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# Taming human brain organoids one cell at a time

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## Abstract

Human brain organoids are self-organizing three-dimensional structures that emerge from human pluripotent stem cells and mimic aspects of the cellular composition and functionality of the developing human brain. Despite their impressive self-organizing capacity, organoids lack the stereotypic structural anatomy of their *in vivo* counterpart, making conventional analysis techniques underpowered to assess cellular composition and gene network regulation in organoids. Advances in single cell transcriptomics have recently allowed characterization and improvement of organoid protocols, as they continue to evolve, by enabling identification of cell types and states along with their developmental origins. In this review, we summarize recent approaches, progress and challenges in resolving brain organoids complexity through single-cell transcriptomics. We then discuss emerging technologies that may complement single-cell RNA sequencing by providing additional readouts of cellular states to generate organ-level view of developmental processes. Altogether, these integrative technologies will allow monitoring of global gene regulation in thousands of individual cells and will offer unprecedented opportunity to investigate features of human brain development and disease, across multiple cellular modalities and with cell-type resolution.

### Keywords

human brain organoids; single-cell transcriptomics; single-cell multi-omics; integrative technologies; cell-states; cell-type

## 1. Introduction

Human brain development is an astonishing process. Starting in the third gestational week and within a relatively short timeframe, a homogenous population of neural progenitor cells differentiates into the billions of interconnected cells that make up the human brain. Diverse combinations of thousands of genes identify the many cell types of the human brain, arguably the most diverse and complex of all biological systems. How does this remarkable diversity of human brain cell types arise? What is the function of each type? How do different types interact, and how does the transcriptome of individual cell changes in disease state? Although much has been learned using model organisms, many of these fundamental questions are still unanswered given the limited availability of developing

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human tissue. Human pluripotent- stem cell-derived brain organoids have opened, for the first time a window into aspects of human brain development and disease that were not accessible before. However, this young field is challenged by difficulties in data analysis and interpretation that result from a lack of reproducible anatomy across individual organoids [1]. Classic analysis techniques, including immunohistochemistry and bulk sequencing, are low resolution and not effective for elucidation of cellular composition [2] and for localization and targeting of selected cell types in organoids. Recent advances in single cell-transcriptomics have revolutionize our ability to resolve cellular heterogeneity and to access cell-type specific gene expression in organoids. Here we summarize recent findings in the brain organoid field that have leveraged advances in single cell-genomics, and we discuss how emerging technologies that complement single-cell transcriptomics will provide additional information across cellular modalities to substantially advance our understanding of human brain development and disease.

## 2. Overview on single cell transcriptomics

Up until the last few years, bulk sequencing has been the primary approach to obtain a low-resolution, average molecular RNA profile of a given sample. However, the advent of single cell sequencing has revolutionized the study of the transcriptome by allowing researchers to analyze the composition of a sample at single cell resolution. This single cell approach has given researchers insight to the diverse cellular heterogeneity that exist with an unprecedented level of detail.

The process of obtaining a single cell transcriptomic profile starts by (1) isolating single-cell or single-nuclei from the sample into discrete compartments. The sequential steps following the isolation remain well conserved between methodologies: (2) RNA extraction, (3) reverse transcription, (4) amplification, (5) and finally, next-generation sequencing of library. Each cDNA molecule in the expansive library is barcoded with short sequences to give it a unique identity and to further identify the single cell it came from. Unfortunately, there is no gold standard method when it comes to the analysis of resulting single cell RNA sequencing (scRNA-seq) expression data. However, the success of these various analysis techniques is determined by their ability to classify the cell types present, to determine the developmental trajectory of each cell, and to infer the putative receptor-ligand regulatory networks involved. In this review, we provide an overview on how the use of this technology has shaped the study of brain organoids and cortical developmental.

## 3. ScRNA-seq pipeline to deconvolute brain organoids

In the past five years, there has been an immense increase in wet lab techniques used to capture and reverse transcribe the RNA of single cells, which has been thoroughly annotated in previous reviews [3–5]. Each of these techniques comes with their own advantages and disadvantages. These methodologies vary by transcript length coverage (full length, 3’-only, or 5’-only), use of unique molecular identifier (UMI), and strand specificity. Droplet-based methods (Drop-seq and Chromium 10X Genomics) and multi-well sorting methods (Smart-seq, Smart-seq2, BD-Resolve/Rhapsody) are platforms that have been used to characterize brain organoids (Figure 1). Various papers have been published testing an array of scRNA-

seq techniques to determine the pros and cons of each platform [6–10]. This thorough analysis gives insight into the potential applications of each of the various scRNA-seq techniques and what questions can be answered from them. With all of these different platforms, one must consider a few options: intensity of throughput, depth of sequencing (how many transcripts per cell), and the range of the captured transcriptome.

