

Identifying Single Cell Transcriptomic of Cerebellar Cells Using Drop-Seq Technology

By
Mahsa Rohani

Supervisor: Shayan Moghimi

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Department of Biology
Faculty of Science
Baha'i Institute for Higher Education (BIHE)

Abstract

The cerebellum takes 10 percent of the brain and contains more than half of the total number of neurons in the brain. Single-cell RNA sequencing is the capable technology to identify and function of diverse cell types in the cerebellum. Traditionally, RNA sequencing was achieved by employing in-plate techniques. This class of approaches, however, suffer from low efficiency and low throughput. The vast diversity of cell types in the cerebellum required novel high-throughput techniques in order to discover new cell types and understand how they function. RNA sequencing with droplet-based technologies has recently been developed to aim this goal, subcategorizing in Drop-seq, inDrop, and 10X genomic techniques.

In this literature review, the similarities and differences between droplet-based techniques are discussed. Also, some case studies have been summarized in each of which a droplet-based technique has been used to discover new cell types in the cerebellum.

Overall, Drop-seq has proven to be a low-cost and fast method to analyze mRNA expression in thousands of cells simultaneously, and it is expected to accelerate biological discoveries in near future.

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List of Abbreviations

CNS: Central Nervous System

CEL-seq: Cell Expression by Linear Amplification and Sequencing

DCN: Deep Cerebellar Nuclei

Drop-seq: Droplet-based Sequencing

ECN: Excitatory Cerebellar Neurons

FACS: Florescent Activated Cell Sorting

GCPs: Granule Cell Precursors

GCs: Granule Cells

MACS: Magnetic Associated Cell Seperation

PCs: Purkinje Cells

RL: Rhombic Lip

RNA: Ribonucleic Acid

RT: Reverse Transcription

SCRB-seq: Single Cell RNA Barcoding and Sequencing

SCRNA-seq: Single Cell RNA Sequencing

SMART: Switching Mechanism at the End of the 5'-end of the RNA Transcripts

SnRNA-seq: Single Nucleus RNA sequencing

STAMPs: Single-Cell Transcriptions Attached to Microparticles

UBCs: Unipolar Brush Cells

VZ: Ventricular Zone

UMI: Unique Molecular Identifier

1. Introduction

The brain is a complex organ of nuclei and neural pathways and it regulates the activity of visceral, endocrine, and musculoskeletal functions and makes higher mental functions that include memory, imagination, and creative thought. The average weight of an adult human brain is about 1400 gr, nearly 2% of total body weight. The central nervous system (CNS) consists of two main parts, the brain and the spinal cord, which contains as many as 100 billion neurons. The three characterized parts of the brain are the cerebrum, brainstem, and cerebellum. The brain has five major subdivisions formed in the embryonic stages of development that include myelencephalon (medulla oblongata), metencephalon, mesencephalon (midbrain), diencephalon, and telencephalon (cerebral hemisphere). The cerebrum consists of cerebral hemisphere and diencephalon, the brainstem consist of midbrain, pons, and medulla oblongata¹ (Figure 1).

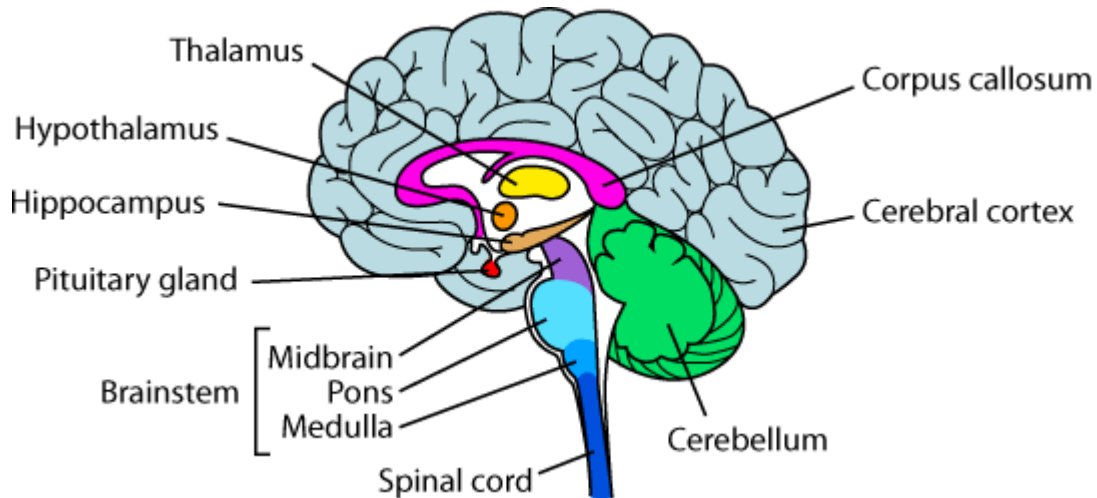


Figure 1. Parts of the Brain. This figure is taken from ².

1.1. Anatomy of the cerebellum

The cerebellum contributes to only 10% of total brain volume and it consists of more than half of the total number of neurons in CNS in mammals. The cerebellum is the first brain structure to emerge in embryogenesis and developed until 2 years in humans and 3

weeks in mice³. The cerebellum structure derived from the dorsal part of the most anterior hindbrain⁴ and is separated from the cerebrum by tentorium. It contains 2 lateral hemispheres and a narrow midline zone and there are many parallel thin transverse folds called folia. There is an extremely convoluted gray matter in the outer layer of the cerebellum, called the cerebral cortex, and it surrounds a highly branched body of white matter known as the arbor vitae (tree of life). The arbor vitae surround 3 pairs of deep cerebellar nuclei in the central cerebellar white matter that are called corpus medullare. The cerebellum consists of 3 lobes by 2 fissures. The anterior is separated from the posterior lobe by the primary fissure. The posterolateral fissure is located between two lobes, posterior and flocculonodular. The cerebellum connects directly to the various structures including the brainstem, spine, and diverse cerebral subcortical and cortical regions. 3 pairs of peduncles inferior, middle, and superior, attach the cerebellum to the brainstem. These peduncles are caused that all efferent and afferent of cerebellum reach to their target. The middle peduncle is the largest and composed of pontocerebellar fibers that arise from pontine nuclei⁵.

From an anatomical point of view, the cerebellum is divided into 3 major lobes, flocculonodular (vestibulocerebellum, a small part of the cerebellum), the medial (vermis) and intermediate (paravermis) lobes that commonly called the spinocerebellum, and the lateral zone (hemispheres) considered as the cerebrocerebellum. A unique feature of flocculonodular is that it sends outputs directly to the vestibular nuclei. Vestibulocerebellum lobe regulates eye movement and coordination of eye and head movements by controlling medial vestibular nuclei activity. Spinocerebellum receives somatosensory information directly and indirectly from the spinal cord and is important in motor control and balance. The lateral zone (cerebrocerebellum) is the largest part of the cerebellum and in connection with the cerebral cortex. This part has expanded tremendously in humans and was recently involved in various cognitive functions⁵ (Figure 2)

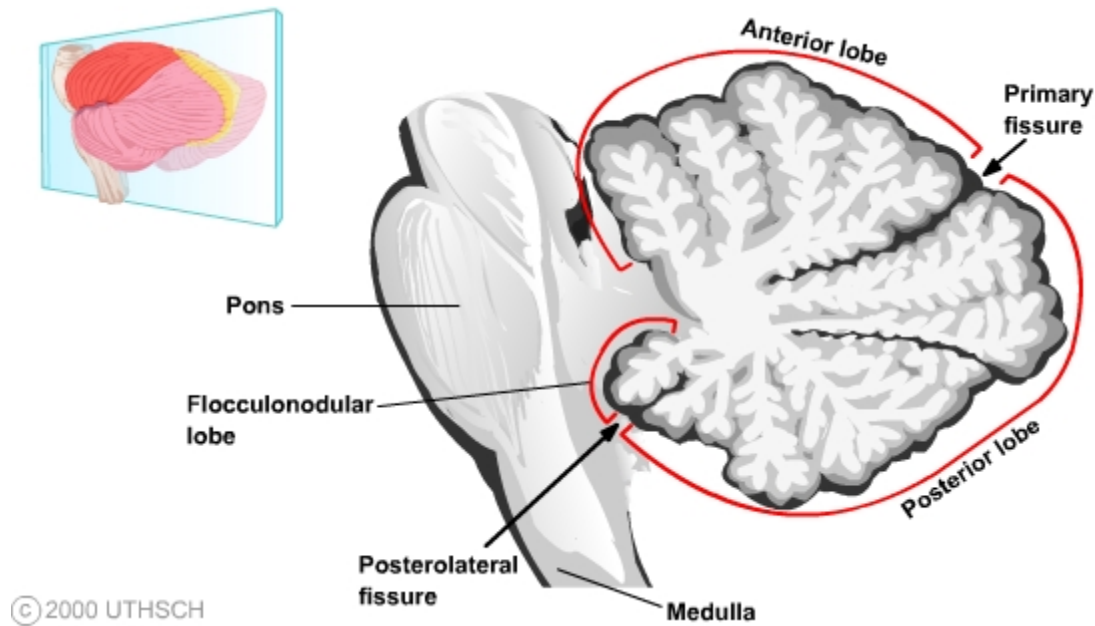


Figure 2. Parts of the Cerebellum. This figure is taken from ⁶.

1.2. Functions of the cerebellum

The cerebellum has critical roles such as motor coordination, motor learning, posture balance, and visual functions^{7,8}. The cerebellum is important in the control of smooth and skillful movements and controls cognitive and emotional functions⁴. The cerebellar dysfunction could lead to cognitive affective syndrome and neurological and neuropsychiatric symptoms, such as executive dysfunction, impaired working memory, disorders in visuospatial, language deficits, personality behavior, ataxia, tremor, autism spectrum disorder and schizophrenia^{5,8}.

Previous studies were performed for identifying cell types of developing cerebellum in mice. The major neuron types in the cerebellar cortex included Purkinje, stellate, Golgi, basket, and granule cells⁹. In the following sections, detailed explanations of classifications methodologies of these neuron types are presented. Broadly speaking, three main types of cerebellar neurons were classified into excitatory glutamatergic (granule cells) and inhibitory gammabutyric acid (GABAergic) and several types of interneurons^{8,10,11}. The granule cells (GCs) are glutamatergic and the other neurons are all GABAergic⁹. These neurons are generated during late embryonic and early postnatal life¹², deep cerebellar nuclei (DCN) neurons are developed at embryonic day E10-E17,

Purkinje cells at E11-E13, Golgi cells at E12-E15 and stellate/basket cells postnatally¹³. The Cerebellar structure emerges from the ventricular zone (VZ) and rhombic lip (RL) in the early embryonic stage⁸. Granule cells produced by the rhombic lip and the other neurons produce from the cerebral VZ⁹.

1.3. Cell Types in the Cerebellum

1.3.1. Purkinje Cells

The GABAergic Purkinje cells (PCs) are the principal neurons in the cerebral cortex with a complex dendritic tree that extends into the molecular layer¹⁰. The pancreas transcription factor 1-a (ptf1-a) precursors of the ventricular zone (VZ) produce Purkinje cells¹². The cerebellar interneurons include stellate and basket cells that are in the molecular layer, candelabrum cells in the Purkinje cell layer, and Golgi cells in the granular layer⁸. GABAergic interneurons are produced from Pax-2-positive cells and appear at E13.5 in the ventricular zone and migrate into cerebellar parenchyma¹⁴. The sonic hedgehog (SHH) is secreted by PCs after E17.5, which manages the great postnatal neurogenic phase by stimulation proliferating of granule cell precursors (GCPs) and nestin-expressing progenitors. PCs have two functions as a developmental regulator of postnatal cortex growth driven by proliferation and as a synaptic bridge between the two main of glutamatergic neuronal subtypes¹⁵. Development of PCs causes the formation of dendritic arbors and synapses onto granule cells in their final maturation phase and probably Wnt3 is involved in this phase¹⁶. Wnt3 is expressed in Purkinje cells during this period, and its expression depends on granule neurons. There are various growth factors that are necessary for PC survival that include nerve growth factor, acetylcholine, neurotrophin 4/5, brain-derived neurotrophic factor, and ciliary neurotrophic factor¹⁶.

