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APPLICATION NOTE

Single-Nuclei Sequencing of Mouse Cerebellum Using the Singulator™ Platform Demonstrates the Preservation of Rare Cell types using an Automated Method

Abstract

Advances in neuroscience research increasingly demand high-quality, reproducible isolations of single nuclei to facilitate the investigation of rare cell types using genomic applications such as single-nuclei RNA sequencing (snRNA-seq), epigenomic and multiomic studies. The Singulator™ Platform offers unmatched precision and reproducibility in isolating single nuclei from various neural tissues, including brain regions critical to development, neurodegeneration and psychiatric research. Here we isolated nuclei from three biological replicates of adult mouse cerebellar hemispheres using the Singulator Platform. We performed snRNA-seq on isolated nuclei and determined the cell types and their relative proportions. The expected proportions of rare cell types (less than 2% of total population), such as Bergman glia, astrocytes, molecular layer interneuron 2, unipolar brush cells, Purkinje layer interneurons, microglia, macrophages, Purkinje cells, oligodendrocyte precursor cells, fibroblasts and endothelial cells, were observed across all replicates. Furthermore, we demonstrate that nuclei isolated using the Singulator Platform are more diverse than manual methods. Finally, we demonstrate the reproducibility and precision of the Singulator Platform using standard bioinformatics comparisons. These data demonstrate that the Singulator Platform is an invaluable tool for isolation of nuclei from neural tissues, while preserving the relative abundance of cell types and the gene expression signatures of those cell types.

S2 genomics

S2 Genomics, Inc.
7683 Southfront Rd. Suite 200
Livermore, CA 94551
USA

WEB: s2genomics.com
EMAIL: inquiries@s2genomics.com
PHONE: 1-925-292-8243

Introduction

Understanding the cellular and molecular mechanisms that underpin brain function and dysfunction remains a formidable challenge. Traditional bulk RNA sequencing averages the gene expression profiles across large populations of cells, masking the diversity and heterogeneity of individual cells within complex tissues. This limitation hinders our ability to fully comprehend the intricacies of brain function, disease mechanisms, and the impact of therapeutic interventions.

Single-cell RNA (scRNA-seq) sequencing has emerged as a revolutionary tool that allows researchers to dissect the transcriptomic landscape at the resolution of individual cells. By capturing the gene expression profiles of thousands to millions of cells, scRNA-seq provides an unprecedented level of detail, revealing the heterogeneity and distinct cellular subpopulations that comprise the nervous system. This high-resolution approach is critical for mapping cellular diversity, understanding developmental processes, and identifying cell-type specific gene expression changes involved in neurological diseases.

One bottleneck that has emerged in scRNA-seq is the limitation of standard sample preparation. Individual users can introduce bias in cell preparation, which may influence the ability to detect rare cell types and changes in gene expression. The Singulator Platform addresses these challenges head-on with its automated, user-friendly, reproducible and precise sample preparation for single-cell genomics applications. By automating the sample preparation process, the Singulator Platform minimizes human error and variability, ensuring that every sample is processed in a consistent manner. This leads to reproducible results, which are essential for interpreting single-cell genomic data.

In this Application Note, we used the Singulator Platform to isolate nuclei from three mouse cerebellar hemisphere samples, for gene expression profiling. The Singulator Platform enabled rapid and high yielding isolation of nuclei while maintaining the gene expression signatures characteristic of the major cell types found in cerebellum. When we compared the Singulator Platform to published manual methods, we found that the Singulator Platform preserves cellular heterogeneity and recovery of rare cell types better than manual methods. Finally, we demonstrate the Singulator platform is highly reproducible across biological replicates.

Methods

Nuclei were isolated from three mouse cerebellar hemisphere samples using the Singulator™ Platform, following the protocol 'Demonstrated Protocol: Nuclei Isolation and Cleanup from Frozen Mouse Brain Tissue for Single Nuclei Sequencing Applications' (S2 Genomics, 100-272-080), with the NIC+ Isolation Bundle with RNase Inhibitor V2 (S2 Genomics, 100-289-152) (**Figure 1**).