Once the single cell RNA transcript counts has been assembled into an expression matrix, the dataset is subject to normalization [11] and possibly batch correction [12] prior to decoding the cell types present based on the count matrix [13]. A commonly used method based on the variance is principal component analysis (PCA), which determines patterns of gene expression that vary between single cells. This information can also be subject to other forms of dimensionality reduction as a t-distributed stochastic neighbor embedding (t-SNE) or uniform manifold approximation and projection (UMAP) [14]. Although the way to identify the biologically proper number of different cell subtypes from these graphs is somewhat arbitrary, the use of other algorithms such as K-means [15] to determine the “nearest neighbor” allows for an approximation for the number of cell types available in the sample of interest. Further details about clustering techniques are found in a recent review by Petegrosso et al. [16]

Cells are captured within a snapshot of their developmental trajectory when they are dissociated for scRNA-seq. Within an organoid, the various cell states, from a progenitor to a fully differentiated cell are found within this single time frame. Pseudotime is a way of inferring the actual temporal differentiation trajectory by ordering the cells in a “pseudo”time that tries to capture the progression of transcriptional changes for one cell to the next. The various lineage trajectory platforms can be categorized into seven trajectory types: cycle, linear, bifurcation, multifurcation, tree, connected graph, and disconnected graph [16]. A thorough analysis on the various platforms and their categorization has been examined in Saelens et al. [17]. One of the “tree type” platforms, Monocle2 [18] has been most commonly used to study brain organoids. Specifically, it was used to study the developmental trajectory of all cell types in dorsal forebrain [19], oligodendrocytic [20], and astrocytic [21] organoids. The previous version, Monocle1 [22] was incorporated into Camp et al to study the lineage trajectory from apical progenitors all the way to the final resting place of the cortical plate [23]. Another lineage trajectory techniques named RNA velocity, looks into the directionality of the lineage progression by observing the levels of certain spliced isoforms of a transcript. [24]. In addition to these platforms, probabilistic models including Diffusion Pseudotime (DPT) [25] and Waddington-OT [26] could also potentially be used to study organoid cell type trajectories based on the probability of a certain cell’s fate. In addition to the cell type identification, uncovering the lineage trajectory gives further insight into human brain development through the use of these techniques on brain organoids. The use of single cell transcriptomics to characterize brain organoids has only been conducted in a handful of papers. In the following sections, we highlight these papers, discuss emerging technologies and determine how these techniques can move the brain organoid field forward.

## 4. Progress in understanding brain organoids through scRNA-seq

The progress made in characterizing human brain organoids through the use of scRNA-seq has been exponential over the last five years. Camp et al. pioneered the use of scRNA-seq technology to characterize brain organoids [23]. This work used the single cell RNA-sequencing platform called SMARTer [27] to compare individual cells generated in cortical organoids ranging from 33–65 days old with cortical cells derived from 12–13-week-old human fetal tissue. The organoids were developed using the whole brain organoid protocol established by Madeline Lancaster in the Knoblich laboratory [28] while a reference transcriptomic atlas was built using cell composition and lineage trajectory of human fetal cortical cells at time points comparable to the organoid developmental stage. Due to the low throughput method, only 333 cells were captured by this technique, but nonetheless the authors were able to provide the first evidence that brain organoids recapitulate gene expression program of human fetal cortex development.

Quadrato et al. carried on the first systematic study of the cellular composition of whole brain organoids after extended time in culture. Using high-throughput droplet based microfluidics technique known as Drop-seq [29], they analyzed more than 80,000 individual cells, from 31 individual organoids, providing the first molecular map of the diversity and reproducibility of cell types generated in brain organoids. They discovered that organoids can make a large diversity of cell classes from distinct regions of the brain and from the retina, and that that the cellular composition of organoids diversifies over time in culture and displays progressive levels of maturity. This was reflected in the acquisition of structural traits characteristic of mature neurons, including dendritic spine-like structures, which have been notoriously difficult to generate by directed differentiation in culture. In agreement with an advanced state of maturation, they showed that whole brain organoids progressively generate spontaneously-active neuronal networks and that during the same period of time, photoreceptor-like cells mature substantially and become responsive to non-invasive, light-based sensory stimulation that appears capable of affecting neuronal activity.

