Slide-Seq with 10 micron resolution) in tissues. Hence, to perform cell-specific analysis akin to single-cell transcriptomics, they require computational deconvolution of cell type-specific information. This is often based on cell-type specific gene expression signatures extracted from reference sc/snRNAseq studies[43,44,46]. Hence, it is important to choose a reference that matches the biological characteristics of the ST dataset such as brain region, cell type composition and disease states. For disease studies, paired single-cell/nucleus and spatial datasets might be necessary for accurate deconvolution. Recent developments such as VisiumHD and StereoSeq provide higher spatial resolution and could address this limitation, though computational pipelines that can segment these data to single cells are not well established. Some low level ST methods can have computationally enhanced resolution using Baysian statistical tools like BayesSpace[184].

Tissue quality and assay performance: Many sequencing-based ST methods are best applicable to fresh frozen tissue samples with high RNA integrity, which has been limiting for disease studies based on archival patient-derived samples. Yet, recent developments (*e.g.* Visium) extend sequencing-based ST to formalin-fixed and paraffin-embedded (FFPE) samples through targeted sequencing of probes. There is no strong consensus yet on key tissue quality control metrics: RNA integrity, histological stains and correlation of ST data with bulk or single-cell/nucleus RNA-seq are generally used in the field. Given the variations in human brain biopsy quality and autopsy protocols, standardization and benchmarks are needed to assess tissue quality and compare different technologies. While many of the sequencing-based ST methods aim to reach whole transcriptome and gene coverage, in many cases the number of features per pixel/area is limited to few thousand unique reads and/or genes, which might thus give limited insights on the cellular and molecular composition of the areas investigated.

Imaging-based ST technologies utilize imaging to enable targeted analysis of transcripts in tissues. To image transcripts at high resolution, most methods utilize probe-based detection approaches derived from single-molecule fluorescent in situ hybridization (smFISH) or custom sequencing chemistries. As with sequencing-based ST, there are many imaging-based methods and they provide different levels of target gene multiplexing, detection sensitivity and specificity. For example, RNAscope *in situ* hybridization (ISH)[184] can detect the expression of a few genes at high sensitivity, while high-multiplexed methods like MERFISH[36] and in situ sequencing (ISS)[186], and STARmap[186] use iterative cycles of labelling and combinatorial barcoding to simultaneously distinguish transcripts from hundreds to thousands of genes.

A major benefit of imaging-based ST is the high spatial resolution that can resolve single cells in tissues and even subcellular localization of targeted transcripts. Imaging-based methods are truly orthogonal to sequencing based sc/snRNAseq technologies for validation of transcripts of interest, such as novel cell type markers or differentially expressed genes in disease. Another benefit of imaging-based ST is direct 3D intact-tissue imaging of thick samples when combined with hydrogel-tissue clearing techniques[186]. The major limitations of these methods are summarized below:

Ease of use: High-multiplexed methods such as MERFISH and In Situ Sequencing (ISS) require specialized automated microscopy equipment and extensive image analysis (*e.g.* barcode decoding and cell segmentation) expertise[36,186]. Hence, their community uptake has been limited compared to more convenient methods like RNAscope ISH or sequencing-based Visium. Yet, several imaging-based ST methods were recently commercialized as end-to-end workflows automating data collection and low-level image analysis, such as the MERSCOPE system based on MERFISH[5,9,86], Xenium system based on ISS[188,189], and Plexa system based on STARmap[186]. These commercial solutions will likely play important roles in democratizing access to these technologies.
Method of choice: Different methods present different trade-offs. MERFISH provides high detection sensitivity that requires high resolution imaging, and in turn, long image acquisition times for large tissue samples[36]. In contrast, ISS detects fewer transcripts per cell, likely due to the enzymatic steps used for signal amplification, yet can be performed at low resolution in a more scalable manner[186]. STARmap has higher signal-to-noise ratio than smFISH and higher detection efficiency than ISS, but requires high-end confocal microscope for 3D imaging. Finally, imaging-based technologies provide different error correction and detection capabilities[36,186] which greatly influence the specificity of transcript assignment and false discovery rate.
Probe selection: The curation of the probe panel is a critical step. Ideally, probe selection is guided by a sc/snRNAseq dataset of that tissue or condition to avoid optical crowding by highly expressing genes (*i.e*. the labelling of numerous

transcripts in a given cell that obstructs optical identification of individual RNA spots) and include markers of diverse cell types or pathological cell states[36]. In the absence of prior sc/snRNaseq data, probe selection could involve trial and error. Furthermore, this process also needs to be tailored to the sensitivity of the given ST technology. While there are several computational tools to automate probe selection from sc/snRNA-seq references[189,190], there is no strong consensus on panel curation approaches and it is often done in a hybrid fashion involving both manual and automated curation.
Protocol optimization: While it is cumbersome, it is important to optimize ISH protocols (*e.g.* proteinase treatment, autofluorescence removal) on new tissue types and sample sources. Human brain tissue, given wide variability in tissue quality and high autofluorescence, could be challenging[192].

Box 3. Shortcomings of spatial transcriptomics approaches to inferring cell-cell interactions (CCIs).

Resolution of sequencing-based ST: For CCI analysis, it is important to consider that most sequencing based ST methods do not provide cellular resolution and profile multiple cells at each spot. Hence, these data are not equivalent to sc/snRNAseq data for interaction analysis and should be treated as such. Cell type deconvolution preceding CCI detection (*i.e.* where interactions are inferred from spatially co-located from sc/snRNAseq profiles) or focused analysis of receptor-ligand transcript spatial co-localization are more appropriate analysis avenues for these datasets[43,85].

Multiplexing levels of imaging-based ST: While these methods provide single cell resolution in situ, targeted probe panel selection often imposes limits on CCI analysis as often only selected receptors and ligands are profiled in these experiments. In addition, targeting of a limited number of genes means that the data should not be treated as equivalent to sc/snRNAseq so subsequent interaction analysis will also need to be treated as such. For example, CCI analyses that rely on the assumption that most genes are not interacting are best applicable to single cell full transcriptome datasets, where null distributions can be directly generated from the data. However, for imaging-based ST experiments, users may specifically select for receptors, ligands, hormones, and other proteins that are expected to be interacting.

Computational models: Most CCI analysis tools were originally developed for suspension sc/snRNAseq data and do not incorporate true spatial information. Whereas ST can be used to prioritize CCI analysis results from sc/snRNAseq by identifying spatially co-located cell type pairs[88], an active area of computational development is focused on inferring spatial effects of CCIs in ST such as the identification of neighbor dependent gene expression patterns[193]. Lastly, almost all models focus on discovering correlations of gene-expression. However, models which build in causality (*i.e.* GRNs) may allow for more accurate hypothesis.

Long-range cellular interactions: Short-range interactions can be robustly captured by ST but medium to long-range interactions, especially at axonal/dendritic processes of neurons or oligodendrocyte processes, complicates cell communication analysis in the nervous system. This challenge could be addressed by integration of ST with viral tracing methods.

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