8-week-old ICR (CD-1®) mice were sacrificed following IACUC-approved protocols, the cerebellum was dissected from the mouse and cut in half along the vermis, and immediately snap frozen on dry ice. Frozen cerebellar hemispheres were loaded into a pre-cooled NIC+ cartridge with RNase Inhibitor V2 and processed using the Low Volume Nuclei Isolation Protocol V2 on a Singulator 100™ instrument. Following nuclei isolation, myelin was removed using Debris Removal Reagents Stock Reagent (S2 Genomics, 100-253-628). Nuclei were counted and the viability was determined using AO/PI (acridine orange/propidium iodide) staining with the Nexcelom K2. Next, nuclei were loaded into the 10x Genomics Chromium GEM-X Single Cell 3' Kit v4 at a concentration of 1,000 nuclei/µL, targeting 10,000 nuclei.

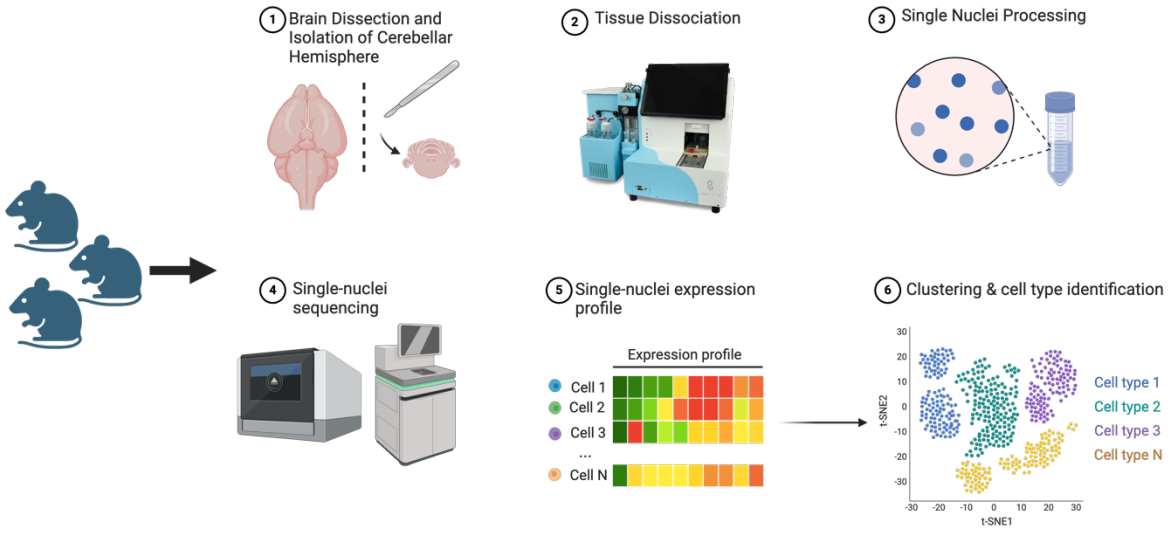

Figure 1. Experimental Design. Normal lung and tumor tissues were dissociated into a single-cell suspension with a Singulator™ 100. The yields and viabilities of the cells were measured. Tumor cells were then used to isolate tumor infiltrating leukocytes (TILs) via EasySep™ Release Human CD45+ Selection Kit (Stemcell™ Technologies). 10,000 cells were targeted with the 10x Genomics™ Next GEM Single Cell 3' Kit v3.1. Sequencing data were analyzed with 10x Genomics' Cell Ranger v7 and Seurat v5.

Gene expression libraries were prepared following the 10x Genomics User Guide[1] and sequenced on an Illumina NovaSeq X Plus with approximately 20,000 read pairs per cell. Data was processed with Cell Ranger v8 using default settings to generate raw and filtered feature matrices.

The raw data were preprocessed using SoupX[2] to remove ambient RNA contamination and DoubletFinder[3] to identify and exclude potential doublet cells. After preprocessing, the data was subjected to quality control using Seurat v5.0[4]. Cells were filtered out if they had mitochondrial gene expression over 5%, had fewer than 500, or more than 6,000 unique feature counts. Following QC, the data was then normalized using SCTransform V2 to prepare for integration. Following normalization, the three cerebellum samples were then integrated with each other via Harmony[5]. Dimensionality reduction was performed, a UMAP was generated, and cells were clustered in Seurat to a resolution of 1.0 using default parameters. Cell types were manually annotated using marker genes from published literature[6,7].

Results

The Singulator Platform Isolates High Yields of Nuclei

The yield per mg for the three cerebral hemispheres was 166,666.67, 187,407.41, and 224,090.91 nuclei per mg for sample 1, 2, and 3, respectively. The average yield across these samples was 192,721.66 nuclei per mg, with a standard deviation of 29,078.63. These yields demonstrate reproducibility in the isolation process.