By shifting focus from whole brain organoids, Birey et al [30] and Xiang et al 2017 [31] generated assembloids by fusing patterned dorsal and ventral forebrain organoids derived from human pluripotent stem cells (hPSCs). The aim was to better recapitulate early development and migration of interneurons from ventral forebrain to dorsal forebrain as seen in vivo. Birey et al used the BD-Resolve platform [32], a non-droplet based microwell system, while Xiang et al 2017 used the 10X Genomics system [33] to obtain cells from these dorsal and ventral organoids. These cells, through downstream analysis techniques, were identified as having proper transcriptional profiles of early developing dorsal and ventral forebrain. In addition to dorsal and ventral forebrain organoids, telencephalic organoids, which contain both excitatory and inhibitory neurons, have been characterized in recent studies using the 10X Genomics system as well [34,35].

One key question still remained unanswered in the brain organoid field. Can these organoids be reproducibly made? This point was addressed by Yoon et al. that showed a reliable and consistent generation of cortical spheroids collected at 4 different stages of differentiation across 6 hiPSC lines over 100 days in cultures. Velasco et al. brought this characterization to

the next level by analyzing 166,242 cells with the 10X Genomics system from 21 patterned dorsal forebrain organoids generated accordingly to a modified version of the Kadoshima protocol published by the Sasai lab in 2013 [36]. They found that organoids generated across different batches and cell lines, reproducibly generate a large variety of progenitor and postmitotic cell types of the human cerebral cortex. Through pseudotime analysis, cells in organoids follow a precise developmental trajectory that is consistent between organoids and comparable to the endogenous cerebral cortex.

In addition to cortical neurons, glial cells, a late born cell type of the developing brain, grown in 3D neural spheroids were thoroughly analyzed in Sloan et al. and Marton et al. using large scale single cell transcriptomics. Specifically, Sloan et al. analyzed the development and maturation of astrocytes cultured for up to 590 day and demonstrated functional changes and overtime through synaptosome phagocytic assays and calcium imaging on co-cultured neurons purified from human cortical spheroids [21]. Following comparison with human primary samples, single cell analysis of cortical spheroid-derived astrocytes with SMART-seq2 [37] revealed a shift in their transcriptional profile from fetal to postnatal-like. The publication by Marton et al. focused on the differentiation and maturation of another glial subpopulation, the oligodendrocytes, cultured in 3D neural cultures [20]. Using SMART-seq2, the authors captured 295 cells derived from neural spheroid containing oligodendrocytes and showed similarity in both molecular identity and timing of developmental progression to human primary oligodendrocytes. Over time, oligodendrocytes in culture successfully myelinated neuronal axons [20]

Despite the vast amount of information that one can obtain on the identity of individual cells from a comprehensive RNA profile, epigenetic heterogeneity plays a fundamental role in establishing the diversity of cell states found in transcriptionally identical cells. Amiri et al. conducted the first epigenetic characterization of organoids by comparing the transcriptome and the epigenome of cortical organoids at 0, 11, and 30 days in vitro to previously published data as well as to human primary fetal tissue ranging from 15 and 17 PCW [38]. Although this pioneering work on investigating brain organoids with assay for transposase-accessible chromatin sequencing (ATAC-seq) was not at a single cell resolution, it identified autism gene modules by investigating patient derived organoids. Recently, Kanton et al. conducted the first foundational tour de force to characterize putative regulatory mechanisms over the human and chimpanzee organoid differentiation trajectory using bulk and single cell ATAC-seq (sc-ATACseq). In addition to discovering that human development occurs at a slower pace relative to chimpanzee and macaque, the authors revealed human specific chromatin accessibility dynamics during cortex development and evolution [39]

This seminal work builds on an elegant study by Pollen and Bhaduri et al., that for the first time employed scRNAseq to establish brain organoids as models of human-specific brain evolution. Specifically, the authors identified 261 differentially expressed genes in human cortical development compared to non-human primates using brain organoids [40]. Of those differentially expressed genes, key players of the PI3K-AKT-mTOR signaling were upregulated in human outer radial glial cells compared to other non-human primates.

Although the main focus of the brain organoid field has been on thoroughly characterizing forebrain organoids, Xiang et al. [41] recently took on the challenge to generate and characterize a new protocol to generate organoids with a thalamic regional identity. With the 10X Genomics scRNA-seq platform, they showed generation of distinct thalamic lineages, however, these and organoids of other brain regions including, hippocampus [42], midbrain [43,44], hypothalamus [43] and cerebellum [45] still necessitate a thorough analysis of their cell type composition and lineage trajectory.

## 5. Disease modeling using brain organoids coupled with single cell transcriptomics

To date, a number of studies have modeled neurodevelopmental disorders including microcephaly, autism, schizophrenia and zika virus infection, as thoroughly addressed in recent reviews [46–48]. However, large scale single-cell multiomics approach combined with brain organoid models could revolutionize our understanding of neurodevelopmental disease by revealing cell-state specific changes along progenitor to neuron lineages. To our knowledge, only two papers have applied scRNA-seq to characterize disease phenotypes in brain organoids to date.

