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No brain no gain: single-cell RNA sequencing in the study of brain tissue

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LAYMAN'S SUMMARY:

The genetic information present inside a mammal eukaryotic cell is transcribed from double-strand DNA molecules into single-strand RNA sequences, which can already have a specific function in the cell or be further translated into functional proteins. The expression of genes into RNA format can be quantified and individually analyzed for each single cell inside a tissue sample. This molecular biology technique is called single-cell RNA sequencing (scRNA-seq), and it has gained popularity during the last decade thanks to the evolution of the technology and its multiple applications to gain insight into the characteristics of different tissues and organs in the cellular and molecular levels. The simultaneous analysis of the unique gene expression profiles of thousands of cells present in a tissue sample by scRNA-seq allows deep characterization of the cellular composition in the tissue by cell type and also cellular state. Combined with tissue acquisition at different time points, scRNA-seq becomes a powerful technique to study organ development. Developmental biology of mammal brains, and specifically the human brain, has advanced with scRNA-seq technology. Several scientific articles have already been published and reviews have been made compiling the main findings of scRNA-seq analyses of human and other animal brain samples. However, brain tissue availability is limited and the study of the human brain is currently supported by different biosynthetic models made up of cells aggregated in the lab that mimic brain tissue architecture and other functional characteristics. ScRNA-seq has also been performed in these so called *in vitro* brain models, but no review has been published covering the contribution of scRNA-seq technology applied to *in vitro* models in the study of the human brain development, aging and associated diseases. This review compiles a representative collection of publications with scRNA-seq data application in *in vitro* brain models. It demonstrates how scRNA-seq data alone or in combination with other molecular techniques has proven useful to validate fidelity and reproducibility of *in vitro* models of the human brain, as well as for the identification of novel cell types or gene expression signatures associated with neurodevelopmental or neurodegenerative disorders. ScRNA-seq is a technology still in development, but holds a great potential in future research in developmental biology and translational medicine.

ABSTRACT:

Single-cell RNA sequencing (scRNA-seq) technology is applied in molecular biology to quantify and analyze gene expression of individual cells present in a tissue sample. During the last 14 years, the technology underwent an exponential evolution towards automation and high-throughput. Current platforms enable the simultaneous analysis of hundreds of thousands of cells, requiring large computational power to analyze the sequencing data output and increased complexity of data-processing. However, scRNA-seq contribution to tissue heterogeneity characterization outweighs its high technical demands, especially in the study of the brain. ScRNA-seq application in human samples and animal models has been reviewed several times, but no review collects the contribution of scRNA-seq technology applied to *in vitro* models in the study of the human brain development, aging and associated diseases. This review compiles a representative collection of publications with scRNA-seq data application in *in vitro* brain models. It demonstrates that scRNA-seq data alone or in combination with other single-cell omics has proven useful to validate fidelity and reproducibility of *in vitro* models of the human brain, as well as for the identification of novel cell types or gene expression signatures associated with neurodevelopmental or neurodegenerative disorders. Single-cell transcriptomics are still in development, but hold a great potential in future research in developmental biology and translational medicine.

KEYWORDS:

scRNA-seq; brain models; neurodevelopment; single-cell omics; *in vitro*

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1. Introduction on single cell RNA sequencing (scRNA-seq) technology

Nucleic acids' sequencing started nearly 50 years ago with Frederick Sanger and colleagues (Sanger & Coulson, 1975). Since then, DNA sequencing and RNA sequencing (RNA-seq) have scaled-up and evolved towards high-throughput methods. Based on reverse transcription of RNA sequences into complementary DNA (cDNA) for its posterior sequencing, RNA-seq progressed hand in hand with DNA sequencing technologies. However, RNA-seq and DNA-seq offer different information.

Cells transfer and express information transcribing DNA sequences into RNA and translating it into proteins to enable cellular processes. DNA sequencing information indicates the shared genetic load present in the cells that compose an organism. Genomic studies, although useful for genetic studies with evolutive or disease-based research questions, are not enough to explore the impact the expression of different genes can have in a tissue or organ at a cellular or molecular level. This is where transcriptomics come into play. Tissues can be classified in a certain developmental stage and physical condition depending on their set and quantity of transcripts. Studying transcriptomics in bulk is useful to quantify overall genetic expression present under specific conditions, such as healthy versus diseased, as well as to map in gross the genetic output of a specific tissue. However, the intrinsic cellular heterogeneity present inside each tissue is obscured when pooling all cells together as an average in bulk studies. To fully study the development and characteristics of a tissue, the diversity of cellular types and states present on it and the influence that inherent variability or intrinsic and environmental interactions may have on the tissue, analysis must deepen to the single-cell level. Single-cell RNA sequencing (scRNA-seq) can further our comprehension in the complexity of developmental biology and associated diseases.

ScRNA-seq methodology was first developed in 2009 in the Surani lab (Tang et al., 2009). The mRNA of a single mouse blastomere was reverse-transcribed into cDNA and amplified using poly(T) primers, then fragmented (as sequencing technologies give out reads of small lengths between 30-400bp (Z. Wang et al., 2009)), ligated to specific SOLiD sequencing platform adaptors and amplified to prepare the required library, and sequenced. The resulting reads were mapped to the mouse genome and known transcripts. The information regarding genetic expression and transcript isoforms acquired by the analysis of this single cell outstripped any other previous methods, ushering an era of in-depth transcriptomic analysis. ScRNA-seq technologies have been developed and improved over the recent years along with the

simultaneous evolution of bioinformatic analysis to reduce costs and increase automation and throughput by scaling up the number of cells that could be single-analyzed in parallel. From a hundred cells, using microfluidics and multiple wells, to thousands of cells in pico-wells, up to the simultaneous analysis of hundreds of thousands of cells using nanodroplet and *in situ* barcoding technologies. In the most recent years, the field of spatial transcriptomics has enabled the parallel analysis of a million or more cells (Jovic et al., 2022). However, the amount of data that can be handled bioinformatically has practical limitations, which makes the number of cells analyzed in parallel be inversely correlated to the sequencing depth or amount of RNA information that can be extracted from each cell. Current high throughput techniques can be classified in two groups. Droplet-based techniques typically enable the parallel analysis of a higher cell number but give out a lower read-depth sequencing (like 10xGenomics). Plate-based methods (like iCell8) sequence full-length transcripts of fewer cells in parallel. Each method has its advantages and limitations: deeper read-length enables the detection of lowly expressed genes, allele-specific expression, isoforms, and splicing variants, whereas lower read-depth correlates with higher cell numbers analyzed, gaining cell-heterogeneity data at population level.

For more information regarding the different commercial scRNA-seq platforms available now, an explanatory table that includes cell numbers and sequence preparation details is included in the review by Jovic and collaborators (Jovic et al., 2022).

All scRNA-seq platforms follow the same basic wet lab and bioinformatic procedures, which involve single cell (or single nucleus) isolation, RNA purification, reverse transcription of the RNA into cDNA, cDNA amplification, fragmentation, and library preparation. Afterwards, the sequencing data output must be preprocessed and analyzed using bioinformatic tools. A graphical representation of the process of different RNA sequencing methodologies is depicted in Figure 1 for a clearer understanding.