Nuclei Isolated Maintain Cell Type Specific Gene Expression Profiles

SnRNA-seq libraries were generated from single-nuclei suspensions. The sequencing metrics demonstrate the nuclei isolated by the Singulator Platform were of high quality. The nuclei maintain their RNA integrity and gene expression diversity through the isolation process with little ambient RNA contamination. These metrics demonstrate the utility of the Singulator Platform for isolating nuclei intended for downstream analysis by snRNA-seq (**Table 1**).

Sample	Cells Detected	Mean reads per cell	Median genes per cell	Total genes detected	Median UMI counts per cell	Confidently mapped reads in cells
Cerebellum Sample 1	12,828	17,775	2,137	25,853	5,115	71.4%
Cerebellum Sample 2	17,268	16,221	2,006	26,062	4,550	71.27%
Cerebellum Sample 3	13,457	17,801	2,129	25,510	4,854	68.34%

Table 1: Sequencing metrics for each sample type by sample.

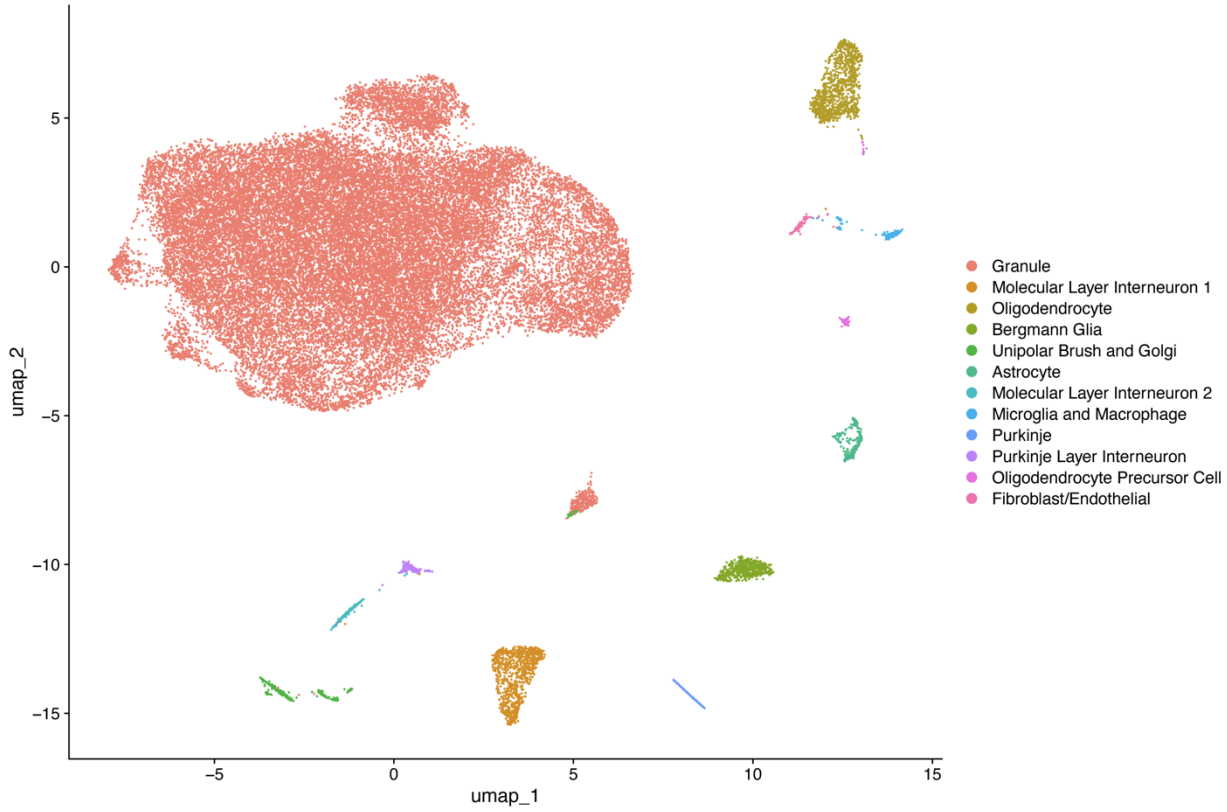

Figure 2: The Singulator Platform isolates nuclei that represent the 12 major cell types found in adult mouse cerebellum. UMAP projection of integrated cerebellum nuclei colored by clustering. Nuclei from each sample were integrated, visualized as UMAP, and clustered. Cells are clustered by gene expression profile and each cluster is assigned a different color. Cell types were identified by canonical marker gene expression.