1.3.2. Golgi cells

large and most numerous interneurons of the granular layer are Golgi cells¹⁷. Golgi cells include an irregular soma that gives out a series of basal dendrites, a wide axon, and two

or three apical dendrites (Figure 3)¹⁸. Camillo Golgi described the Golgi cells for the first time. Golgi cells dendrites extend in all directions and are in contact with thousands of granule cells as well as unipolar brush cells. Golgi cells are inhibitory interneurons that observed in the granular layer and use GABA as their neurotransmitter. stellate/ basket cells give inhibitory projections to Golgi cells¹⁹. These cells have a central role in the regulation activity of granular layer¹⁸.

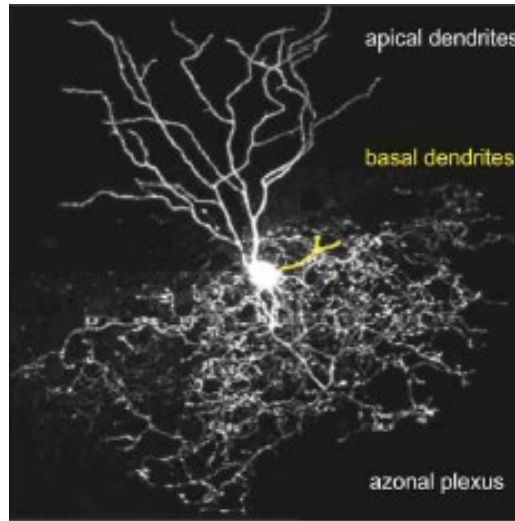


Figure 3. The microscopy reconstruction of Golgi cells. This figure is taken from ¹⁸.

1.3.3. Basket and Stellate Cells

The small GABAergic interneurons in the molecular layer of the cerebellum are basket and stellate cells. The basket and stellate cells are born at the second postnatal week. In rats, the basket cells are born between P2 and P17 in the inner molecular layer and the stellate cells between P4 and P19 in the outer molecular layer¹⁷. Excitatory synaptic input is received by the basket and stellate cells from granule cell axons (parallel fibers) and their axons produce an inhibitory synapse with Purkinje cells. The majority of basket cells are located at the bottom half of the molecular layer but the majority of stellate cells are located in the top half²⁰.

1.3.4. Granule Cells

Glutamatergic neurons consist of granule cells, unipolar brush cells, and excitatory projection neurons. The smallest and most numerous cerebellar neurons are granule cells¹⁰. After proliferative cycles, precursors of Granule cells are located in the outer part of the external granular layer and exit the cell cycle¹⁷. Unlike all other cell types in the cerebellum, which emerge from VZ, the granule neurons are derived from rhombic lip between E15 and P15. The location of the rhombic lip is between the fourth ventricle and the neural tube^{16,21}. Granule cells receive inputs from mossy fibers and the process of maturing of Granule cells is induced by secreting Wnt family ligands¹⁷.

1.3.4. Excitatory Projection Neurons

Excitatory projection neurons are the largest section of all neurons in the cerebellar nuclei. The excitatory cerebellar neurons (eCN) are the first neurons that produce in the embryonic cerebellum (E9.5-E12.5) by Atoh1-expression rhombic lip and then unipolar brush cell directly¹⁵. These neurons are followed by PCs and local CN interneurons. They are produced from neural progenitors in the dorsal telencephalon^{10,22}. The GABAergic inhibitory interneurons modulate the activity of these projection neurons²².

1.3.5. Unipolar Brush Cells

Unipolar brush cells (UBCs) are small interneurons in the cerebellar cortex, as the main recipient of mossy fibers^{10,23} and generated from the rhombic lip at E15.5 and P2^{17,24} and migrate along pathways through the cerebellum²⁴. S. Hockfield described the morphology of this new cell type for the first time and then called mono dendritic cells. The size of UBC cells is between granule cells and Golgi cells¹⁹. These cells have a critical role in cerebellar functions such as consolidating motor learning and controlling²⁵.

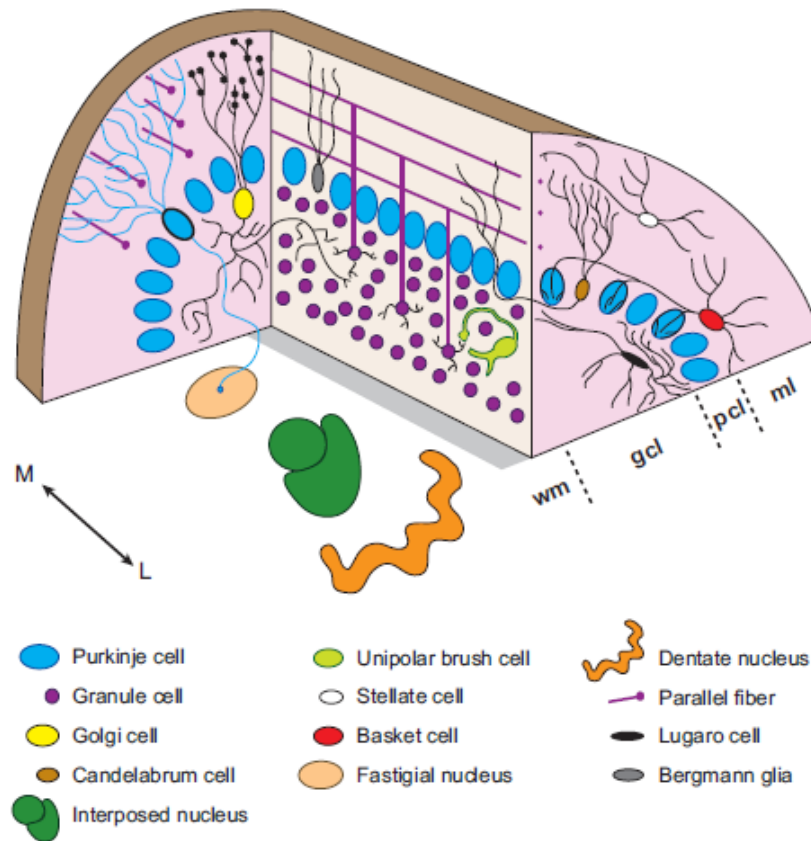


Figure 4. The cell-type in the cerebellum. Acronyms: pcl, Purkinje cell layer; ml, molecular layer; gcl, granule cell layer; wm, white matter; M, medial; L, lateral. This figure is taken from ²⁶.

1.4. Connections between neurons of the cerebellum

As we know the cerebellum has a central role in our lives and it acts as a coordinate center and our movement and balance are dependent on the cerebellar system and connection between neurons. The Purkinje cells are inhibitory and used from GABA (γ -aminobutyric acid) as their transmitter and primary output from the cerebellar cortex is provided by Purkinje neurons, whereas granule cells are excitatory neurons. The Stellate and basket cells are involved in the modulation of Purkinje cell output and the Golgi cells, which include GABA and glycine and act as an inhibitor of granule cells (Figure 5)¹⁶.

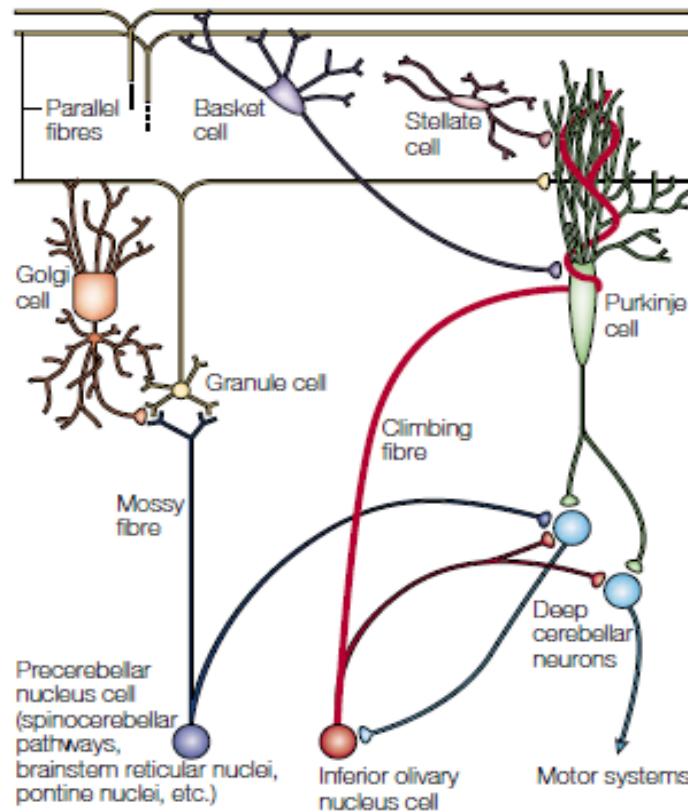


Figure 5. Neurons in the cerebellum and their connections. This figure is taken from ¹⁶.

Our knowledge about cell type complexity of the brain is unknown and widely discussed. We should learn the functions and responses of each cell type to understand how complex tissues work²⁷. The transcriptome is described as the entire RNA transcripts of an individual cell. Recent studies have also shown that obtaining information on single-cell transcriptomes is possible by RNA-sequencing analysis²⁸. Further understanding of the molecular characterization of different brain cell types can be obtained by Single-cell RNA sequencing (scRNA-seq) method⁸. Focusing on cerebellar development helps to future investigations of its functions and diseases³.

1.5. Single-Cell Transcriptomic Analysis

Single-cell transcriptomic data have rapidly grown public in genomic science²⁹. Single-cell transcriptomics analysis was pioneered in 1990 by Norman Iscove and he used from exponential amplification of single-cell cDNAs by PCR³⁰. James Eberwine used linear

amplification of cDNAs by T7 RNA polymerase-based in vitro transcription¹³. Single-cell transcriptomics has a crucial role to characterize cell-type-specific functional modules, intercellular signaling pathways, and cellular diversity by profiling thousands of cells in a single experiment³². Single-cell RNA sequencing was first introduced in 2009³³. It is a powerful tool in biomedical research because it is capable of identifying cell types and subtypes in tissue and also in a lesion of disease to identify molecular dynamic and cell types³⁴.

There are many current applications of scRNA-seq: 1) identifying cell composition of tissues, including rare and unknown populations, 2) knowing about cell differentiation and progeny from stem cells, 3) understanding about clonal evolution of cancer and cell of origin resulting in cancer extension, 4) determining the relationship between cells through cell receptor-ligand networks, 5) investigating about cellular response to manipulation of DNA or drugs that used in the development of new treatments in future³⁵.

ScRNA-seq has many advantages including a resolution at a single nucleotide, ability to distinguish different isoforms and allelic expression, possibility to identify new transcripts, low sample quantity requirement, and lower cost³⁴ than microarrays applications and is expected to replace them³⁶. RNA-Seq is the first sequencing-based method that has been used extensively for in transcriptomic studies that allows the entire transcriptome to be investigated in a very high-throughput and quantitative manner^{36,37}.

Generally speaking, there are two categories of ScRNA-seq, namely plate-based and droplet-based. Plate-based methods capture and sort individual cells in multiwall plates or tubes. The main drawback of plate-based techniques is the limited number of cells that can be collected. Additionally, they require cell classification, making it impossible to maintain the heterogeneity of central nervous system cells. In droplet-based techniques, on the other hand, thousands of cells can be captured and sequenced simultaneously. This high-throughput approach is advantageous to identify variations in specific cell populations for each condition. With sufficient statistical power, we will be able to develop understanding of pathological changes in cellular responses³⁸. In the following sections, the main plate-based and droplet-based techniques are introduced.