Several techniques have been developed for single cell isolation, all based on capturing each cell in an isolated solution where the next steps would be taken. An alternative to single cell capture is single nucleus RNA sequencing (snRNA-seq), useful for tissue samples which are difficult to dissociate to obtain intact cells and minimize artificial transcriptional stress responses, such as brain tissue (Grindberg et al., 2013). However, this method only allows analysis of nuclear processes, losing all cytoplasmic RNA information. Once the RNA of each single cell to analyze is isolated by cellular lysis and specific methods to retain mRNA, a first strand of cDNA is obtained by reverse transcription. This cDNA contains unique molecular identifiers (UMIs) as the first barcodes to recognize each initial sequence and avoid amplification-associated biases. Then,

each cDNA is pre-amplified, either exponentially by PCR (most common) or linearly by *in vitro* transcription (IVT) to get enough material to prepare the DNA fragments in libraries depending on the final sequencing platform. Taking the 10XGenomics platform as an example, they use microfluidics to capture at most one single cell on each nanodrop of reaction; this droplet-based technique enables the processing of tens of thousands of cells in a single experiment; beads present inside each droplet capture mRNA using barcoded poly-A complementary sequences, and isolated mRNAs get reversed transcribed into cDNA which is amplified in bulk. Then amplified and tagged DNA material with specific sequencing platform adaptors is pooled in libraries and sequenced (Zheng et al., 2017). For 10XGenomics as well as for most scRNA-seq technologies, wet lab kits are commercially available to ease the whole procedure.

The obtained sequencing data must undergo quality control and be pre-processed before analysis. Depending on the sequencing platform used and the sample type, different pipelines (sets of connected algorithms that help process and analyze next generation sequencing data) are used, like Cell Ranger for 10xGenomics. Secondary computational methods can be then applied depending on the final desired product from the data, like cell clustering, data sets comparison, etc. Once data has been preprocessed, it can be analyzed by any researcher with a basic knowledge in bioinformatics.

For data pre-processing, raw sequencing data in FASTQ format is first ran for quality control before an aligner tool (like STAR aligner inside the Cell Ranger pipeline used for 10xGenomics) is used to map high-quality reads to a specific reference genome. Reads can be categorized into exons, introns and intergenic depending on the confidence of the alignment. Pre-processed data can then be used for general analysis, consisting of different steps that can all be followed using a single bioinformatic tool like *Seurat* (Stuart et al., 2019). Additional quality control tools are used to filter low quality gene expression interferences based on control indicators like expression of mitochondrial genes and number of transcripts. Then the data from each single cell, treated as one independent sample, is normalized to remove technical non-uniformities among the sequencing data and be able to compare data among samples. A simple and popular normalization method is *Reads per Million* (RPM), which standardizes total number of reads between cells (Luecken & Theis, 2019); however, many normalization methods have been specifically designed for scRNA-seq and yield good results (Vallejos et al., 2017). Normalized data undergoes feature selection to reduce the volume of material received from the sequencing, discard unnecessary data, and accelerate downstream analysis. A selection is made of subsets of features showing high variation among cells: the highly variable genes (HVGs), excluding housekeeping genes without significant expression changes among all cells of the sample. At

this point, the dataset is still highly dimensional, with sequencing data of tens of thousands of cells, each individual cell expressing thousands of genes with specific transcriptional signatures. Dimensional reduction of the data is needed to make it manageable. Correlations are eestablished among genetic information to reduce the number of dimensions present in the sample dataset and therefore ease the computational work in downstream analyses. After all this processing, data can now be used for cell clustering and annotation based on transcriptional signatures, followed by different exploratory analyses depending on the goal of the experiment in question. The basic analysis chapters of "Orchestrating Single-cell Analysis with Bioconductor" book by Robert Amezquita, available online (*Basics of Single-Cell Analysis with Bioconductor*, n.d.) give a more detailed explanation of all bioinformatic procedures on scRNA-seq data.

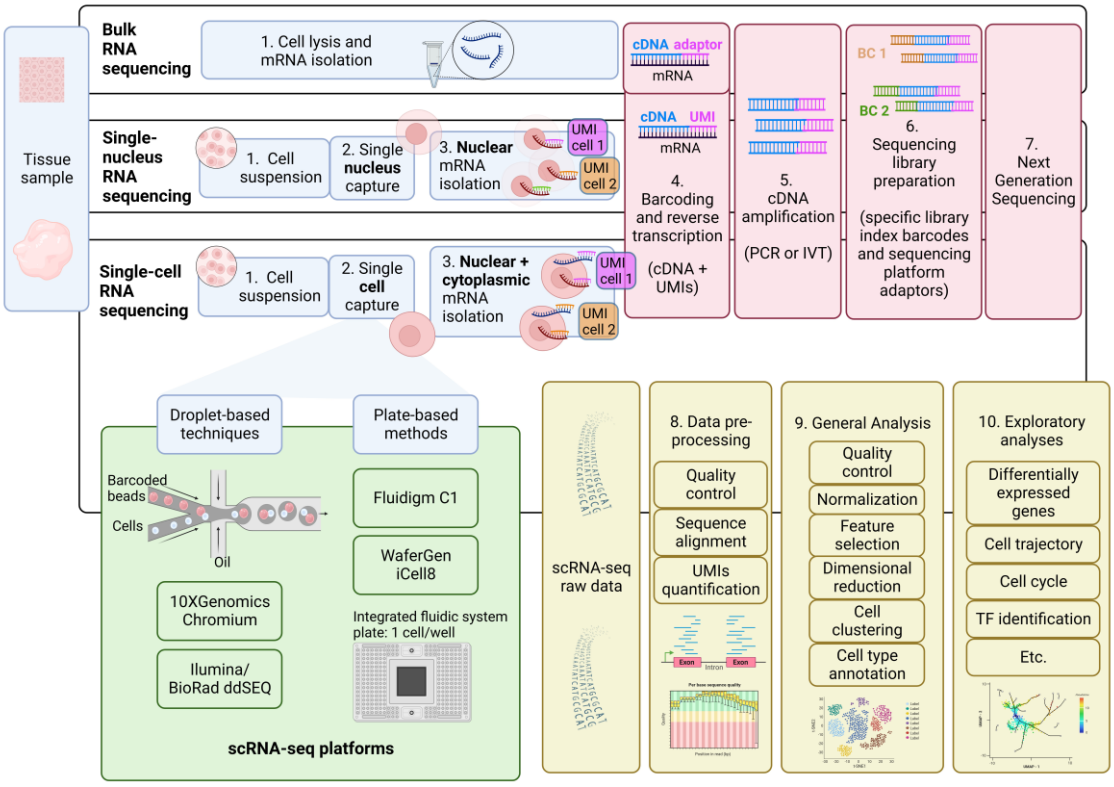

Figure 1: Graphical representation of the methodological process of bulk, single-cell and single-nucleus RNA next generation sequencing technologies. Single-cell and single-nucleus RNAseq methodologies begin dissociating the tissue sample into a cell suspension, whereas in bulk RNAseq the sample is directly treated for mRNA purification of all cells in one pool (1). SnRNA-seq basis is the capture of individual nuclei (2) and distinction of nuclear mRNAs isolated from each cell by unique molecular identifiers (UMIs) (3). Similarly, in scRNA-seq techniques nuclear and cytoplasmic mRNAs from single cells are captured from processing of the cell suspension using microfluidics (2-3), either by bonding to uniquely barcoded beads in oil droplets (drop-based techniques) or by placement of each individual cells into a micro-well carrying unique identifiers (plate-based methods). Captured mRNA molecules undergo reverse transcription into

complementary DNA (cDNA) carrying UMIs or primer adaptors (4). Amplification of the cDNA (5) and library preparation (6) introducing specific platform adaptors and index barcodes (BC) is required before sending the sample to be sequenced (7). Received scRNA-seq raw data is pre-processed (8) and primarily analyzed (9) bioinformatically until a manageable dataset is generated to be used for different exploratory analyses (10). Figure created with BioRender.com.

This review provides a concise overview of the basis and different applications of scRNA-seq technology and then focuses on its utility in the study of the brain, brain tissue development and associated diseases. The application of scRNA-seq technology in different brain models is discussed, making special emphasis on *in vitro* model systems, its implications, its potential for basic neuroscience research as well as future perspectives on translational medicine.