Impairments of molecular mechanisms involved in human corticogenesis leads to a broad range of gross morphological defects ranging from microcephaly or lissencephaly to subcortical or periventricular heterotopia (PE) [49–53]. Recently, Klaus et al. [54] showed that mutations in the protocadherins DCSH1 and FAT4 result in cortical heterotopia phenotype associated with PH. Through scRNA-seq and pseudotime analysis they identified a subset of neurons with defective neuronal migration pattern that was not generated in the control organoids, likely due to impaired morphology of progenitor cells. This subset of neurons had a unique transcriptional profile defined for its upregulation of axon guidance and migration genes and downregulation of synapse formation genes, suggesting a causal link between mutations in the DCHS1 and FAT4 genes and dysregulation of the proteins essential for cell guidance and cell-cell communication.

Lopez-Tobon et al. [55] studied the role of GSK3 pathway in human cortical organoid development. Disruption of this signaling pathway is of great interest as it has been associated to multiple neurodevelopmental disorders including fragile-X, autism, bipolar syndrome and schizophrenia. Despite this, few advances have been made in untangling the impact of GSK3 activity during human brain development. In this study, Lopez-Tobon et al. found that chronic inhibition of the GSK3 pathway leads to morphological alteration of cortical organoids. Through scRNA-seq they identified a cell-type specific role of GSK3 signaling in human corticogenesis. Specifically, followed GSK3 inhibition they found impairment of NEUROD1/2 lineages and overall decrease in outer radial glia (oRG) production. To our knowledge this is the first paper that attempts mechanistic dissection human corticogenesis in brain organoids, which demonstrates the potential of this technology to uncover cell type specific disease phenotype in brain organoids.

## 6. Limitations of single cell RNA transcriptomics

Despite the ability to probe cellular heterogeneity at a single cell resolution, some considerations need to be made into the limitations of this method prior to investing time, energy, and finances into its utilization. Some technical limitations of single cell RNA sequencing are low capture efficiency, high dropout, low depth, and dissociation bias. In addition to these technical issues, a lack of spatial context, proteomic, and splicing isoform variation information inherently limit the types of questions that single cell RNA transcriptomics can answer.

One of the main technical limitation is the high dropout rate, a feature of scRNA-seq experiments whereby only a small portion of an entire transcriptome from a single cell is captured [4]. Some reasons for a high dropout rate includes the inability to properly capture the mRNA from the cell (only 10–20% of transcripts are reverse transcribed) [58], the low levels of the transcript, as well as the highly stochastic nature of mRNA expression within each cell [59]. A high number of dropouts can skew the data set by artificially inflating the number of zero expression genes. This could be a major issue when opting for low depth sequencing to decrease cost and increase practicality of the experiment. The low depth, in combination with the high dropout rate could prevent the identification of rare cell types and detection of subtle changes in mRNA levels.

Another limitation that may skew the data is the dissociation bias. This comes about from samples undergoing an enzymatic isolation that results in a selection against certain cell types due to the higher stress experienced by these cells due to the digestion conditions. The selection of more resilient population of cells could give a skewed perspective on the actual organoid composition. Finally, it is important to recognized that single cell disassociation leads to the loss of spatial context within the organoid, and it is this important piece of information showing direct cell-to-cell interactions within the organoid that could reveal how communication between key partners regulate various processes including maturation, pruning, and migration. Currently, putative cell-cell, and receptor-ligand interactions based on around scRNA data is inferred information through computational modeling.

In addition to the inherent limitation to directly probe cell-cell and receptor-ligand interactions, scRNA seq does not offer any information about the proteome within these single cells. Due to the lack of high throughput proteomics conducted on human brain organoids, it is difficult to determine whether the levels of a certain receptor or ligand are at comparable levels based only in the RNA presence. Previous work has shown that the levels of mRNA do not necessarily linearly correlate to protein levels [60]. The variable relationship between mRNA and protein levels forces only inferences to be made about the complex interactions at the protein level.

Finally, current techniques are mostly aimed toward polyT priming, which initiates the coverage of a transcript from the 3’ end in order to avoid capturing ribosomal RNA. This method excludes certain isoforms and thus results in a biased view of the transcriptome. However, efforts have been made to address this particular issue by modifying platforms to capture various isoforms of RNA, which indicates that the field is heading in the right

direction [61](cite Hagemann-Jensen et al). As the field develops, it is clear that these limitations must be addressed in order to take advantage of the full potential of scRNA-seq.