1.5.1. Plate-Based Techniques

Single-cell RNA barcoding and sequencing (SCRB-seq) is a promising tool for the identification of heterogeneous cell populations. This method enables the efficient characterization of thousands of single cells at the time with minimal cost. The SCR-seq acts on a template-switching process and based on the reverse transcript that converts poly(A)+ mRNA to cDNA from isolated single cells. Every single cell includes a well-specific barcode and unique molecular identity (UMI). SCR-seq is optimized for studying mRNA from large cell numbers and it is a cost-efficient method and covers deep and full-length transcriptome³⁹.

SMART reaction (Switching Mechanism At the end of the 5'-end of the RNA Transcript) has been developed for the improvement of transcriptome coverage⁴⁰. Picelli et al. has shown that Smart-seq is able to analyze from both single cells and quantitative amounts of purified RNA. Smart-seq2 improved reverse transcription and template switching and is able to obtain an increased cDNA yield from single cells with higher sensitivity and fewer technical biases⁴¹. A recent study explained details of Smart-seq and Smart-seq2 and these methods had much higher sensitivity compared to other methods⁴⁰. Smart-seq is a robust and efficient method for ScRNA-seq that employs the technology of SMART template-switching and also amplification of cDNA using 12 to 18 cycles of PCR to create full-length cDNA⁴². Smart-seq and Smart-seq2 are similar protocols to generate full-length libraries and they are different in isolation cells and reaction volume⁴³ so, Smart-seq2 has improved in accuracy, coverage, and detection compared to Smart-seq⁴⁴.

Another method in single-cell transcriptome is CEL-seq that can be used for the identification of unknown cells. Past studies showed the CEL-seq (Cell Expression by Linear amplification and Sequencing) had certain advantages over the PCR amplification, mainly in sensitivity, reproducibility, and linearity. To analyze complex tissues, in which a diverse population of various cell types exists, CEL-seq is a suitable technique⁴⁵.

1.5.2. Droplet-Based Techniques

There are many limitations such as time and cost for preparing libraries of many individual cells, using the abovementioned plate-based techniques. Recently three new, fast, easy, and low-cost technologies to profile thousands of cells have been developed, called 10X genomic, Drop-seq, and inDrop⁴⁶. These are droplet-based technologies that are currently and commonly used as high-throughput approaches⁴⁷. A brief introduction to of these three methods is given below.

Drop-seq is a low-cost and high-throughput method to analyze mRNA expression in thousands of cells by encapsulating cells into droplets^{27,48}. In this platform, cells are lysed and the uniquely barcoded poly (dT) primers on the bead capture the RNA of cells. In the next step, cDNA libraries are generated to produce single-cell transcriptomics attached to microparticles (STAMPS).

In June 2016, inDrop (indexing in droplet) system with a high-throughput instrument started at Harvard University. Inspired by the drop-seq system, cells are encapsulated with hydrogel beads and UMI-barcoded primers are carried and reverse transcription (RT) occurred. After ultraviolet radiation is caused by the release of primer and cDNA is generated in each droplet. Finally, droplets are broken and the cells are amplified for sequencing and are enabled for barcoding 40000 cells⁴⁹. inDrop technique differs from the Drop-seq mainly by the numbers of barcodes; a fewer number of barcodes in inDrop are used compared to Drop-seq, leading to limited identifiable cell types but results in 10 percent gain in the efficiency by using soft hydrogel beads³⁸.

Chromium system (10x genomics), launched later in 2016, is a scalable method for the characterization of transcriptomes of thousands of cells at once⁴⁹. 10X is able to distinguish scarce cell populations⁵⁰. This method includes beads, barcoded primers, and UMI in droplets and the kit for cDNA generation. This method is capable of increasing the number of transcripts with a very low doublet rate. The company of 10x genomics (Chromium Software Suite, 10x Genomics, Pleasanton, CA, USA) has commercial applications for analysis and visualization of single-cell gene expression data⁴⁹. The 10x system has higher precision and molecular sensitivity with less technical noise and is a

safe choice for the majority of applications. The main downside of 10X is its significantly higher cost³³.

2. Discussion

In this thesis, we narrowed down the scope of the discussion to the Drop-seq technology only. Elaborate details of the Drop-seq method are given in the following section.

2.1. Drop-seq Technology

Drop-Seq is a complex and multi-step technique. Here, we enumerate the steps involved in any Drop-Seq study. The first step is the isolation of single-cells extracted from a target tissue and their suspension in the droplets with distinct beads. When each cell is lysed in the droplet its mRNA binds to microparticles. Then, they are reverse-transcribed into cDNAs and a set bead forms that are called "single-cell transcriptomes attached to microparticles" (STAMPs) (Figure 6A). Using barcoded STAMPs for analyzing individual cells results in high-throughput mRNA-seq. Microparticles (beads) are applied to determine a large number of differently barcoded primer molecules in droplets. Each bead contains oligonucleotide primers. Each oligonucleotide has four parts (Figure 6B): "PCR handle" that is a common sequence and used in PCR amplification, "cell barcode" similar across all the primers, a Unique Molecular Identifier (UMI) that is different on each primer and 30-bp oligo dT sequence to capture the mRNAs and priming reverse transcription. Macosko et al. developed a "split-and-pool" DNA strategy to generate great numbers of beads (Figure 6C) after the UMI is synthesized²⁷.

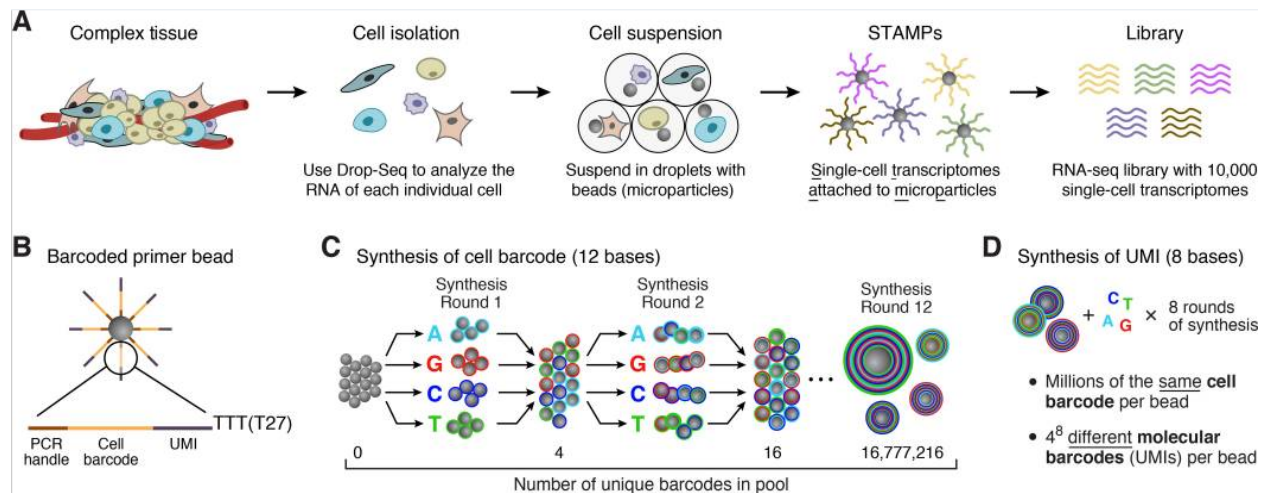


Figure 6 Molecular Barcoding of Cellular Transcriptomes in Droplets. This figure is taken from ²⁷.

Isolating single-cell from the tissue is the first step in single-cell sequencing technologies. The classical method to isolate cells from the suspension is Micromanipulation and using microscopes and capillary pipettes to isolate single cells (Figure 7A). This method is time-consuming and low throughput⁵¹. Microfluidic technology is a popular technique for single-cell isolation and performed with low sample consumption and low cost. Recently, developing in microfluidic technology has transformed research ability in basic scientists and clinicians (Figure 7B). Another technique for single-cell isolation is microdroplet-based microfluidics that enables screening of thousands to millions of cells with high throughput and low cost⁵¹.

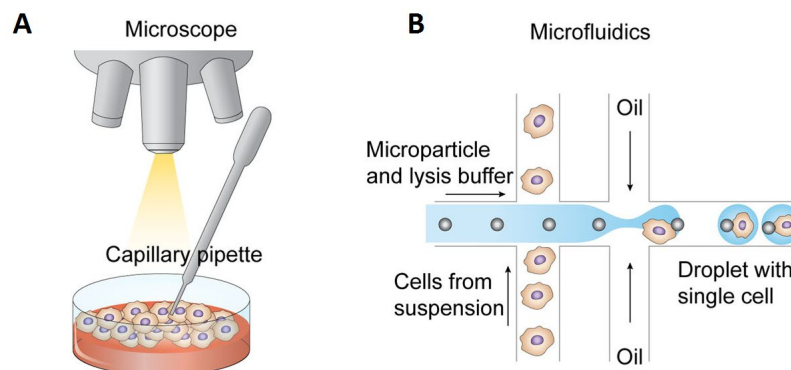


Figure 7. Single Cell Isolation. A. Micromanipulation used from microscope-guided capillary pipettes. B. Microfluidics Technology (e.g. Drop-seq). This figure is taken from ⁵¹

High quality of single-cell RNAseq depends on conditions of primary cells from tissues including fixing cells or tissues in 80% methanol and storage -20 or -80 °C for several months. In addition to these conditions, three additional factors that can drastically change the outcome are sample quality of ScRNA-seq technique containing the number of dead cells, debris cells in the sample of solid tissues, and high level of a rare population of cells. Also, flow cytometry could provide a characterization of the sample using fluorescent-labeled monoclonal antibodies. When there is a high level of dead cells, debris and a rare population of cells, it is suggested to use two of the most common cell separation techniques, Fluorescent Activated Cell Sorting (FACS) and Magnetic Associated Cell Separation (MACS) to improve the quality of the sample as described below³⁵.

Fluorescent Activated Cell Sorting (FACS) is used for isolating highly purified single cells. First, the cells are tagged with a fluorescent monoclonal antibody that identifies specific markers and enables the sorting of different populations. Then, on the basis of fluorescence, cells are separated. FACS removes the dead cells and residues, deletes cell mass and defective sample particles, and purifies target cells that are characterized by markers labeled with fluorescent antibodies ³⁵. Generally speaking, the sorting methods utilize electrostatic, physical, piezoelectric, or magnetic fluidics. FACS, in particular, uses droplet-based electrostatics to sort cells. FACS needs a conductive solution that is usually isotonic phosphate-buffered saline. There are two conditions before droplet breakoff: 1) the presence of a target cell and 2) lack of a non-target cell³⁵. There are some limitation around the FACS: isolation of single cells requires large starting volumes because isolation is difficult from low input numbers $<10,000$, laser capture micro dissection needed to have the laser system and computer to isolate cells from solid samples⁵¹ (Figure 8).

MACS is a cell separation method that labels target cells with paramagnetic iron oxide nanoparticles that are attached to antibodies on cell surface receptors in the high gradient magnetic field. MACS is less effective than FACS but MACS is more time effective³⁵ (Figure 8).

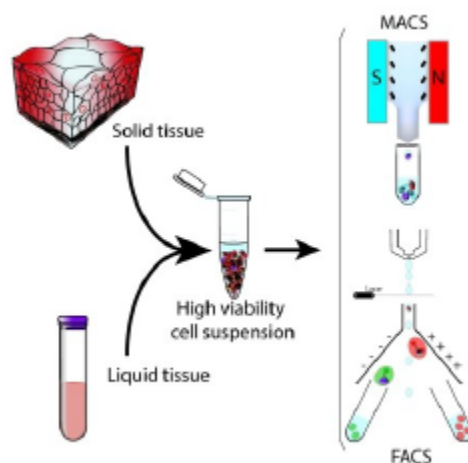


Figure 8. Sample Preparation. This figure is taken from ³⁵

To generate the cell barcode, the first step is "split, and pool" DNA strategy (Figure 1). Many microparticles are divided between equally sized groups, one of the four DNA bases (A, G, C, or T) is added to each, after participated together in each cycle and occurred 12 split-pool cycles after there are 4^{12} (16,777,216) possible sequences and each microparticle have different sequences (Figure 1C). Next, for generating a unique molecular identifier (UMI), eight rounds occur and generate the 4^8 (65,536) UMI to each bead and dT (T30) attached to the 3' end of each oligo (Figure 1D)²⁷.