2. Applications for scRNA-seq

The data acquired by scRNA-seq gives information regarding gene expression signatures of each individual cell analyzed in one sample. Such information can be applied to characterize at the transcriptomic level the cellular populations and subtypes present in a tissue. The uses given to the preprocessed scRNA-seq data are referred to as exploratory analyses. After cell clustering and annotation, typical exploratory analyses are the classification of specific cell populations by biological significance or functional bias of differentially expressed genes, followed by pseudo-time analysis to establish a pattern of dynamic processes experienced by the cells in the tissue, such as cellular differentiation or apoptosis. Associated with pseudo-time, cell cycle status of the analyzed cells can also be determined. Anther extended application is the identification of enriched transcription factors (TF) in each cell cluster and the combination with pseudo-time to comprehend dynamic changes in gene regulation mechanisms among cell types. Exploratory analyses can be conducted using commercially available computational packages, like *SCENIC* for TF identification, *IRIS3* for TF dynamics, *Monocle* for pseudo-time analysis, or *CellCycleScoring* function inside *Seurat* tool (Haque et al., 2017; Jovic et al., 2022). Diverse bioinformatic tools have been and are being designed and upgraded to analyze scRNA-seq datasets alone or in combination with other single cell omics for different purposes. The combination of proteomics and transcriptomics datasets is used in tools like CellPhoneDB or CellChat to research on cell-cell interactions and communication for tissue homeostasis, immune activity, or cellular differentiation (Armingol et al., 2020). CRISPR technology is also being used in combination with scRNA-seq for lineage tracing (Raj et al., 2018), or CRISPR

screenings with deeper resolutions (Replogle et al., 2020). Recent studies are pairing transcriptome sequencing with chromatin accessibility sequencing (ATAC-seq) (Uzquiano et al., 2022) to understand the influence epigenetics have on gene expression and further characterize cell subtypes. Most interesting is the combination of single-cell transcriptomics with spatial data obtained by tissue imaging enables the generation of a 4D map of the tissue development comprising spatial situations along with cellular characterization during a range of time points (R. Wang et al., 2023). Generally, the trend among users of single-cell omics technologies seems to be the combination into single-cell multiomics to corroborate and link results to better comprehend tissue development and associated pathologies at a deeper level. All these combinatorial assays are already becoming more accessible and reproducible. Last year 10XGenomics launched a commercially available kit to perform spatial transcriptomics (Visium Spatial Gene Expression) which also allows protein co-detection. A great graphical explanation of the process is available on their website (*Spatial Gene Expression - 10x Genomics*, n.d.).

ScRNA-seq data, alone or in combination with other omics, was first conceived for developmental studies, given its fundamental use for cell type identification. The goal of most scRNA-seq studies is the characterization of the cellular composition of a tissue, and curation of a detail dataset or cell atlas of that tissue. Single cell atlases are being constructed for tissues, organs, and even whole organisms, including plants, animals and humans, most of them being publicly accessible to facilitate understanding and speed up important discoveries at genetic and cellular levels. However, scRNA-seq application has even further expanded after the usefulness of the technology has been discovered for other purposes, such as characterization of tissues and organs not only in healthy, but also in diseased conditions, making scRNA-seq a common tool in many disciplines involving basic biology, biomedical and clinical investigations (Svensson et al., 2020), even paving its way into precision medicine. The European initiative LifeTime is a good example of the application of single-cell multi-omics combination with artificial intelligence and machine learning in personalized diseased models like organoids to uncover new insights of human cells' behavior during disease origins, progression, and response to therapy at single cell resolution (Rajewsky et al., 2020).

As of 2020, more than 1700 single cell RNA sequencing studies have been published, comprising more than 180 different tissues. These numbers are only growing. Interestingly, the brain is by far the most investigated tissue using single cell transcriptomics (Svensson et al., 2020).

3. ScRNA-seq in the study of brain development and associated diseases

ScRNA-seq has been used to study the brain in its evolutionary, developmental, and pathophysiological context. The transcriptome of the human brain and the brain of other animal models that resemble it in some extent, like mouse (Vinsland & Linnarsson, 2022), zebrafish (Raj et al., 2018), salamanders (Lust et al., 2022) and primates (Kanton et al., 2019), has been studied from postmortem tissue samples. However, in the inaccessibility of human brain samples, inter-species differences on brain development and structure and the rise of reproducible *in vitro* model systems that resemble the human brain tissue have made the latter the best alternative in which to invest in and develop in other to elucidate neurological research questions without jeopardizing any ethical values. Still, fresh and frozen tissues are the main source of single-cell data recollection and used as reference to validate *in vitro* models.

a. Animal models

The brain tissue of many animal species has been analyzed using scRNA-seq to characterize its cellular heterogeneity, developmental dynamics, and to generate informative gene expression datasets to use in further studies. Mouse, human, dog, fly, salamander and fish brain single cell transcriptome studies are examples, and the main application of scRNA-seq technology in most publications is cellular characterization. Cell atlasing through single-cell omics has become a research area on its own, with major focus on the brain tissue due to its complexity and low accessibility. Numerous databases on single cell studies in animal brain have been generated and made publicly accessible, some of them covering several species and brain regions for comparative studies. In May of this year a new single cell omics database launched that covers brain cell types and associated genetic markers of 14 species (Chi et al., 2023). ScBrainMap allows search by gene, cell type, species, brain region, developmental period and disease state, of nearly 5 thousand cell types covering 124 brain regions across 128 developmental stages and 20 kinds of diseases. Out of these 14 species, most information belongs to mammalian brain studies.

The most studied mammalian brain is the mouse brain, given its similarity on cellular types and tissue architecture to the human brain and the accessibility of the model. The number of single cell studies conducted in mouse models double those conducted on human samples. Acquisition of human brain tissue samples from second and third fetal semesters is difficult and postnatal

samples are limited. However, there is scRNA-seq data of all murine prenatal stages of development. In 2022, the Linnarson lab published a thorough review on mammalian brain development that covers all available scRNA-seq datasets of different brain regions and developmental stages of the mouse and human brain (Vinsland & Linnarsson, 2022). In this review, two figures clearly illustrate currently available scRNA-seq datasets of human and mouse nervous system development, showing how many human brain regions and developmental time points are yet to be studied using single-cell omics.

There is a big information gap still to cover in the understanding of the human brain. To fully understand brain development, data must be recollected not only from all neuroanatomical regions and developmental timepoints, but also from cephalic tissues such as choroid plexus and meninges and from the cerebral vasculature. Scarce single-cell transcriptomic studies cover these tissues when the study on systemic roles on brain development and homeostasis could make a difference. A human brain vasculature atlas was generated in 2022 and first analysis already found presence of diverse mediators of Alzheimer's risk, suggesting vascular and perivascular involvement in the disease (Yang et al., 2022). These findings portray the importance of data integration at a whole-body level. Brain organogenesis should be placed into systematic context to fully understand the extent to which brain development and associated diseases are related to other body regions.

However, human tissue samples from any organ and specially from brain tissue are highly inaccessible compared to other animal models. Therefore, several initiatives have arisen to integrate bulk and single-cell data of the human brain on cell atlases. STAB, a spatio-temporal cell atlas of the human brain (Song et al., 2021), covers the analysis of more than 140k cells from 20 different human brain regions across 11 distinct developmental periods of the human brain including some postnatal and aged brains. Another relatively complete atlas is The Allen Brain Cell Types Database based on snRNA-seq (*Cell Types Database: RNA-Seq Data - Brain-Map.Org*, n.d.), which includes mouse and macaque brain datasets for comparative analyses. The current most ambitious initiative is the Human Cell Atlas in which 650 labs around the world are collaborating and sharing multi-omics data, including scRNA-seq data (*Single Cell Expression Atlas*, n.d.), to map the human body at the cellular level.