## 7. Multi-omics approach to complement brain organoid characterization

Although attempts are being made to amend some of the previously mentioned limitations, alternative methods are also being devised that can be used in conjunction with scRNA-seq to get a clearer picture of what a human brain organoid is made up of and how it functions. The next step forward in characterizing brain organoids would be to understand the epigenetic modifications that regulate key transcriptional programs. Another step would be to probe alternative RNA sequencing techniques that incorporate other aspects such as spatial, functional, or lineage information. Finally, incorporating single cell proteomics, and ligand-receptor interaction via the use of synthetically engineering receptors/ligands will be essential to answering our question concerning cellular functionality and connectivity within organoids (Figure 2).

### 7.1 Probing the Epigenetic Landscape

DNA methylation and histone modifications are two mechanisms that contribute to a complex epigenetic landscape. The combinatorial activity between these modifications and transcription factors control DNA accessibility and ultimately govern the plethora of states that a near homogenous ball of pluripotent stem cells can achieve. It is thus essential to characterize how epigenetic regulation contributes to generating the highly diverse cell types found in human brain organoids. Amiri et al used transposase-accessible chromatin sequencing (ATAC-seq) to look into the epigenetic landscape governing chromatin accessibility of their brain organoids. However, more recently a method known as single-cell transposase-accessible chromatin sequencing (scATAC-seq) aims to address this broad stroke approach offered by ATAC-seq by peering into the “regulome” at a finer resolution [62,63]. Kanton et al. used scATAC-seq to identified human specific differences in chromatin accessibility by comparing human vs chimpanzee derived brain organoids [39]. Through measuring the genome-wide chromatin accessibility, the variability between cell-type specific epigenomes could be determined and thus aid in identification of cell types. The field is also moving toward integrating a spatial component to single cell ATAC seq (sciMAP-ATAC) [63,64], as well as combining the ATAC-seq platform with single cell RNA-seq to obtain the transcriptome and the DNA chromatin accessibility by sequencing (SNARE-seq) [65].

Other methods for studying DNA methylation signatures have also come forward. MethylC-seq [66], a methylome characterization technique, has been used to identify the high degree of similarity between the epigenetic signature of human fetal brain compared to cerebral organoids developed using the Lancaster protocol [67]. As interest in this field grows, newer, emerging techniques, such as snmC-seq2 [68] and sci-MET [63] will be used to study the methylome of single cells within brain organoids.

In addition to the methylome, histone modifications can be interrogated through single cell chromatin immunoprecipitation sequencing (scChIP-seq) [69] or single cell chromatin immunocleavage sequencing (scChIC-seq) [70], which offer the potential to study cellular

subpopulations at a single cell resolution. In the study conducted by Rotem et al, the use of droplet based technology combined with CHIP allowed for the identification of cell types in a mixed population of mouse embryonic stem cells (ESC), embryonic fibroblasts (MEF), and hematopoetic progenitor cells (EML) based on of known epigenetic landscapes [71]. By bringing these technologies to the organoid field, cell type identification and characterization at the single cell level could be observed from an epigenetic standpoint.

### 7.2 Combining RNAseq with Functional, Spatial, and Lineage Information

Classical functional analysis techniques have involved measuring the electrical activity of living neurons by recording the flux of intra- and extracellular ions. Patch Clamp recordings are a type of intracellular functional assays done to record the firing patterns of neurons. Given the vast amount of information that can be obtained from this functional analysis in term of characterizing different cells based on their firing patterns, integrating this technique with current methods of characterization cell types based on scRNA-seq would be a great step forward for the field. Patch-seq addresses this point by combining the elegant recording techniques of Patch Clamp with scRNA-seqto capture the transcriptomic information from within a patched cell [72–74].

One major drawback to scRNAseq, which include the techniques listed above, is the loss of spatial information. The ability of combining the data between the spatial orientation or morphology of a specific cell with that same cell and its transcriptomic profile is not a trivial task. Modified versions of fluorescence in situ hybridization (FISH) such as smFISH [59], seq-FISH [75], and osmFISH [76] have the potential to overcome this issue by capturing multiple transcripts within a cell without losing its spatial context. As technology developed, the ability to capture more RNA with a lower error rate and lower off-targets emerged as was demonstrated with MERFISH [77–80]. Next, STARmap [48] came along, and afterward to improve the ability of MERFISH, which only captures long RNA species. Further development has led to improvements to previously published platforms such as seqFISH+ [81], which addresses the limitation of the previous method to capture a lower number of genes within a cell. One of the most recent studies, Slide-seq [82], aimed to provide single cell spatial transcriptomics information by placing a sample on a slide covered in DNA barcoded beads. As newer platforms of higher resolution, lower cost, and increased specificity come about, the ability to clarify the spatial orientation of cell types within brain organoids will become more commonplace.