The droplet strategy reduces the cost and increases the reaction high-throughput. These methods are the same in generating droplets and use the unique molecular identifier (UMI) and beads primer with barcodes to differentiate individual cells but there are differential approaches between them that include bead making, barcode design, and cDNA amplification. The material of beads in Drop-seq are brittle resin whereas 10X and inDrop techniques utilize hydrogel beads. The main difference between these two materials is that hydrogels are capable to immobilize the primers throughout the beads while the resin beads can only capture the primers on their surface (surface attachment). The performance of the three approaches has been analyzed and compared. The results determine the strengths and weaknesses of each method³³.

The main advantage of the Drop-seq technique is the cost that is the most inexpensive method^{33,43}. As illustrated in Table 1, one can see that the instrument cost of Drop-seq is 60 percent of that for inDrop and 10X techniques. The main difference between inDrop and 10X is the running cost per cell, 0.10, and 0.50 dollars, respectively. By approximating the instrument and sequencing costs, the overall cost per cell for these methods is estimated. In conclusion, the cost per cell of 10X is by far the most expensive one, followed by inDrop and Drop-seq³³ (Table 1).

Table 1. Approximate cost of three main droplet-based techniques³³.

	inDrop	Drop-seq	10X
Instrument price	\$50,000	\$30,000	\$50,000
Instrument cost per cell ¹	\$0.07	\$0.04	\$0.07
Running cost per cell	\$0.10	\$0.10	\$0.50
Sequencing cost per cell ²	\$0.30	\$0.30	\$0.30
Overall cost per cell	\$0.47	\$0.44	\$0.87

1. Estimated for 1000 samples per day and two years of constant service.
2. 50000 reads per cell on average

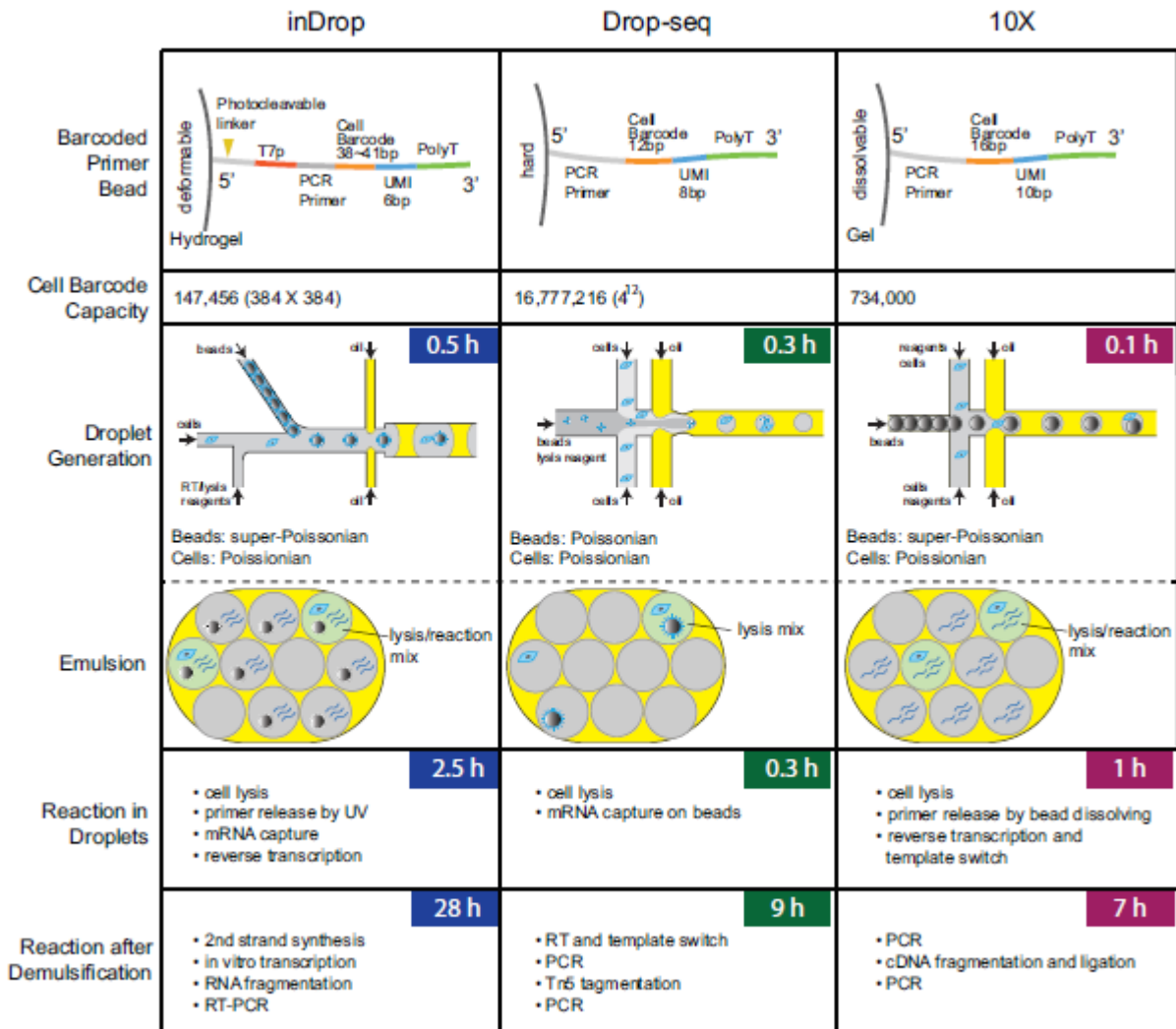


Figure 9. Compared the Features of the Three Systems. This figure is taken from ³³.

2.2. On comparing Drop-seq and Plate-based methods

Ziegenhain et al in 2017 performed a comparative analysis of ScRNA-seq methods. These methods included Drop-seq, SCRB-seq, Smart-seq, Smart-seq2, and CEL-seq. Smart-seq is further optimized in cost, accuracy, sensitivity, and full-length transcripts in Smart-seq2⁴³. Smart-seq2 is a low-throughput plate-based method⁵², a low-cost technique that generates full-length cDNA from RNA transcripts⁵³. It is the most sensitive method and it's able to detect the most genes per cell and across cells so it's the most accurate method and is the best method for measurement and count per million. It cannot use UMI and shows more amplification noise than other methods. Smart-seq2 is

preferable when quantifying transcriptomes of few numbers of cells whereas, Drop-seq is preferable for large numbers of cells with low sequencing depth. Drop-seq method has minimal cost calculation and is the most efficient method and followed by SCRB-seq and Smart-seq2. SCRB-seq has the highest ability to detect differentially expressed genes and uses PCR amplification. CEL-seq is a UMI method and relies on cDNA amplification by in vitro transcription^{43,54}.

2.3. Applications of Drop-seq in Identifying Cerebellum Cell-types

Recently, few studies performed scRNA-seq (Drop-seq) to identify cell-types of mouse brain development. One of them is “the identification of distinct neural and non-neural cell types by methanol-based cell fixation of mouse brain cells” by Alles et al in 2017⁵⁵. Parts of cerebellum and hindbrain of a newborn mice was dissected for the experiment since they are composed of a mixture of different cell types including neurons, glia, and non-neuronal cells. Then they used methanol and fluorescence-activated cell sorting (FACS) and obtained a mixture of neurons, glia, and non-neuronal cell types into ice-cold methanol. They identified 12 clusters of 4366 fixed cells into cell populations (Figure 10B). Cell population allows identifying gene expression of, neurons, and glial cells. They were able to identify a wide range of neuronal cell types: “granule cells (proliferation marker Mki67; neuronal marker stathmin-like 2, stmn2), excitatory neurons (glutamatergic neuronal marker Slc17a6/VGLUT2 and neuronal markers Stmn2 and tubulin beta 3, Tubb3), inhibitory neurons (GABAergic neuronal markers GAD67, Gad1 and VGAT, Scl32a1; Tubb3; transcription factor AP2 beta, Tfap2b) and cerebellar neurons (Cerebellin, Cbln1; lncRNA Meg3; Stmn2)”. They also identified “oligodendrocyte progenitors (oligodendrocyte transcription factor 1, Olig1; fatty acid-binding protein 7, Fabp7), myelinating oligodendrocytes (myelin basic protein, Mbp; Olig1), microglia (chemokine receptor Cx3cr1), astrocytes (gap junction protein alpha 1, Gja1; aquaporin 4, Aqp4; Fabp7) and astrocyte/neuronal progenitors (vimentin, vim; Aqp4, Fabp7) and a subtype of myelinating glia that expressed myelin protein zero (Mpz), Schwann cells”. As far as non-neural cells are concerned, they detected “endothelial cells expressing

platelet/endothelial adhesion molecule 1 (Pecam1) and endothelial progenitors (vitronectin, Vtn)⁵⁵.

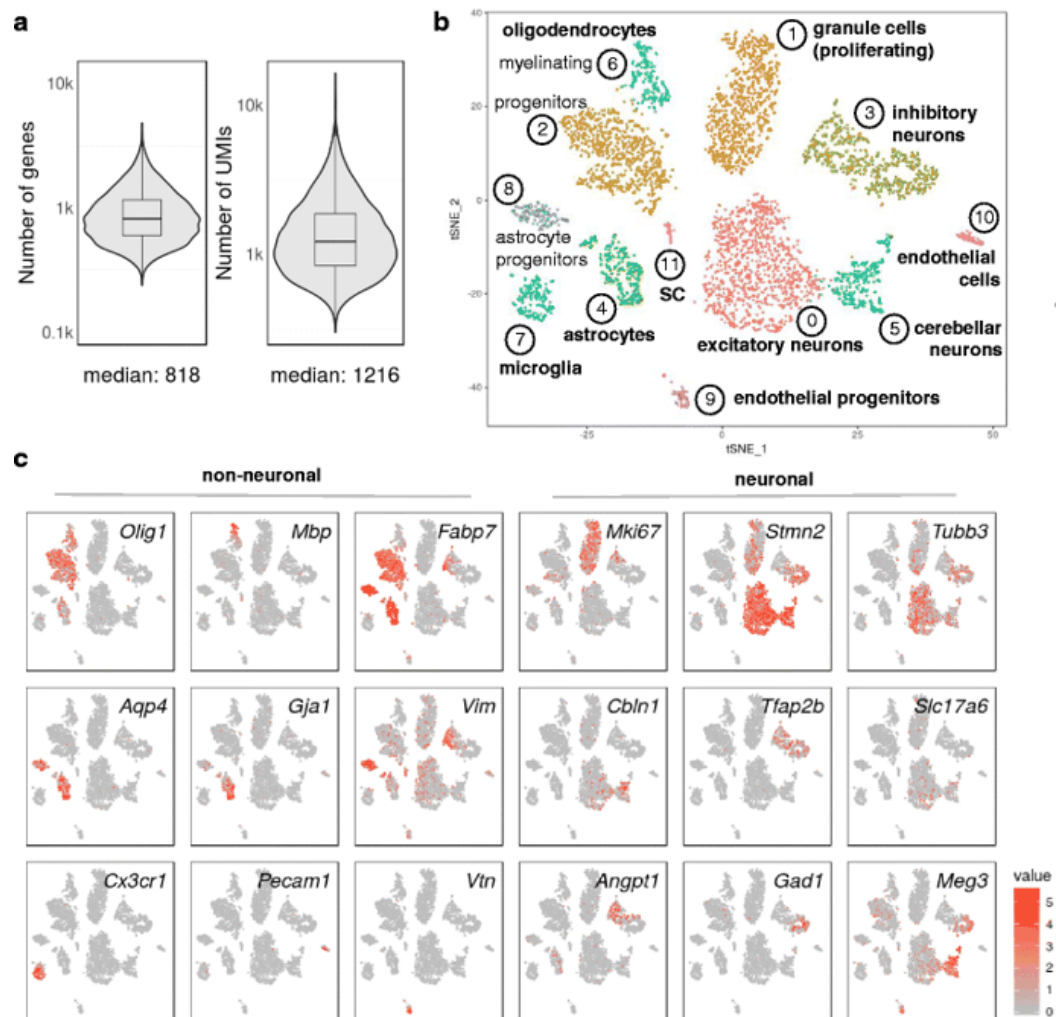


Figure 10. Cell populations of mouse brain (A) The median number of genes and UMIs detected per cells in Drop-seq data. (B) 12 clusters of 4366 distinct cells and cell populations marked by color. (C) Known marker gene expression clusters of brain cells. This figure is taken from ⁵⁵.