These databases allow the exploration of cell dynamics during human brain development, mapping expression trajectories and identifying aberrant cell subtypes related to neurodegenerative diseases. However, sequencing depth differences among studies and sample inhomogeneity among datasets results in the presence of batch effects and incorrect definition

of certain cell types. What is more, none of these atlases covers all brain developmental stages and brain regions, and there is the sparse data available of mature and aged human brains. Time, process standardization and maybe other research models are needed to collect and better integrate single cell data from the human brain.

b. *In vitro* models

So far, several 2D and 3D neuronal cultures presenting different complexities have been developed as *in vitro* model systems to study the human brain. ScRNA-seq has been performed in those models believed to closely resemble certain functional and cellular organization properties to human brain tissue, like brain spheroids, organoids, or long-term explant cultures. In depth transcriptomic analysis of these *in vitro* models serves in the first instance as a characterization assay for and analytical comparison to real human brain samples, so that in the second instance the collected data from validated models can provide insights into developmental cues, from expression signatures conserved throughout evolution to cellular identities and dynamics of different brain regions during their development. Many reviews have been written bringing together scRNA-seq studies of human and animal models, but there are no such reviews on scRNA-seq applied on *in vitro* brain models.

Using PubMed and RDiscovery as literature search tools and focusing on research in labs known for their use of *in vitro* human brain models to study the human brain like Paola Arlotta, Sergiu Paşca, Jörgen Knoblich or Madeline Lancaster, several publications were found in which scRNA-seq technology is used on their studies. Subsequent extensive search was conducted towards other labs that combine different single-cell omics, use alternative *in vitro* systems or whose final fundamental scRNA-seq application diverts from the main course of studies. Overall, a compendium of 27 representative published studies has been curated for this review to cover the general trends followed by scRNA-seq application on *in vitro* brain models (Table 1).

In vitro *brain models generally exhibit genetic expression profiles found on the prenatal human brain.*

Over the last 8 years, brain organoids, spheroids and assembloids have become the main human brain *in vitro* models in which scRNA-seq studies are applied, followed by bioengineered brain-like tissue models, being their focus of studies on the prenatal brain. Most *in vitro* models are generated from directed differentiation of pluripotent stem cells (PSCs), and they recapitulate

early developmental stages of brain tissue in terms of morphology, cell types, neuronal activity and migration only to a certain extent. Brain organoids being the most studied models only manage to recapitulate brain developmental transcriptional profiles up to 24 weeks after conception (Chiaradia & Lancaster, 2020). Even though *in vitro* systems are mainly used to model brain development during gestation there is research in progress into other brain aspects such as diseases or brain aging. Only one study from the Geschwind lab (Gordon et al., 2021) analyzed bulk RNA-seq data of long-term cultured cortical organoids (300 days old) demonstrating reproducibility of *in vivo* postnatal brain development, cellular composition and other components like RNA editing. Single-cell level characterization of these long-term organoids would better validate the cellular composition similarities claimed on these findings.

Prenatal cortex is the most studied brain region.

Regarding brain areas, the cerebral cortex is the most studied *in vitro*, alone or together with other forebrain structures, followed by midbrain, retina, and unspecific whole-brain tissue analyses. It correlates with published human brain scRNA-seq datasets, with the cortical area being the most studied with more developmental time points covered, followed by the retina (Vinsland & Linnarsson, 2022).

Cellular characterization is the main application of scRNA-seq technology.

General applications of the scRNA-seq data analysis are usually model characterization and comparative analysis to human fetal brain tissue. As previously mentioned, single-cell transcriptomic profiling of the *in vitro* models serves as an exhaustive control and validation of the level of similarity they present to human brain regions, being model validation the first step to fulfill before its application for specific neuroscience research questions. Single-cell transcriptomic profiling, in combination with other techniques, has played a pivotal role in the clarification on the level of fidelity of *in vitro* models to cell types and organization in human brain areas and subsequent upgrade.

Literature analysis by lab shows the evolution undergone by brain organoids and other models to perfectionate fidelity to primary tissue. Protocols to generate brain organoids have been developed and modified by several research labs. Initial unguided whole-brain cerebral organoids created by Lancaster and Knoblich gave rise to a mélange of ectoderm-derived neural tissues and mesoderm tissue. The Treutlein lab made an initial single-cell transcriptomic comparative analysis of cerebral organoids to fetal human neocortex postmortem samples to

characterize which features of human cortical development they recapitulate, including zonal and cellular hierarchy and genetic expression signatures of extracellular matrix (ECM). Findings made paved the direction of future studies of the lab towards biomechanosensing and ECM impact on organoid development (Camp et al., 2015; Jain et al., 2023). The Arlotta lab also characterized unguided cerebral organoids (Quadrato et al., 2017) realizing their potential to model high-order functions of the human brain like cellular interactions and neural circuit function. However, scRNA-seq data also served to identify transcriptomic differences present among batches and culture conditions. Aiming for inter- and intra-organoid variability reduction, research in the Arlotta lab turned towards the implementation of cortical and dorsal forebrain organoids generated by guided differentiation, which characterization demonstrates improved reproducibility as well as fidelity in developmental trajectories (Uzquiano et al., 2022; Velasco et al., 2019).

Using different patterning techniques, organoids have been developed to accurately mimic specific brain regions, like forebrain, retina, hippocampus, or choroid plexus (ChP), being the most studied cortical organoids. In the reviewed literature, more than half the publications use cortical organoids as a model. Arlotta along with Lancaster, Knoblich, Kriegstein and Vaccarino labs made important contributions to the transcriptome characterization of guided cortical brain organoids using either snRNA-seq (Amiri et al., 2018) or scRNA-seq data (Bhaduri et al., 2020; Kelava et al., 2022; Li et al., 2022). Lancaster lab even perfectionated cortical organoids culturing them in an air-liquid interface, achieving differentiation of intracortical and subcortical neuron clusters with gene expression profiles associated with axon outgrowth and neural circuit formation (Giandomenico et al., 2019). Organoids patterned into other brain regions have also been characterized using scRNA-seq data. The Reh lab made a comparative transcriptomic analysis of retinal organoids with fetal human brain tissue and retinal cultures (Sridhar et al., 2020). Retinal organoids matched main cellular composition with fetal tissue, excluding some genetic expression differences in specific cell types, which made room for organoid protocol modifications. A posterior higher resolution study on their retinal organoids and implementing single cell transcriptomic and epigenetic data (Finkbeiner et al., 2022) identified TF cascades that define each major retinal cell type as well as correct recapitulation by retinal organoids of chromatin accessibility dynamics of human fetal retina, but also discovered differences on gene regulation of inner retina interneurons. On another note, Lancaster and collaborators generated and transcriptionally characterized human ChP organoids (Pellegrini et al., 2020) demonstrating high similarity in cell composition and transcriptomic signature to *in vivo* ChP. Midbrain has also been recapitulated using patterned organoids. The Parmar lab compared transcriptomic

signatures of ventral midbrain (VM) organoids bioengineered or not with spider silk microfibers functionalized with human laminin to validate the better model for the study of the developmental trajectory of dopamine neurons (Fiorenzano et al., 2021). Different parts of the human brain have been recapitulated in brain organoids, as well as brain tissue structure from other species. Single-cell RNA-seq data from macaque and chimpanzee brain organoids has been atlased and analyzed to uncover specific features of human brain development (Kanton et al., 2019).

Although brain organoids are the most characterized *in vitro* model at molecular resolution, they are not the only one. Analysis of the genetic expression profile of the brain-on-a-chip model from the Fischer lab indicates how the 3D co-culture of neurons, astrocytes, and oligodendrocytes (OLs) manages to recapitulate neuronal cell maturation with neural network development, mature axons and microglia (Enright et al., 2020). Like organoids, stem cell-derived spheroids self-assemble following a distinct protocol, being able to pattern specific human brain regions or developmental transition stages of cellular types present in the brain. The research group of Sergiu Paşca generated human OLs spheroids and used scRNA-seq analysis to prove similarity on gene expression profile of OLs developmental transitions into mature axon-myelinating cells (Marton et al., 2019). The same group fused spheroids that patterned different brain areas, like cortex (hCS) and subpallium (hSS), into so called "assembloids" to study neuronal migration and inter-brain connectivity and functions. ScRNA-seq analysis of hCS-hSS assembloids served to identify transcriptional signatures associated to interneuron migration and the dataset has been used in a subsequent study for gene selection of a CRISPR screen conducted in assembloids to reveal neuro-developmental disorders-risk genes (Gordon et al., 2021; Meng et al., 2022).