In conjunction with spatial transcriptomics, lineage tracing can be done with a technique called memory by engineered mutagenesis with optical in situ readout (MEMOIR). This is a system that stably integrates 28 barcoded scratchpads into a target genome that is susceptible to editing by the expression of an inducible sgRNA and a degradable Cas9 protein. This unique system is coupled with the spatial transcriptomic readout obtained with seqFISH, which is based on sequential in situ hybridization using a scratchpad oligo probe set, a barcode-specific oligo probe set and the corresponding set of transcript-specific probes [81,83,84]. Other recent lineage tracing techniques that make use of Cas9 “scars” include Lineage tracing by nuclease-activated editing of ubiquitous sequences (Linnaeus) [85], ScarTrace [86], and Single-cell genome editing of synthetic target arrays for lineage tracing

(scGESTALT) [87]. These techniques offer the ability to directly track the fate of a specific cells and overcomes the post hoc strategy offered by pseudotemporal analysis, which limits the identification of the causal factors that drive a cell down to a specific trajectory [22]. In Linnaeus, a red fluorescent protein (RFP) transgene is integrated into 16–32 different loci to ensure that Cas9 “scar” created on this transgene by injecting the Cas9 protein and targeting sgRNA is not deleted or overwritten. This “scar” is detected once the sample undergoes two rounds of amplification during sc-RNAseq with primers specific to the RFP transgene. The ScarTrace method uses 8 copies of the green fluorescent protein (GFP) repeated in tandem within the construct are scarred upon the injection of the Cas9 protein and sgRNA targeting the GFP. This method tries to overcome the ability of a scar to be overwritten or deleted by sorting by robot-assisted transcriptome sequencing (SORT-seq) into a 384 well-plate and has the ability to detect both RNA and DNA scars. ScGESTALT, like Linnaeus, has the ability to make use of scRNA-seq and can edit at two time points. While the previous methods allow for scaring with one initial injection, this technique allows for tracking heritable changes and progression at later stages with an inducible second scarring.

Non-Cas9 barcoding techniques such as transposon-based barcoding, (TracerSeq) [88], or a viral delivery approach, Cell Tagging [89], offer an alternative to Cas9 “scars”. TracerSeq integrates GFP fused to a scratchpad of 20 nucleotides into the genome and generates higher combinatorial diversity of barcodes compared to Cas9 methods. Cell Tagging transduces viral vector containing a specific library of an 8 nucleotide random sequence in the 3’ UTR of GFP and delivered at targeted developmental time points. The amalgamation of scRNA-seq into these two techniques results in the emergence of a map of a cell’s divergent or convergent paths toward a committed cellular fate.

### 7.3 Investigating Cell-Type Specific Translatome and Proteome

The critical step of translation from mRNA to protein offers the potential to explore cellular diversity by capturing ribosomes that are in the process of generating the proteome of a cell through a technique called Riboseq or active mRNA translation sequencing (ARTseq). [90,91]. There are papers that have been published that make use of this technology in order to better understand the translational changes found in fragile X syndrome model mice [92,93]. Taking this one step further, Furlanis et al. was able to combine this platform of Riboseq with ribosomes conditionally tagged in known cell types (RiboTRAP), which gives a clearer picture of the translatome of specific cell populations [94]. Unfortunately, this technology does not exist at the single cell level yet. One l future avenue is to combine it with a labeling technique that would allow for the identification and FACS sorting of cell types [95,96]. This would then lead to the ability to uncover the cell type specific “translatome”.

Given the discrepancy found between mRNA and protein levels, proteomics takes Riboseq one-step further by characterizing cells by their protein composition. CITE-seq is a step in the right direction by integrating transcriptomic and cell surface protein measurements into a single single cell output [97]. Other antibody linked oligonucleotide techniques such as REAP-seq [98] and proximity extension assay (PEA) [99–101] also make use of this multiplexing technique by combining the proteomic and transcriptomic profiles to get a

better picture. FACS sorting for cell surface proteins is also proving to be a useful tool with a recent study sorting 7000 proteins across 12 immune cell populations and elucidating more cell type specific proteins in the process [102].

## 8. Conclusions and future outlook

Despite the excitement and increased popularity of brain organoid as a model system, their complex structure, heterogenous cell types, and lack of reproducible anatomical organization, poses an incredible challenge to scientists wishing to interrogate developmental process and disease phenotypes. In recent years, advances in single cell-genomics have given us the means to partially tame this complexity, and we anticipate that in the coming years integration of single-cell multi-omics techniques will became commonplace. For example, a much-needed characterization of the wiring diagram of monosynaptic connections in the organoids could be achieved by combining pseudotyped rabies virus tracing with large-scale spatial transcriptomics.