In 2018, cellular populations during murine cerebellar development were analyzed and used 10x Genomics. The scientists investigated 39,245 single cells from twelve time points that included embryonic 10 (e10) through e17 (e10, e11, e12, e13, e14, e15, e16 and e17) and four postnatal time points (P0, P4, P7 and P10) (Figure 10A). They performed hierarchical clustering and used dynamic Tree Cut application and identified 48 distinct clusters (Figure 11B). They calculated the cell populations in each cluster that

expressed each gene and they found the top 100 genes for each cluster. For assign cluster identify, they used different marker genes, for example, Hes5 to indicate progenitor cells, Slc1a3 to mark astrocytes, Pax6 to mark GNPs and/or granule neurons, Lhx5 to identify GABAergic progenitors, Pax2 to identify GABAergic interneurons, Meis2 to identify glutamatergic CNs and Calb1 to mark PCs. Cell types included cells that express markers such as Lmx1a and Msx1 (cluster 14), early progenitor populations that express Id1 and Msx3 (clusters 6, 16, 18, 20, 3, 30, 8, 17, 38, 36), glutamatergic neurons comprising GNPs and/or granule cells that are marked by Atoh1 and/or Pax6 (clusters 21, 10, 2, 7, 37, 26, 11, 9, 4, 13, 32, 22) and glutamatergic CNs that uniquely express Meis2 and Lhx2 (clusters 5, 39, 23) (Figure 12C). Identified GABAergic progenitor clusters (15, 1, 27, 28) that express Lhx1 and Lhx5 and two clusters, 34 and 40, that like GABAergic interneurons that express Pax2 and Lbx1. Cluster 12 consists of differentiating PCs that are characterized by expression of PC marker genes Calb1 and Car8. Glial populations (clusters 19, 25, 29, 31, 35) that contain a cluster of oligodendrocytes that express Sox10 and Olig1 (cluster 29), Bergmann glia in clusters 19 and 31 that express high levels of Hopx and Gdf10 and microglia (cluster 44) that high expression of Tmem119 and Itgam (Figure 12C). Identified two midbrain clusters (24, 41) that express Tlx3, erythrocytes clusters (33, 43, 48) marked by Hba-a2, Alas2, Hbb-bt, and Eech expression, endothelial cell precursors (cluster 42, 45) that express Cd34 and Pecam1, meninges (cluster 46) that express Foxc1 and a cell population that express cilium marker genes (cluster 47) such as Dynlrb2 and Meig1 (Figure 12C)³.

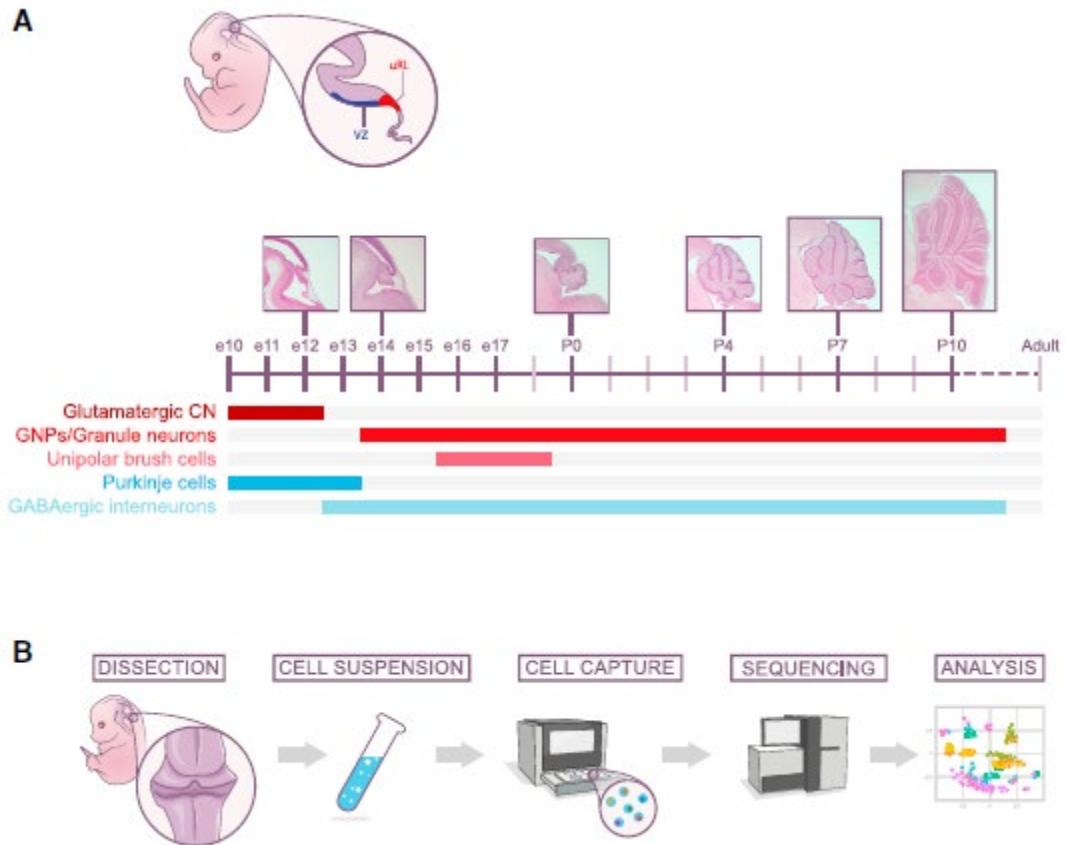


Figure 11. Summary of the Experimental Strategy. (A) Schematic diagram showing the mouse cerebellar development and major neuronal cell types in the several time points. (B) Image of using the 10x genomics chromium platform. This figure is taken from ³.

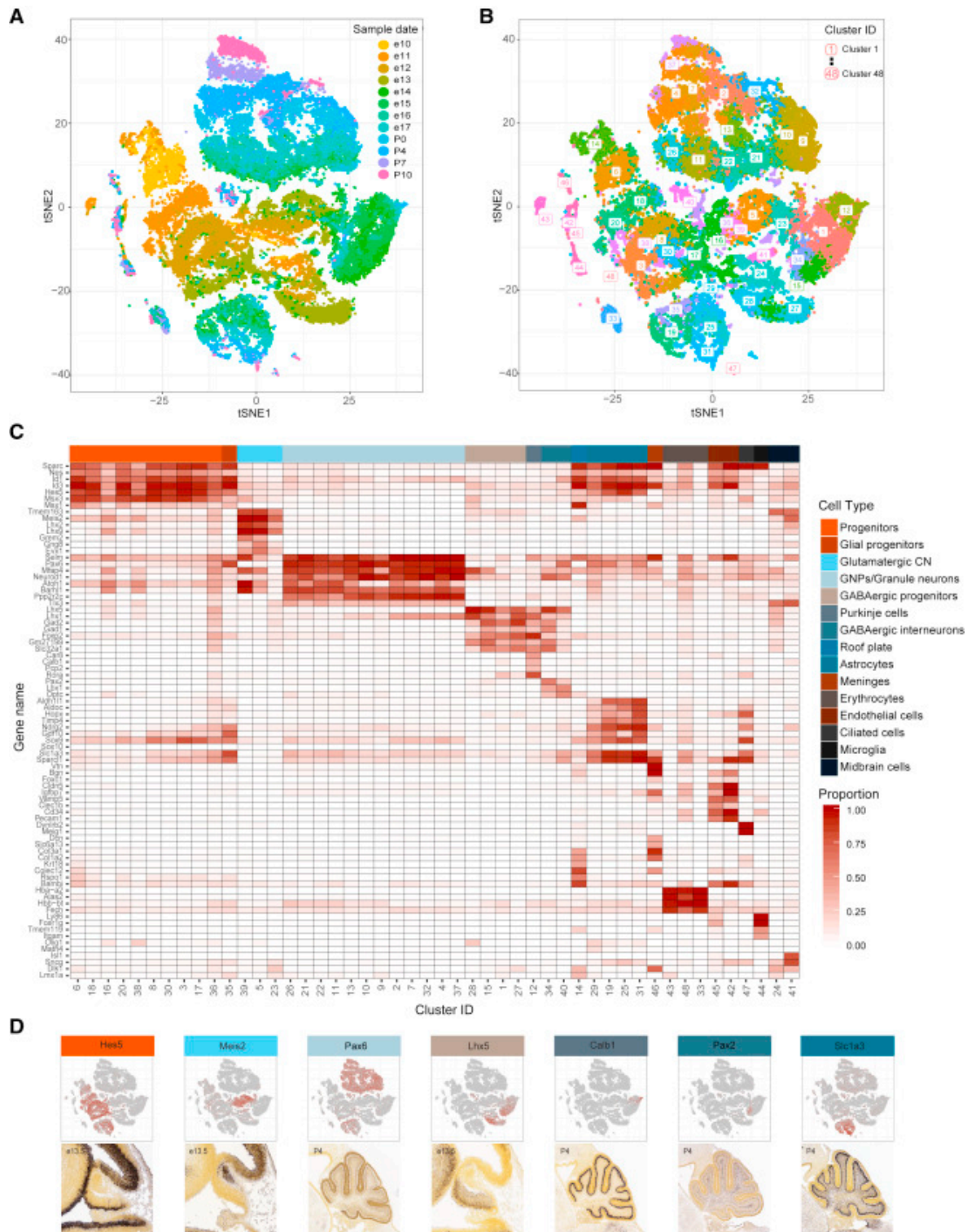


Figure 12. Identification of the Main Cerebellar Lineages by scRNA-Seq. (A) t-SNE showing the single-cell clusters and colors represent a specific time point from e10 to P10. (B) Used dynamic Tree Cut based on the top 1000 genes for the clustering of cells. (C) Clusters of cerebellar cell population and heat map showing a selection of genes from 100 top genes for each cluster. (D) t-SNE showing the expression of known lineage markers in upper panels and in lower panels, their expression in situ in the developing cerebellum. This figure is taken from ³.

Recently, a study was performed to identify single-cell types and their full-length RNA isoforms without FACS in mouse cerebellum at postnatal day 1 (P1) that used single-cell isoform RNA-seq (ScISO-Seq) strategy (Figure 13a)⁵⁶. They sequenced 17,885 reads per cell according to 10x genomic statistics. They had 3875 unique molecular identifiers (UMIs) and 1448 genes per cell and 6627 cells classified into 17 groups (Figure 13b). High-expression of cell-type-specific markers identified many clusters. High-expression of *Clu* and *Apoe* identified a cluster of Bergmann glia (BG). High-expression of *Pdgfra*, *Olig1*, and *Olig2* identified a cluster of oligodendrocyte precursors. High expression of *Neurod1* and *Ccnd2*, included cells in several stages of differentiation that identified the external granular layer (EGL) cell cluster. Purkinje cells, expression of *Pcp4*, *Gad1*, and *Gad2* in the Purkinje cell layer (PCL). Deep cerebellar nuclei (DCN) cluster and internal granular layer (IGL) neurons expressed *Pnoc*, *Snhg11*, *Tcf7l2*, *Gad1*, *Gad2*, and *Lhx9*. Probably DCN and IGL neurons involved in embryonic origins. Three clusters of neural progenitor cells including *Ccnd2* (expressed in the postnatal EGL), *Atoh1* (glutamatergic neuron precursors from the rhombic lip and EGL), or *Ptf1a* (GABAergic neuron precursors from the ventricular zone). High expression of myeloid-associated genes, for example, *C1qa*, *C1qb*, *C1qc* and, *Tmem119* that identified microglia population and endothelial and circulatory-system cells. The blood cells had the lowest gene number and were the smallest clusters and EGL, IGL-DCN cells and astrocytic cells are the largest clusters and Purkinje cells had the highest number of reading, UMI, and gene counts⁵⁶.