All reviewed publications on model characterization agree on the fact that actual *in vitro* models of the human brain are useful but further protocol improvements are needed to make them recapitulate more features of the human brain, like co-presence of all glial cell types, endothelial cells, specific zonal architectures, or complex organization of myelin sheets.

Cell atlasing is the second most recurrent application for scRNA-seq data.

Apart from cellular characterization, the most extended general application of scRNA-seq methodology is the generation of gene expression atlases at single-cell resolution, usually combined with other omics data and with diseased models or other animal models to be used as a tool to study human-specific evolutionary traits in neurodevelopment and associated

diseases. The Arlotta lab generated in 2022 a transcriptomic and spatial atlas of human corticogenesis reproduced in brain organoids that integrates scRNA-seq, spatial imaging data, transcription factor proteomic data and chromatin accessibility data (Uzquiano et al., 2022). Also last year, the Reh lab generated a single cell methylome and transcriptome dataset of human retina development obtained from organoids and postmortem tissue samples (Finkbeiner et al., 2022). The Knoblich lab created a developmental and cell-type specific phenotypic database of ASD high-risk genes based on LOF research in cortical organoids (Li et al., 2022). Treutlein and Gray Camp labs collaborated in the generation of a temporal cell atlas of great ape forebrain development using macaque, chimpanzee, and human cerebral organoids back in 2019 (Kanton et al., 2019). And some months ago, the Vaccarino lab published an extensive single-cell level dataset modeling idiopathic autism spectrum disorder (ASD) in cortical organoids (Jourdon et al., 2023).

Open Science is key for scRNA-seq data utility.

Even if atlasing is not the main goal of the study, most datasets of the reviewed publications are publicly accessible online or available upon request (datasets availability of reviewed publications is indicated in Table 1). Open access to published datasets saves time and expenses to other labs working in the field and accelerates model and technology improvement as well as discovery rates. A good example of open science potential is the debate on the fidelity of cortical brain organoids on cellular subtypes opened by a publication of the Kriegstein lab (Bhaduri et al., 2020). Their results suggest that the 3D culture of PSCs induces endoplasmic reticulum (ER) stress response as well as a glycolysis expression profile in cells. These activated stressed pathways affect transcriptomic analysis and impair cell type specification. However, other studies in brain organoids from Arlotta and Lancaster labs differ on these findings. The Arlotta lab has compared transcriptomic, proteomic, and epigenetic data of cortical and whole brain organoids with fetal brain tissue samples and argues how, although glycolysis and oxidative phosphorylation metabolic pathways are enriched in organoids dependent on the culture conditions, only certain cell types express this stressed transcriptomic signature. Additionally, they counter argue how glycolytic metabolism switch towards mitochondrial oxidative phosphorylation is observed in neural progenitors upon differentiation during fetal brain development, which suggest this stress-activated pathways are also common in the developing human brain to some extent, and that organoids can consistently generate accurate cell identities independent of this metabolic state (Uzquiano et al., 2022). Simultaneously, Knoblich lab members made use of internal and published scRNA-seq datasets to identify stress

signatures in brain organoids. They found out the stress signature is expressed by a defined cell subpopulation not present in fetal brain samples that does not alter neuronal maturation or differentiation of non-stressed cells, and they subsequently developed a computational algorithm to identify and remove this specific stressed cell type from scRNA-seq datasets, improving bioinformatic analysis of cell heterogeneity and developmental trajectories in brain organoids (Vertesy et al., 2022).

ScRNA-seq application in in vitro *studies of neurodevelopmental and neurodegenerative disorders*

Brain organoids and spheroids have proven to be a valid model for neurodevelopmental disorders. On one hand, organoids mutated on autism spectrum disorder (ASD) genes along with the transcriptomic profiling of their cellular heterogeneity has helped identify cell type-specific developmental abnormalities, like early differentiation of GABAergic neurons and deep-layer projection neurons (Paulsen et al., 2022), or shifted expression of transcription factors that control cell fate during early cortical development (Jourdon et al., 2023). On the other hand, Tuberous Sclerosis Complex (TSC) brain organoids transcriptomic analysis identified a novel cell type responsible for tumor growth and derived neurological defects part of the disease pathogenesis (Eichmüller et al., 2022). Additionally, a system combining scRNA-seq and CRISPR technologies in brain organoids identified ASD-associated regulatory genes on a pooled loss-of-function screen in mosaic organoids (Li et al., 2022). Furthermore, scRNA-seq data from assembloids has been used as baseline for a CRISPR screen on neurodevelopmental disorders-risk genes (Meng et al., 2022). These studies exemplify the importance of human *in vitro* models to identify disease mechanisms with processes not conserved in other mammals.

Surprisingly, even in neurodegeneration studies, considering the limited maturity of the current *in vitro* brain models and the late onset of neurodegenerative disorders, molecular profiling of brain organoids has been useful. ScRNA-seq analysis of brain organoids generated from Alzheimer's disease patients with apolipoprotein E deficiency has given new insights into the neurogenesis deficits and metabolism dysregulation caused by the disease (Zhao et al., 2023).

Other engaging scRNA-seq studies in brain organoids focus on differences among male and female human brains. ScRNA-seq data on male and female PSC-derived organoids has been used to study the effect of androgens on excitatory neural progenitors' populations (Kelava et al., 2022).

ScRNA-seq is usually combined with other omics or molecular technologies.

As any other molecular technique, scRNA-seq has its limitations, which can be partially overcome by comparison and supplementation with data obtained from complementary methodologies. Most *in vitro* human brain studies combine scRNA-seq data with other single-cell omics to support their findings and even expand them. Common combinations across the reviewed literature are chromatin accessibility assays, proteomic studies, lineage tracing or imaging techniques for spatial information. Different labs have developed combinatorial tools of scRNA-seq along with other technologies. Treutlein and Gray Camp labs developed iTracer technology for in depth study of cell dynamics (He et al., 2021), a cell lineage tracer and recorder based on CRISPR technology and compatible with single-cell and spatial transcriptomics. Its utility in cerebral organoids was proven for the study of lineages' dynamics of healthy and diseased models. Another interesting combinatorial approach to study neurodevelopmental disorders using *in vitro* models is the CHOOSE system by the Knoblich lab, a technology applied in parallel by Treutelin and Gray Camp labs (Fleck et al., 2022; Li et al., 2022). A CRISPR-human organoids-scRNA-seq system that generates LOF screenings in brain organoids whose transcriptome can be subsequently analyzed at the single cell level.

Throughput evolution on scRNA-seq technology in in vitro *human brain models*

ScRNA-seq technology evolution towards high throughput sequencing is evident when expressed in cell numbers analyzed per sequencing experiment. Using reviewed literature as representative reference, the lowest number of cells analyzed in parallel is 295 cells from spheroids (Marton et al., 2019) and 500 cells from cerebral organoids back in 2015 (Camp et al., 2015) when high throughput sequencers were still being tested, compared to more than 700 thousand cells analyzed in cortical organoids in 2022 (Paulsen et al., 2022). Low cell number scRNA-seq studies are usually complemented with a subsequent higher throughput sequencing experiment conducted afterwards for a deeper characterization. Moreover, scRNA-seq data-based atlases studies do not start to be published until the technology enabled simultaneous analysis of tens of thousands of cells. Over the last 8 years no publication was found on low throughput whole transcriptome scRNA-sequencing, as most studies focus on big cell numbers for cellular heterogeneity characterization of the *in vitro* model.