Recently, approaches to characterize cell-cell communication mediated by ligand-receptor interactions across individual cell-types, using single cell RNA sequencing, have been developed [103]. However, these methods, due to cell dissociation, do not preserve the spatial and morphological information present in the sample. We envision that human brain organoids, in combination with spatial transcriptomics and other rapidly expanding technologies, including tools to engineering human pluripotent stem cells with synthetic receptors and ligands in which communication between cells can be easily monitored, [104] and 4D live-cell imaging techniques [105,106] will, in the near future enable the study of dynamic interactions between individual cell types as well as uncover dysfunctional communication signals in disease states with single-cell resolution.

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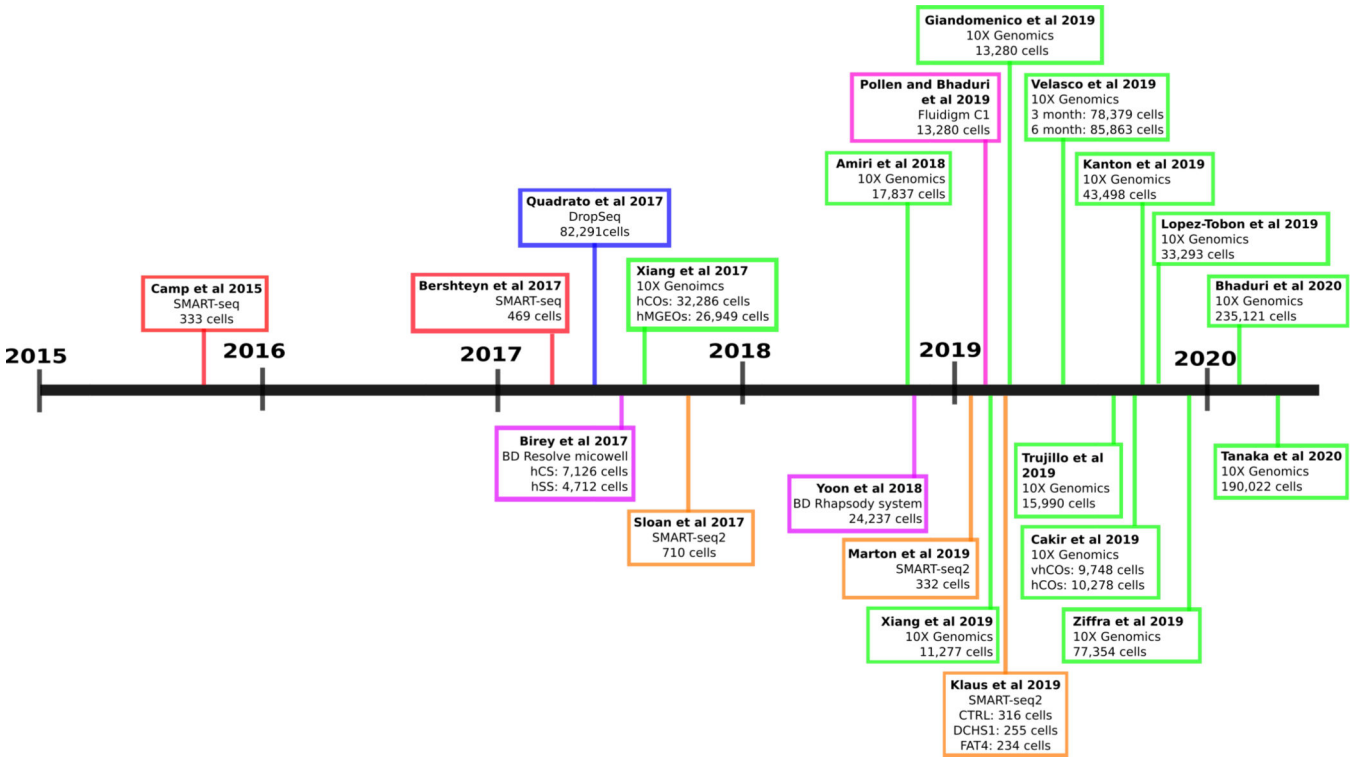


**Fig. 1. Timeline of all papers that have used scRNA-seq to characterize hPSC derived brain organoids.**

Each colored bubble contains author, year of publication, single cell platform used, and number of cells characterized after passing quality control. SMART-seq (red), DropSeq (blue), BD Resolve/Rhapsody microwell (purple), SMART-seq2 (orange), Chromium by 10X Genomics (green), Fluidigm C1 (pink). Human Cortical Spheres (hCS); human Striatal Spheres (hSS); human Cortical Organoids (hCOs); human Medial Ganglionic Emience Organoids (hMGEOs); vascularized human Cortical Organoids (vhCOs).