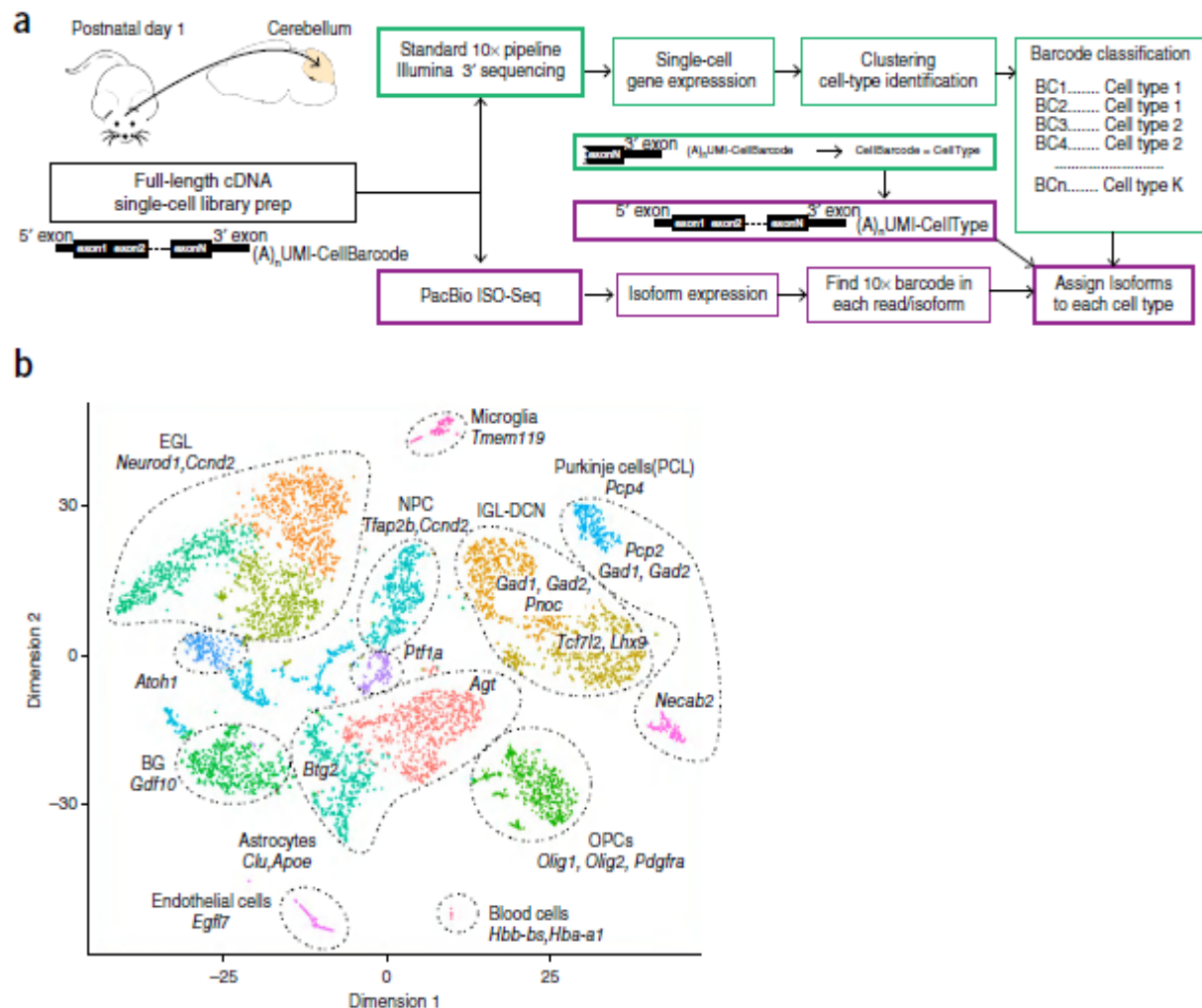


Figure 13. Cell type and barcode identification of mouse brain “a) Outline of ScISO-Seq strategy. b) t.SNE showed cell clusters and marker genes of mouse cerebellum”. This figure is taken from ⁵⁶.

To characterize cell-types in mice cerebellum, chromium system (10x Genomics) was used in E13.5 mouse cerebella and 9,326 cells and 15,823 genes were analyzed. They identified 19 main cell groups and their molecular features (Figure 14A) and known markers divided into four categories: 1) VZ neural progenitors cells (NPCs) that express Hes1 and Hex5; 2) GABAergic neurons and their precursors that express Lhx1 and Lhx5; 3) glutamatergic neurons and their precursors that express Atoh1 and Barhl1; 4) non-neural cells, including endothelial, pericytes and erythrocytes (Figure 14B)⁵⁷.

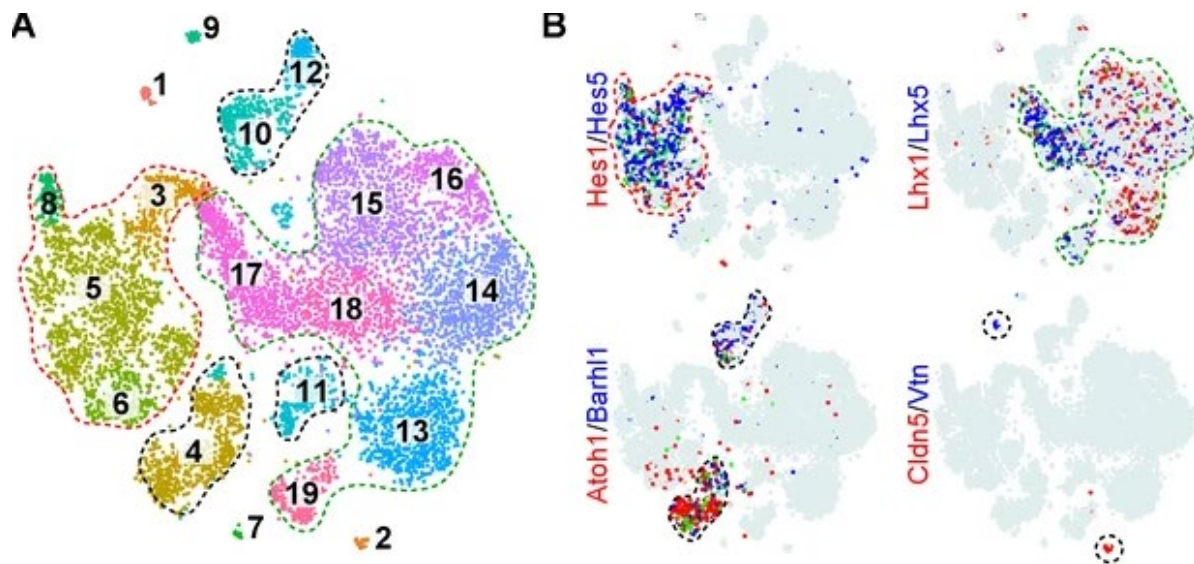


Figure 14. Identification of major cell types in 13.5 mice cerebellar by scRNA-seq. (A) t-SNE showing 19 major classes of cells. (B) Expression of known markers (red and blue, expression of individual markers; green, co-expression; azure, no expression). This figure is taken from ⁵⁷.

GABAergic neurons including PCs and interneurons are born in mice between E10.5 and E13.5. GABAergic cells are divided into nine cell groups. They assigned these nine groups as GABAergic precursors (1-2), immature GABAergic neurons (3), interneurons (4), and PC subgroups (5-9). A further explanation of these 9 groups is given below. Kirrel2, which is a marker of GABAergic precursors, was expressed in the first two groups. The distinguishing factor was that these two cell groups display the different expressions of Ptf1a and proneural genes Ascl1, Neurog1, and Neurog2 in the cerebellar VZ. Some common markers were expressed among the PCs and interneurons (Foxp2 and Gad2 and specifically Pax2, Gad1, Pnoc, and Glra2) whereas the latter expresses Tle1 and Islr2. PCs subgroups (5-9) are newly identified and distinct by strong expression of Etv1, Nrgn, En1, Cck, and Foxp1, respectively, and exhibit the variable level of Foxp2 and only one expresses both Foxp1 and Foxp2. One of the main subcategories of PCs is Foxp1⁺. It expresses RELN receptor genes, Vldlr, Lrp8, and obligatory adaptor Dab1. RELN signaling that controls the migration of PCs is sensitive to Foxp1⁺. The immunohistochemistry (IHC) revealed multiple PC clusters at E18.5 for Foxp1, Foxp2, and Nrgn. Except for the copula pyramids, Foxp1⁺/Foxp2⁺ in the most-anterior region is present in all lobules of the cerebellar hemisphere⁵⁷.

In 2019, scRNA-seq analysis was performed in samples postnatal days 0 and 8 to investigate neuronal cell type in murine cerebellum by 10x Genomics Chromium. 21,119 single cells were detected from one P0 sample and two P8 samples (Figure 15A). 58,994 UMIs and 2,615 genes identified were in each cell (Figure 15B). Peng et al⁸ used the Seurat R package and identified 12 distinct cell clusters. They used series of known cell cerebellar markers to each cluster, such as *Atoh1* marking clusters 0, 3, 4 and 5 as granule precursors (GPs) and *Rbfox3* marking clusters 1 and 2 as Granule cells (GCs) and expression of *Rbfox3* occur in a fraction of *Atoh1*-positive GPs (Figure 15E). Other clusters were appointed to interneurons, astrocytes, PCs, oligodendrocytes, endothelial cells, and microglia (Figure 15F and G). *Sox1* and *Sox 9* are Bergmann glial cells marker genes that are expressed by astrocytes. A type of GABAergic neurons, known as the Purkinje cells (PCs), have expressed *Gad1* and were detected by specific marker genes encoding Calbindin (*Calb1*) and Purkinje cell protein 2 (*Pcp2*) (Figure 15G). GCs and GPs accounted for 78% and PCs accounted for only 1.43% of total cells in the cerebellum of P0 and P8 mice⁸.