Table 1. Recent studies of the human brain* using scRNA-seq in *in vitro* models.

MODEL	BRAIN AREA	scRNA-seq APPLICATION	N.º CELLS	FINDINGS	LAB	YEAR	REFERENCE
Dorsal forebrain and whole-brain organoids	Cortex	Generation of a transcriptomic, epigenetic, and spatial **atlas** of second trimester human corticogenesis *in vitro*. scRNA-seq data combined with Slide-seqV2 for spatial organization, SHARE-seq for TF and chromatin accessibility, and scATAC-seq for epigenetics.	>610k	Trajectories of development in human brain organoids. Organoids' individual cell type signatures are highly reproducible. Glycolysis and oxidative phosphorylation enriched in organoids. Metabolic states do not affect identity acquisition for most cells in organoids. Analysis of pseudo-time trajectories in organoids predicts human-specific regulation. Transcriptional diversity of callosal neurons emerges at early stages of development.	Arlotta	2022	Proper acquisition of cell class identity in organoids allows definition of fate specification programs of the human cerebral cortex - ScienceDirect (Uzquiano et al., 2022)
Brain organoids	Cortex	**Identification** of cell-type-specific **developmental abnormalities** in mutated organoids on autism spectrum disorder risk genes.	>745k	Day 35 organoids mutated on either SUV420H1, CHD8 and ARID1B show early populations of GABAergic neurons and deep-layer projection neurons.	Arlotta	2022	Autism genes converge on asynchronous development of shared neuron classes | Nature (Paulsen et al., 2022)
Dorsal forebrain and whole-brain organoids	Cortex. Dorsal forebrain.	**Characterization** of cellular heterogeneity in organoids and **comparative analysis** to human fetal brain tissue.	166k	Reproducibility of cortical cell types compendium in organoids generated from different cell lines, as well as developmental trajectories with variability levels like *in vivo* tissue. CNS cellular diversity can be established *in vitro*.	Arlotta	2019	Individual brain organoids reproducibly form cell diversity of the human cerebral cortex | Nature (Velasco et al., 2019)
Cerebral organoids	Whole-brain	**Characterization** of cellular heterogeneity of whole-brain organoids, heterogeneity between them and **comparative analysis** to the human fetal brain.	82k	Organoids generate dendritic spines and form spontaneously active neuronal networks. scRNA-seq data showed 7 transcriptionally different cell clusters, six of them neuroectodermal (forebrain and retina cells), but one mesodermal. Transcriptomic differences among batches and culture conditions.	Arlotta	2017	Cell diversity and network dynamics in photosensitive human brain organoids - PMC (nih.gov) (Quadrato et al., 2017)
Brain-on-a-chip: 3D co-culture of neurons, astrocytes, and oligodendrocytes on a multi-electrode array (MEA) system	Brain-like tissue	Transcriptional **characterization** of a new 3D model resembling human brain-like tissue.	--	Transcriptional differences exist between simple (2D) and complex (3D co-culture) *in vitro* systems. Brain-on-a-chip system shows multilayer architecture and further cell maturation with neural network development, mature axons and microglia.	Fischer	2020	Functional and transcriptional characterization of complex neuronal co-cultures | Scientific Reports (nature.com) (Enright et al., 2020)

MODEL	BRAIN AREA	scRNA-seq APPLICATION	Nº CELLS	FINDINGS	LAB	YEAR	REFERENCE
Long-term brain organoids, prenatal and postnatal postmortem tissue	Cortex	Bulk RNA-seq and DNA methylation analysis. **Characterization and comparative analysis** between organoid and prenatal and postnatal tissue maturation and progression.	--	Long-term organoids reach postnatal stages after 250-300 days of culture. *In vitro* models can reproduce certain components of *in vivo* postnatal brain development, including RNA editing, cellular composition and switch in ratio of NMDA receptor subunits. Cellular stress pathways activation issue in previous organoids studies is slim and does not infer in progressive stress or dysfunction. Creation of GECO webtool (Gene Expression in Cortical Organoids) to annotate neurodevelopmental and degenerative disease-risk genes.	Geschwind and Paşca	2021	Long-term maturation of human cortical organoids matches key early postnatal transitions | Nature Neuroscience (Gordon et al., 2021)
Healthy and APOE-deficient cerebral organoids	Whole brain	**Molecular profiling** of apolipoprotein E (APOE)-deficient cerebral organoids, strongest genetic risk factor for Alzheimer's disease (AD). Combination with CRISPR/Cas9 KO technology to obtain isogenic APOE-/- iPSC line.	27k	APOE is predominantly expressed in RG and astrocytes. APOE deficiency increases RG, astrocyte and inhibitory neuron populations, and decreases excitatory neuron population. Activated EIF2 signaling pathway leads to altered neuronal differentiation. Deficient APOE and show neurogenesis deficits and lipid metabolism dysregulation. APOE4 cerebral organoids show altered neurogenesis and cholesterol metabolism.	Guojun Bu	2023	APOE deficiency impacts neural differentiation and cholesterol biosynthesis in human iPSC-derived cerebral organoids - PMC (nih.gov) (Zhao et al., 2023)
Brain organoids and fetal human brain data	Cortex, retina	Use of scRNA-seq data from published and own datasets to **identify stress signatures** in brain organoids and to test a new granular functional filtering (Gruffi) algorithm for non-biased removal of stressed cells from scRNA-seq datasets.	190k	Unique stress signatures identified in a defined cell subpopulation only present in organoids and not in fetal samples. This population does not affect neuronal maturation and differentiation of non-stressed cells. Development of a computational algorithm to identify and remove stressed cells from the dataset, improving bioinformatic analysis of developmental trajectories.	Knoblich	2022	Gruffi: an algorithm for computational removal of stressed cells from brain organoid transcriptomic datasets | The EMBO Journal (embopress.org) (Ertesy et al., 2022)
Tuberous Sclerosis Complex (TSC) patients-specific brain organoids.	Cortex	scRNA-seq along with histological validation. **Characterization** of a new *in vitro* model for TSC.	--	Identification of a novel human interneuron progenitor cell type in the caudal ganglionic eminence during mid- gestation: CLIP cells, responsible for tumor growth and cortical lesions in TSC pathology via sensitive mTOR signaling. EGFR inhibition reverts TSC phenotype.	Knoblich	2022	Amplification of human interneuron progenitors promotes brain tumors and neurological defects | Science (Eichmüller et al., 2022)