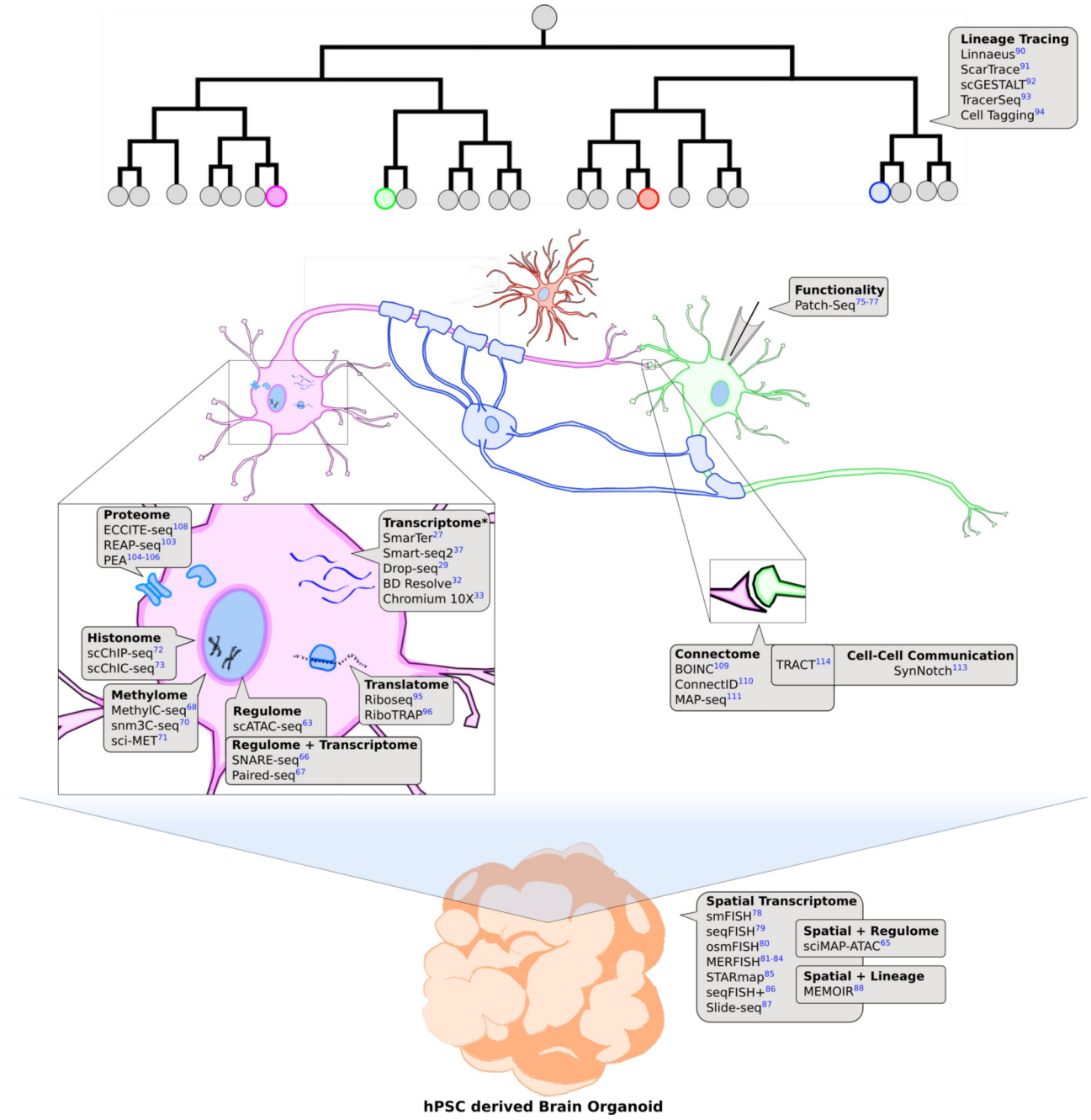


**Fig. 2. Multimodal approach to characterize hPSC derived brain organoids.**

An overview on the current methods that are available for characterizing brain organoids at a single cell resolution. Cell types include astrocyte (red), oligodendrocyte (blue), neuron A (purple), neuron B (green). *the platforms listed under “Transcriptome” only include ones used to study hPSC derived brain organoids