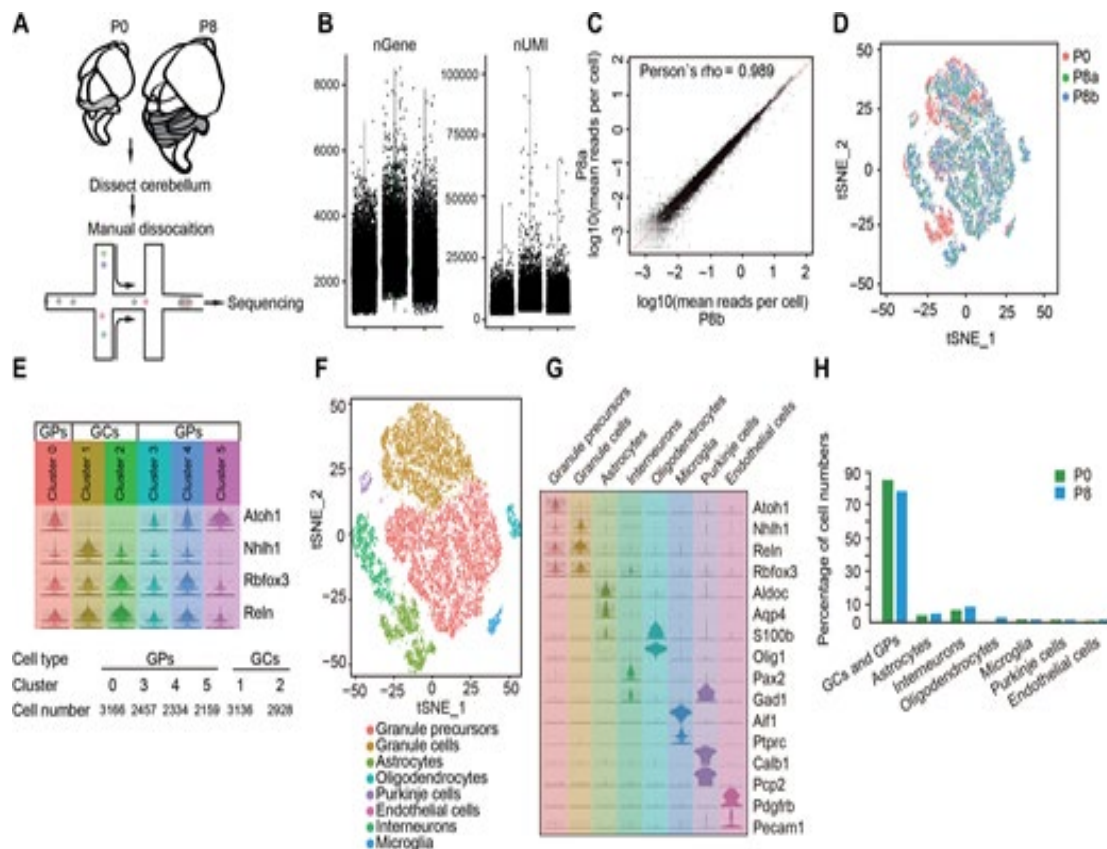


Figure 15. Classification of cell type in the mouse cerebellum. (A) The process of cerebellum dissection, cell dissociation, and droplet-based RNA-seq analysis. (B) Identified the number of genes and UMIs in P0 and P8. (C) Gene expression reads are highly correlated between two P8 samples. (D) Cell clusters in three samples. (E) Series of known cerebellar cell lineage markers to each cluster. (F) Cerebellar cell clusters. (G) Cerebellar cell clusters based on known lineage genes. (H) Percentage of cell types in the mice cerebellum of P0 and P8. This figure is taken from ⁸.

Recently, Peng et al⁸ analyzed 256 PCs from one P0 sample and two P8 samples in their molecular mechanism and development (Figure 16A). PCs at P0 exhibit bipolar morphology and are named as simple-fusiform cells. After development, these cells are planar with fan dendrites (Figure 16B). They detected as many as 618 differentially expressed genes (DEGs) between P0 and P8, using scRNA-seq technique. Gene ontology (GO) analysis showed that DEGs are involved in the mitochondria and ATPase process (Figure 16C-E), also suggested that PCs may contain more mitochondria and mitochondria pathway is related to morphological development of PCs. PCs have critical roles in cerebellar development and circuit formation such as proliferating of GPs, migration of GCs, and synapse formation and refinement. They used Seurat analysis to compare DEGs in PCs and other clusters and identified 815 DEGs at P8. They compared the first 20 DEGs in PCs with other clusters. Their analysis revealed that these selected

DEGs have crucial roles in the development of cerebellum: nuclear receptor ROR-alpha (Rora), genes encoding insulin-like growth factor 1 (Igf1), and Inositol 1,4,5-trisphosphate receptor type 1 (Itpr1), just to name a few. They identified 16 genes with high expression in PCs, including Igf1, Ndnf, Sema 3a, 4g, 6b, 7a, Wnt7b, Hbegf, Pdgfa, Gdf11, Nenf, Vegfb, Fgf11, Fgf3, Nppc, and Shh. Among these 16 genes, some members of Semaphorin family are highly expressed in different subsets of PCs, including Sema 3a, 4g, 6b, 7a, c-type natriuretic peptide (Npcc), neuron-derived neurotrophic factor (Ndnf), and platelet-derived growth factor subunit A (Pdgfa) (Figure 16G). Also, several other genes are only expressed in PCs and their distribution marked by Calbindin. "These genes include Cold shock domain-containing protein C2 (Csdc2), Coiled-coil-helix-coiled-coil-helix domain-containing 10 (Chchd10), inositol-trisphosphate 3-kinase A (Itпка), phosphatidate cytidyltransferase 1 (Cds1) and Adenylate cyclase type 1 (Adcy1)". Adcy1 protein was not only expressed in the soma of PCs and expressed in dendritic processes in the outer external molecular layer. Previous studies have demonstrated a correlation between the mutation of Chchd10 and frontotemporal dementia, cerebellar ataxia, and some other diseases. Elongation factor 2 (Eef2), Ataxin-10 (Atxn10), Nuclear protein 56 (Nop56)⁸ are some of the PCs that are composed of a large portion of ataxia genes.

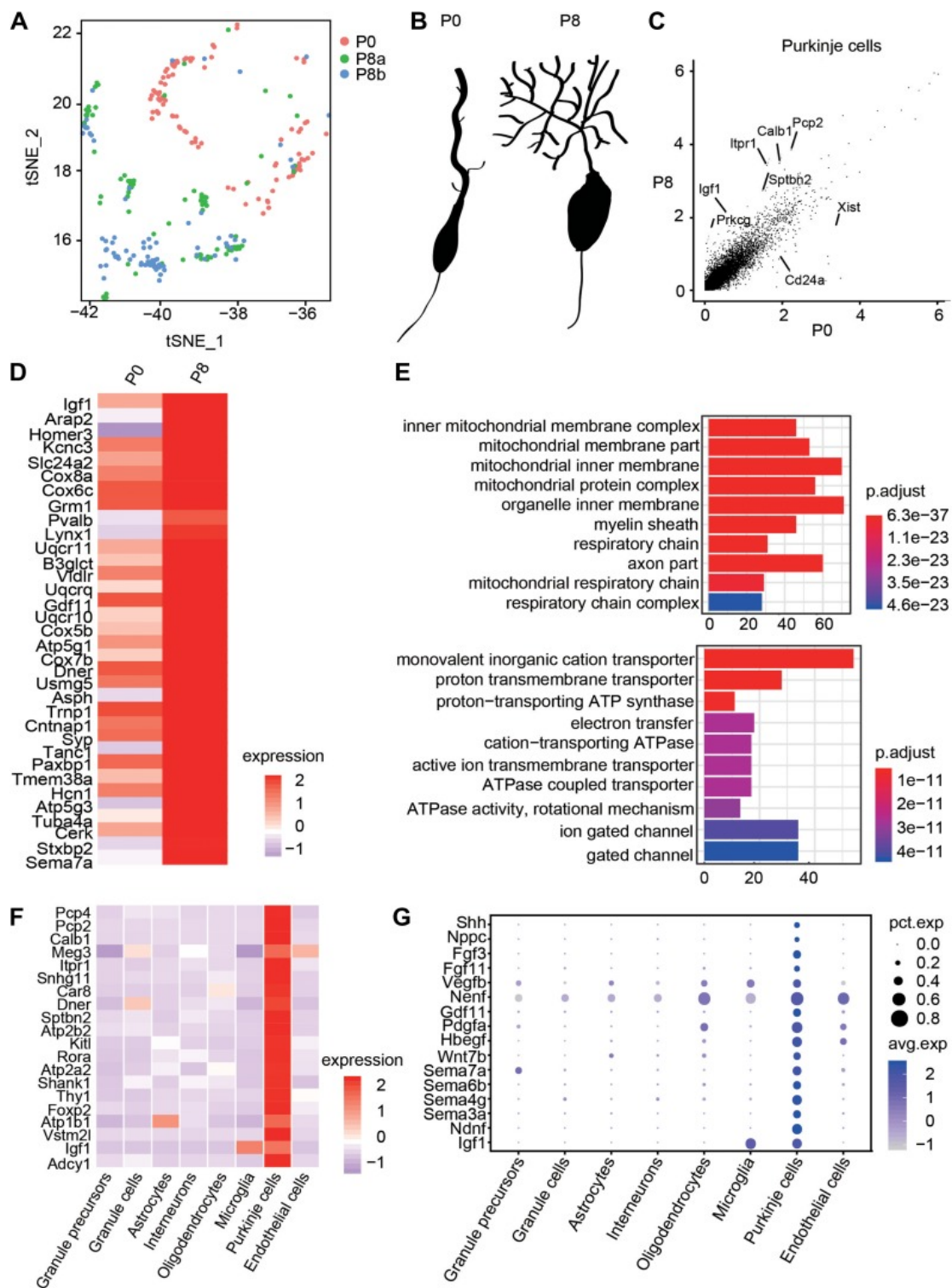


Figure 16. Dataset of PCs of developing mouse cerebellum. (A) t-SNE map showing 256 PCs in three samples. (B) Schematic diagram of PCs morphology from P0 and P8. (C) Analysis DEGs for PCs between P0 and P8. (D) The heat-map showing the expression of DEGs between three samples. (E) Gene Ontology terms in PCs between three samples. (F) The 20 top DEGs in PCs and comparison of them with other cell types. (G) Highly expressed genes that encoding secretory proteins in PCs compared with other cell types. This figure is taken from ⁸.

Approximately 80 percent of neurons in the cerebellum are composed of GCs. The origin of GPs is the rhombic lip region. They move to different parts of outer EGL such as cerebellar anlage and proliferates (Figure 17A). The function of GP genes was associated with cell division and proliferating. On the other hand, GC genes were responsible for cell morphogenesis and axonal development (Figure 17B). Peng et al⁸ analyzed 17,160 single GPs and GCs and divided them into 6 clusters (Figure 17C). Clusters 3 and 4 have high expressions of proliferation genes such as Mki67 and Tpx2. Clusters 0 and 5 have high expressions of differentiation of GCs that include Atoh1 and Hey1. A dense population of maturation genes, namely Cntn2 and Tubb3, were identified in clusters 1 and 2. (Figure 17D). They found the gene regulation network that is involved in GCs differentiation and analyzed these transcription factors (TFs) with GC expression genes. Particularly, Naca (Nascent polypeptide-associated complex subunit alpha), which is a transcriptional co-activator, is involved in the development of skeletal muscles and cardiac. They hypothesized that Naca plays an important role in regulating the differentiation of GCs (Figure 17E). Expression patterns of TFs including differentiation-related genes showed transient up-regulation such as Atoh1, Hey1, Barhl1, and proliferation-related genes showed gradual down-regulation such as Tipin and Cited⁸ (Figure 17F).