MODEL	BRAIN AREA	scRNA-seq APPLICATION	Nº CELLS	FINDINGS	LAB	YEAR	REFERENCE
Mosaic cerebral organoids	Telencephalon	**CRISPR-human organoids-scRNA-seq (CHOOSE) system** for pooled loss-of-function (LOF) screening in mosaic organoids. **Construction of a developmental gene regulatory network and identification of ASD-associated regulatory genes**.	80k	Results support ubiquitin proteasome system's role in ASD when dysregulated. Genes related to Parkinsons disease are also frequently dysregulated. Identification of ASD-associated TF modules during cortical development and two ASD risk genes-enriched gene regulatory subnetworks. Generation of a developmental and cell-type specific phenotypic database of ASD high-risk genes based on LOF research.	Knoblich	2022	Single-cell brain organoid screening identifies developmental defects in autism | bioRxiv (Li et al., 2022)
Brain organoids and fetal samples	Cortex	**Characterization and comparative analysis** of human cortical samples and cortical organoids to evaluate cortical cell types' fidelity in retinal organoids.	Human samples: 189k cells, organoids: 235k cells	Organoids show less outer radial glia (RG) cells. Lack of neuron and glial subtype resolution. Neural and glial cell types occur earlier in organoids than in fetal brain development. Areal signature is strongly defined in neurons. 2D and 3D culture of PSCs induce expression of endoplasmic reticulum (ER) stress and glycolysis genes that affect transcriptomic analysis.	Kriegstein	2020	Cell Stress in Cortical Organoids Impairs Molecular Subtype Specification - PMC (nih.gov) (Bhaduri et al., 2020)
Chimpanzee and human-derived cerebral organoids	Cortex	**Identification of human-specific features** of cortical development compared to the primate brain.	--	Human organoids show 261 human-specific gene expression changes compared to chimpanzees. Human RG has increased mTOR activation compared to chimpanzees. Organoid cell composition can vary across experiments, individuals, and protocols. However, underlying cell types, gene co-expression relationships, and developmental trajectories are preserved in organoid models across individuals, species, and protocols.	Kriegstein	2019	Eestablishing Cerebral Organoids as Models of Human-Specific Brain Evolution - PubMed (nih.gov) (Pollen et al., 2019)
Brain organoids	Cortex	**Comparative analysis** of cell populations among male and female organoids, treated and not treated with androgens.	7k	Androgens cause an increased proliferation of cortical progenitors. scRNA-seq data shows increased excitatory neural progenitors in male and female cerebral organoids exposed to androgens compared to non-treated organoids.	Lancaster	2022	Androgens increase excitatory neurogenic potential in human brain organoids - PMC (nih.gov) (Kelava et al., 2022)
Human chroroid plexus (ChP) organoids with crebrospinal-like fluid (CSF) secretion	Choroid plexus	scRNA-seq along with proteomic studies. **Characterization and comparative analysis** to *in vivo* ChP and CSF.	--	High similarity of ChP organoids in cell composition, transcriptomic and proteomic signature to *in vivo* human ChP. New insights into ChP. Discovery of key human CSF components and new specialized epithelial subtypes that produce them.	Lancaster	2020	Human CNS barrier-forming organoids with cerebrospinal fluid production - PMC (nih.gov) (Pellegrini et al., 2020)

MODEL	BRAIN AREA	scRNA-seq APPLICATION	Nº CELLS	FINDINGS	LAB	YEAR	REFERENCE
ALI-COs (air-liquid interface cerebral organoids)	Cortex	**Characterization** of cell heterogeneity in the new brain organoids model.	13k	ALI-COs show improved survival and morphology, extensive axon outgrowth and axon guidance behavior compared to previous organoids culture systems. scRNA-seq shows intracortical and subcortical neuron clusters with gene expression profiles associated with axon outgrowth and neural circuit formation.	Lancaster	2019	Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output - PMC (nih.gov) (Giandomenico et al., 2019)
Patterned ventral midbrain (VM) organoids and Bioengineered VM organoids (spider silk + human laminin supporting microfibers)	Midbrain	**Study developmental trajectory** of mature dopamine neurons.	Patterned organoids: 91k cells silk-VM organoids: 32k cells	Identification of three human dopamine neurons subtypes highly like adult human midbrain in patterned VM organoids. Silk organoids reproduce dopamine neurogenesis and decrease organoid-to-organoid variability in proportion of cell types. Silk scaffolding reduces necrosis and supports neuronal maturation.	Parmar	2021	Single-cell transcriptomics captures features of human midbrain development and dopamine neuron diversity in brain organoids | Nature Communications (Fiorenzano et al., 2021)
Assembloids of subpallial and cortical spheroids (hCS-hSS)	Cortex, subpallium	Use of previously generated scRNA-seq dataset (Paşca 2021) for **gene selection** of neurodevelopmental disorders (NDD)-risk genes for the CRISPR screen.	--	Map roles of NDD genes in interneuron generation and migration into cortical circuits. ER dynamics play a role in interneuron migration towards the cerebral cortex.	Paşca	2022	CRISPR screens in 3D assembloids reveal disease genes associated with human interneuron development | bioRxiv (Meng et al., 2022)
hCS-hSS assembloids	Forebrain	**Characterization** of assembloids' cellular heterogeneity. Analyze hSS-derived neuronal population migrating into hCS region of the assembloid.	--	hCS-hSS assembloids include functionally integrated glutamatergic and GABAergic neurons. Identification of transcriptional signatures associated with interneuron migration. Assembloids proved to efficiently model disease processes: used to find a neuron migration defect in Timothy Syndrome subjects, which can be rescued pharmacologically targeting a specific calcium channel.	Paşca	2021	Assembly of functionally integrated human forebrain spheroids - PMC (nih.gov) (Birey et al., 2017)
Human olygodendrocyte spheroids (hOLS)	Cortex	**Characterization** of oligodendrocyte-derived cell populations in the 3D cultures.	295	*In vitro* 3D model for long-term study of cellular interactions, cell diversity and cytoarchitecture of oligodendrocytes (OLs). Developmental transition stages of OLs in the spheroids are transcriptionally like primary OLs, maturing morphologically and physically until myelinating nearby axons. Useful for disease modelling of OL-related disorders.	Paşca	2019	Differentiation and Maturation of Oligodendrocytes in Human Three-Dimensional Neural Cultures - PMC (nih.gov) (Marton et al., 2019)

MODEL	BRAIN AREA	scRNA-seq APPLICATION	Nº CELLS	FINDINGS	LAB	YEAR	REFERENCE
Human retinal organoids, fetal postmortem retina.	Retina	Previously generated scRNA-seq data in combination with scATAC-seq to **study human retina development** at higher resolution. **Dataset generation** for future studies.	103k	Identification of transcription factor (TF) cascades that define each major retinal cell type. Retinal organoids recapitulate main chromatin accessibility dynamics of human fetal retina but show differences in Notch signaling pathway and gene regulation of inner retina interneurons.	Reh	2022	Single-cell ATAC-seq of fetal human retina and stem-cell-derived retinal organoids shows changing chromatin landscapes during cell fate acquisition: Cell Reports (Finkbeiner et al., 2022)
Human retinal organoids, fetal postmortem retina, and long-tern retinal cultures	Retina	**Characterization** of human fetal retina development and degree of recapitulation achieved in retinal organoids.	--	Found genetic expression differences in specific cell types of retinal organoids even though main cellular composition matched to fetal tissue. Inner retinal lamination disrupted in a later developmental stage in organoids. Place for culture protocol modifications.	Reh	2020	Single-Cell Transcriptomic Comparison of Human Fetal Retina, hPSC-Derived Retinal Organoids, and Long-Term Retinal Cultures: Cell Reports (Sridhar et al., 2020)
Cerebral organoids	Whole-brain	scRNA-seq in combination with long-term live imaging. **Study extracellular matrix (ECM) impact** on organoid development at single cell level.	>100k	Externally provided matrix modulates gene expression programs involving extracellular matrix (ECM) pathway regulators and mechanosensing. ECM affects organoid morphogenesis, inducing regional guidance and luminal expansion within the developing neuroepithelium.	Treutlein	2023	Morphodynamics of human early brain organoid development | bioRxiv (Jain et al., 2023)
Cerebral organoids and fetal postmortem tissue	Cortex	**Comparative analysis** between human fetal neocortex and cerebral organoids single-cell transcriptomes and **characterization** of features in human cortical development that can be studied using brain organoids as a model.	Fetal tissue: 226 cells. Organoids: 500 cells	Cerebral organoids contain cortical regions with differentiated forebrain identities. Organoids show reduced basal progenitors' population. Cells in the organoid cortex show zonal and cellular hierarchy like fetal tissue. Expression signature of ECM, transcription regulation, RG delamination, notch/delta signaling, and neurite outgrowth-related genes correlates between fetal and organoid cells. Similar differentiation programs *in vivo* and *in vitro*. Differentially expressed genes in the organoids are usually lowly expressed in fetal tissue, so differential expression may come from analytical noise and low cell number analysis.	Treutlein	2015	Human cerebral organoids recapitulate gene expression programs of fetal neocortex development | PNAS (Camp et al., 2015)