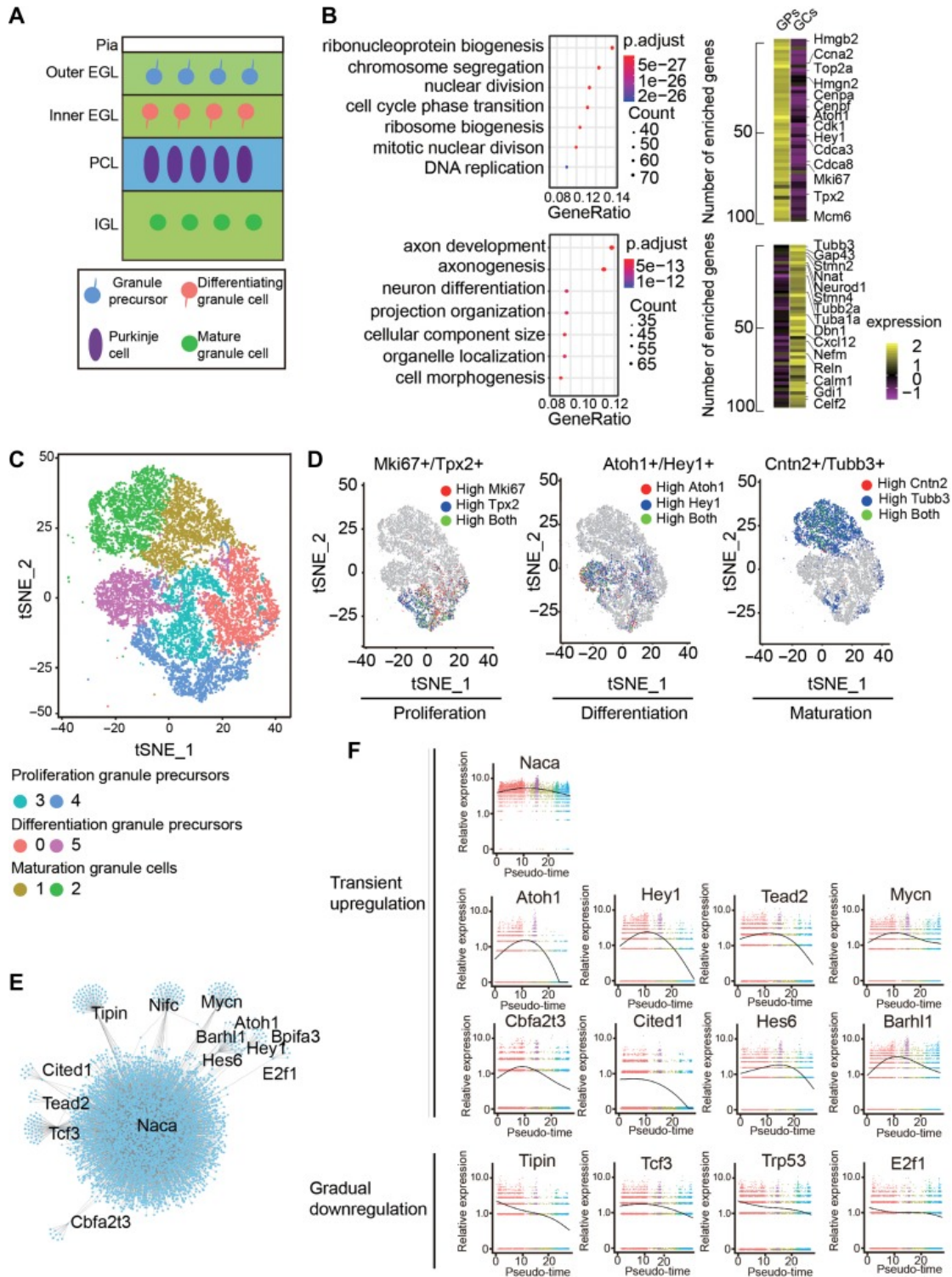


Figure 17. GC development (A) Schematic diagram showing the development of the GCs from proliferation, differentiation, to maturation. (B) DEGs between GPs and GCs and the number of enriched genes at each state. (C) t-SNE map showing the development of GCs in various states. (D) t-SNE visual image for the expression of proliferation-related genes, differentiation markers, and maturation-related markers. (E) Networks of TFs that regulating the development of GCs. (F) Expression patterns of TFs in pseudotime that related with GC development. This figure is taken from ⁸.

Another population in the cerebellum is interneurons that are different in their morphology, connectivity, and patterns of activity. The basket/stellate cells and Golgi cells are inhibitory interneurons derived from a precursor population in the cerebellar white matter. Unipolar brush cells, on the other hand, are excitatory neurons derived from the rhombic lip. 2,034 interneurons were investigated and divided into 5 clusters (Figure 18A). *Ptf1a* and *Ascl1*, which are inhibitory interneurons marker genes, were utilized to distinguish inhibitory interneurons progenitors in clusters 1 and 3, as shown in Figure 18B. For cluster 4, two marker genes, namely *Atoh1* and *Reelin* (*Reln*), signified that interneurons are stemmed from the rhombic lip region. In addition, *Pax2* and *Slc32a1* genes classified cluster 4 as a lineage of inhibitory interneurons. The *Gad1*, *Gad2*, and GABA transporter *Slc6a1* are expressed in the clusters 0 and 2. These expressions cause that these two clusters show differentiated inhibitory neurons (Figure 18B and C)⁸.

The functions of glial cells are important in neural development, nerve growth, and neural migration. Between 2,826 glial cells (2034 astrocytes, 455 oligodendrocytes, 337 microglia), the team identified 233 DEGs among them and Semaphorin family enriched in glial cells, *Sema6d* in astrocytes, *Sema5a* and *5b* in oligodendrocytes (Figure 18E)⁸.

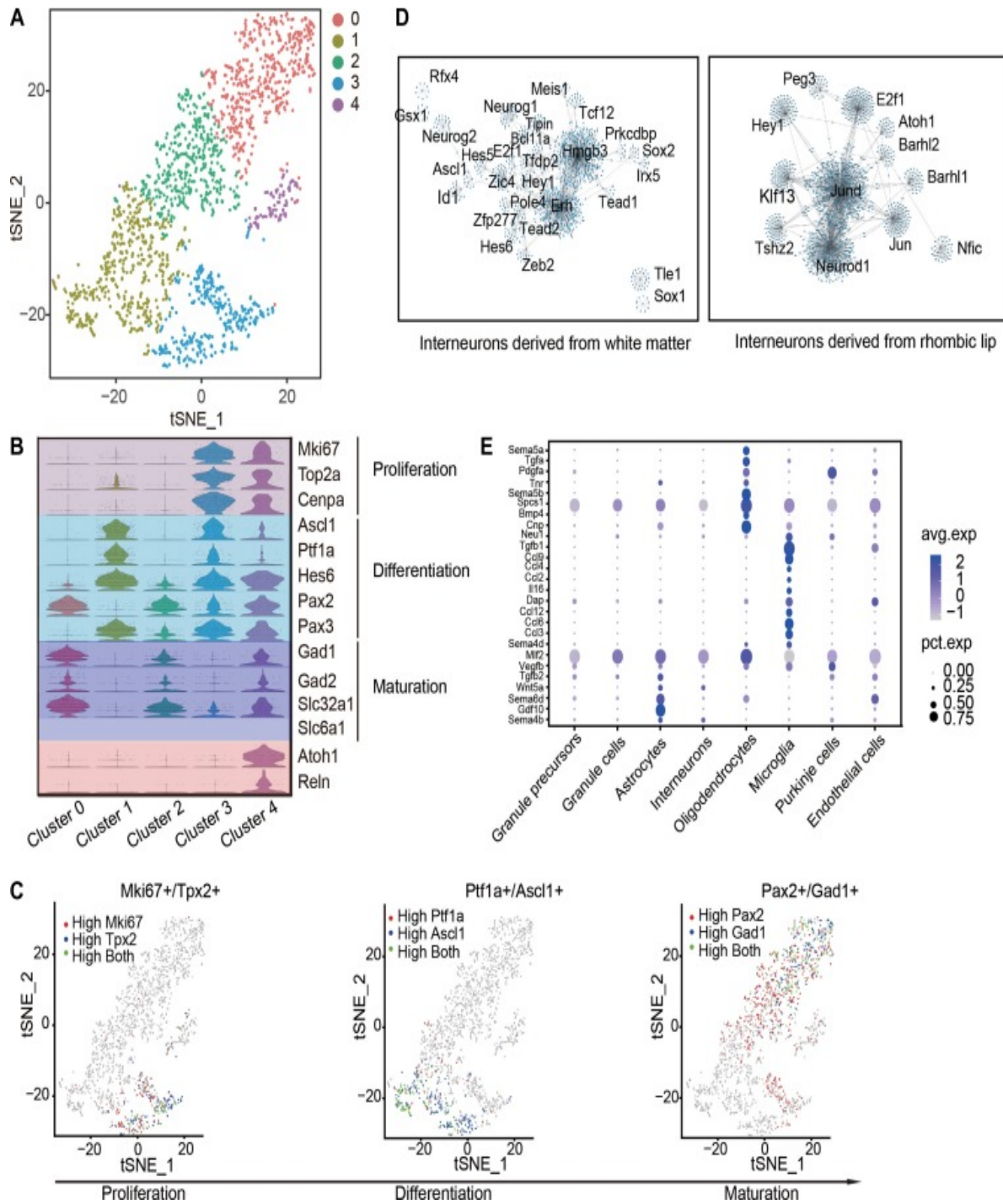


Figure 18. Gene expression of cerebellar interneurons and glia cells. (A) 5 clusters of interneuron lineage cells. (B) Violin plots showing the expression of genes with cell states. (C) t-SNE visualization showing the expression patterns of proliferation-related genes, differentiation-related genes, and maturation-related genes during the development of interneuron. (D) Transcription networks of TFs and origin of interneurons from cerebellar white matter (left) or rhombic lip (right). (E) Several genes encoding secretory molecules in glia cells. This figure is taken from ⁸.

2.4. Current and Future Developments of ScRNA-seq

Single-cell RNA-seq (scRNA-seq) is a high-throughput method to quantify a large number of transcripts, in a parallel manner, for thousands of cells⁵⁸. As a result, scientists have been able to understand many cell types and functional activities in complex tissues. However, the preparation of fresh tissue is the major obstacle in single-cell suspension due to difficulties associated with the dissociation of archived tissues. It is very problematic because the totality of neuronal RNA cannot be conserved in enzymatic dissociation of brain tissues. In addition, scRNA-seq is only viable for samples taken from younger organisms, and cannot be used for dead patients with neurodegenerative disorders⁵⁹. Also, scRNA-seq profiling is biased and does not include all neural cell types because of the vulnerability of some cell types compared to others⁵⁸.

A more recent trend in RNA sequencing is to utilize single nuclei instead of single cells. Nuclei can be isolated from frozen tissue and are also more resistant to mechanical damage during experimentation. The only possible drawback of using the single-nucleus RNA-sequencing (snRNA-seq) technique is that the diversity and number of transcripts in the nucleus are questionable when compared to the whole cell. A study⁵⁸ in 2018 demonstrated that even though the mRNA content of nuclei can vary among cell types, enough transcripts are available in the nuclei to distinguish cell types with resolutions comparable to scRNA-seq. snRNA-seq has good performance in sensitivity and classification of cell types⁶⁰.

Another recent trend in this field is focusing on the determination of the spatial position of cells in tissue. Developing an understanding of spatial position of cells is crucial in the determination of the tissue function. Slide-seq is a valuable method for transferring RNA from tissue segments onto a surface that is packed in DNA-barcoded beads that allows the determination of the RNA locations. In this method, DNA-barcoded 10- μm micro-particles (beads) are located on a rubber-coated glass to form a monolayer (puck). The beads, used in this technique, are identified to those used in Drop-seq technology. In order to spatially index unique barcodes of each bead, sequencing by oligonucleotide ligation and detection (SOLiD) technique has proven to be effective. This method is a promising complementary technique that can be integrated with datasets of scRNA-seq

to spatially discover the pattern of gene expression, in both diseased and normal tissues⁶¹.

3. Conclusion

The Brain is one of the most complex organs, consisting of more than 100 billion neurons. The cerebellum, although it only takes 10 percent of the brain, contains more than half of the total number of neurons in the brain. Thus, understanding the identity and function of diverse cell types in cerebellum remains to be a major challenge in molecular biology studies.

RNA sequencing is a common approach to uncover this topic. But the classical RNA sequencing methods, such as in-plate techniques, are notably slow and are incapable of uncovering all cell types in the cerebellum. The trending way to deal with this problem is the development of high-throughput techniques.

In 2015, a novel high-throughput method was developed at Harvard Medical School, known as Drop-Seq. Basically, cells are isolated in very small droplets with distinct beads. The beads are manufactured in a way that provides libraries of millions of well-defined barcode sequences that only specific mRNAs in the cell can be attached to. Each droplet behaves as an experimental investigation, and thousands of cells can be identified within one experimental run.

This is a cost-efficient and fast technique that was well-received by the scientific community and has been developed and employed extensively. Three versions of this droplet-based approach are Drop-seq, inDrops, and 10X Genomics that differ slightly in the procedure, accuracy, and cost.

In one of the first applications of Drop-seq in studying the cell types in the cerebellum, Alles et al⁵⁵. Studied the brains of newborn mice and humans in 2017. They showed that, using Drop-Seq, they were able to distinguish cell types of different species, with a high degree of confidence. They developed software (dropbead) to analyze and visualize the raw data. They were eventually able to identify and classify 12 clusters of distinct cell types.

10X Genomics, the more advanced and commercialized version of the droplet-based techniques, has particularly been used to identify more cell types. A diverse library of cell types have been identified so far and Drop-seq has offered a transcriptional profiling routine that is expected to accelerate biological discoveries in the years to come.

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