MODEL	BRAIN AREA	scRNA-seq APPLICATION	Nº CELLS	FINDINGS	LAB	YEAR	REFERENCE
Cerebral organoids	Whole-brain	Development of a lineage recorder compatible with single cell and spatial transcriptomics: iTracer, and application in cerebral organoids to **study lineages dynamics** during organoid self-organization. scRNA-seq data coupled with long-term light-sheet microscopy data.	44k	iTracer can trace back clones, record lineages, and detect lineage alterations during normal or perturbed development. Brain regionalization initiates early in development. Cells from the same lineages tend to contribute to the same brain region.	Treutlein and Gray Camp	2021	Lineage recording in human cerebral organoids | Nature Methods (He et al., 2021)
Human, macaque and chimpanzee cerebral organoids and adult prefrontal cortex.	Forebrain, midbrain and hindbrain	scRNA-seq for organoids. snRNA-seq for postmortem samples. Generation of a temporal cell **atlas** of great ape forebrain development and uncover human-specific dynamic gene-regulatory features.	Human organoids: 44k cells. Primate organoids: 37k cells	Human neuronal development follows a slower pace than macaque and chimpanzees. Chromatin accessibility divergence between human and chimpanzee correlates with human specific genetic changes in expression profiles.	Treutlein and Gray Camp	2019	Organoid single-cell genomic atlas uncovers human-specific features of brain development | Nature (Kanton et al., 2019)
Brain organoids	Cortex	Idiopathic autism spectrum disorder (**ASD) extensive study** at single-cell level.	665k	ASD involves balance disruption of excitatory neurons of the dorsal cortical plate population and other lineages, caused by shifted expression of transcription factors controlling cell fate during early cortical development.	Vaccarino	2023	Modeling idiopathic autism in forebrain organoids reveals an imbalance of excitatory cortical neuron subtypes during early neurogenesis - PubMed (nih.gov) (Jourdon et al., 2023)
Brain organoids and fetal brain	Cortex	Used snRNA-seq in combination with bulk RNA-seq and ChiP-seq. Transcriptome and epigenome **characterization** in organoids and assess relevance to model human cortical development and associated disorders like ASD.	18k	Generation of an initial map of enhancer elements and corresponding transcripts active in cortical cell lineages dynamics. Organoids mimic development of early fetal cortical primordium. They match nearly all cell clusters of fetal samples except for one. Human gained enhancers are regulators of the earliest phases of human brain development also in organoids and may be involved in the regulation of RG proliferation.	Vaccarino	2018	Transcriptome and epigenome landscape of human cortical development modeled in organoids - PubMed (nih.gov) (Amiri et al., 2018)

Color background on reference cells indicates datasets accessibility being publicly available (green), upon author's request (yellow) or under controlled access (blue).

*****Most entries refer to human brain studies; additionally, most organoids recapitulate up to the second trimester of fetal brain development. Otherwise, it is indicated on the table accordingly.

4. Challenges of scRNA-seq in Neuroscience

Most of the challenges scRNA-seq technology faces when implemented in Neuroscience and other fields are linked to its scale-up evolution towards high-throughput.

Technical limitations include RNA capture efficiency, sequencing depth, and experimental operation. Current capture efficiency of transcripts from single cells does not reach 60%. Limited detection of the number of transcripts per cell results in the overlooking of low-abundance transcripts. However, complementation with bulk RNA-seq data can give an overall transcript signature analysis including low abundance expression genes. In relation to the study of the brain, some studies contemplate how cell stress caused by single-cell disaggregation techniques used for scRNA-seq leads to artificial transcriptomic patterns and support snRNA-seq methodology to minimize undesired transcriptional artifacts (Ding et al., 2020). Another discrepancy is the sequencing depth required versus the number of cells to analyze due to the limited computational capacity per run and the costs of the library preparation and sequencing. On the one hand, the scRNA-seq analysis of complex tissues should focus on the number of cells analyzed to obtain an unbiased realistic view of the cellular composition. On the other hand, to obtain a detailed genetic expression profile of a specific area of the tissue, deeper sequencing of a smaller cell population shall be prioritized (Haque et al., 2017).

A limiting factor technical and analytical is the expertise required for scRNA-seq technology implementation. Experimental operation may alter environment related genetic expression, cell cycle state and cell fate, creating biological noise that affects library preparation and data analysis. Additionally, data pre-processing and downstream analysis requires computational experience to avoid misinterpretations of the data. The use of multiple data analysis pipelines or combination of data acquired by other techniques is a possible solution to increase confidence in the results. Furthermore, standardized commercial kits are already available for the wet lab part of the methodology, and automatic pipelines and user-friendly interfases are being developed to be used by investigators without proper bioinformatic skills, like the Galaxy Community Single Cell Omics workbench that integrates tens of bioinformatic tools and workflows in one place to simplify single-cell analyses (*Single Cell Omics Workbench - Galaxy Community Hub*, n.d.).

Regarding *in vitro* models of the human brain, their fidelity to brain tissue architecture and function is still in question given the lack of molecular signatures to compare to, but reviewed studies suggest correct culture conditions bring out models with similarities to human brain cell types and organization that validate their use for specific research questions in developmental

biology and associated disorders. However, further research is needed to generate reliable *in vitro* models of the aged brain for neurodegenerative studies. The future paths for *in vitro* models and for scRNA-seq are intertwined with scRNA-seq technology being a great tool for validation assays as well as for the identification of novel gene expression signatures.

Above all and linked to all limitations of scRNA-seq there is cost. The investment required for the application of this technology cannot be supported in many fields including clinical diagnosis and personalized medicine even though it has a great potential as a biomarker detection tool. Hopefully the refinement and standardization of the technology will continue to grow and inversely correlate with its cost (Jovic et al., 2022), making it more reachable.

5. Future directions

Future perspectives of scRNA-seq thought 6 years ago have already become a reality (Yuan et al., 2017). The combination of single cell multiomics to create a full picture of healthy and diseased tissues at cellular and molecular levels is already happening in a small scale, as well as the evolution and implementation of spatial transcriptomics to map gene activity in a tissue, even at subcellular resolution (R. Wang et al., 2023). For instance, CITE-seq technique (cellular indexing of transcriptomes and epitopes by sequencing) detects proteins of interest and their corresponding mRNA levels at single-cell resolution, and the Visium Spatial Gene expression commercial kit from 10XGenomics enables co-detection of proteins along with spatial tissue data and scRNA-seq data. Furthermore, wet-lab kits and software for downstream processing and analysis of the scRNA-seq data are commercially available, some even publicly accessible for free, making the technique reachable to non-experts. Even prediction methods have proven useful in single-cell spatial transcriptomics (Sun et al., 2023), which could lead to advances in prediction methods for other single-cells omics in the long run, reducing experimental costs.

Future directions rely on technology refinement and sharing of data making open science the only science. More complete atlases and data accessibility tools will be generated to accelerate understanding of the available single-cell datasets by organism and tissue to apply to literature search and posed biological questions. The manually curated alive database created by Oxford students in 2020 (Svensson et al., 2020) is a good example of open science application for scRNA-seq data, a categorized collection of single-cell transcriptomic studies that keeps growing by being open to new submissions.

Upgrade and standardization in sample processing and computational analysis tools, followed by better incorporation of whole genome sequencing data, genetic expression profiles, epigenetic markers, proteomics, metabolomics and special data and the translation of the results into different research areas seem the next steps predicted for these technologies. Although interdisciplinary research, based on careful integration of different backgrounds, still requires major changes to work out, especially in the way scientific capitalist society is built on short-term output merits, the outside-the-box thinking mentality achieved by combination of research fields could be the way to novel breakthroughs in Neuroscience and other Medicine and Biology sectors.

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