Cell clustering and cell-type annotation based on transcriptomic data identified twelve major classes of cells: granule cells, molecular layer interneuron 1, oligodendrocytes, Bergman glia, unipolar brush and Golgi cells, astrocytes, molecular layer interneuron 2, microglia and macrophages, Purkinje cells, Purkinje layer interneurons, oligodendrocyte precursor cells, fibroblasts and endothelial cells (**Figure 2**). These results agree with two previously published reports of cerebellar cellular composition in adult mice[6,7].

The Singulator Platform Preserves Cellular Heterogeneity and Recovery of Rare Cell Types Better than Manual Methods

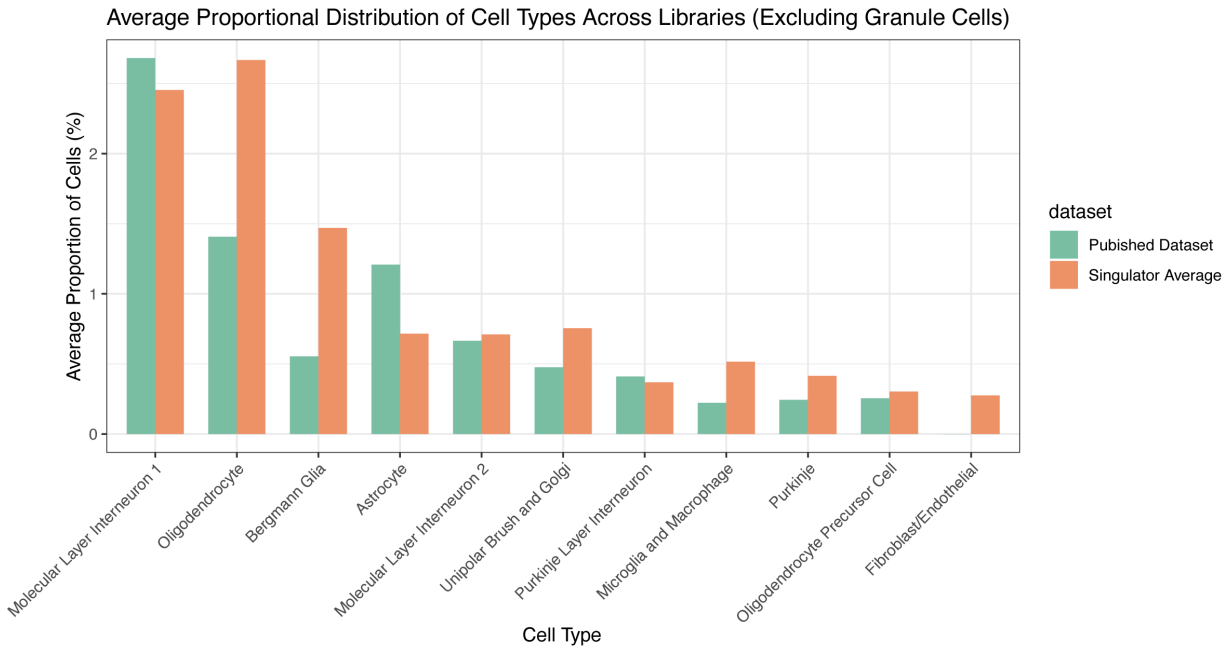

Figure 3. The Singulator Platform outperforms manual methods for isolating most rare cell types. Distribution of cell types comparing average proportion for each cell type with less than 5 % in nuclei isolated by manual methods (n=2) versus those isolated by the Singulator Platform (n=3).

Fully capturing the diversity of cell types, including rare cell types, is critical when studying complex tissues like the mammalian brain. To examine rare cell type populations more closely, we removed the granular cell fraction (88%) and compared the remaining 11 major cell type fractions between our libraries and a recent publication that explores the cell types found in adult mouse cerebellum[7] (**Figure 3**). These cell types make up no more than 2.5% of the total cells recovered and as little as 0.25% of cells recovered. The Singulator Platform was able to isolate and retain more oligodendrocytes, Bergman glia cells, unipolar brush and Golgi cells, microglia and macrophages, Purkinje cells, and fibroblast and endothelial cells, while recovering equivalents proportions of molecular layer interneurons 1 and 2, oligodendrocyte precursor cells and fewer astrocytes[7]. Taken together, these data demonstrate preservation of cell-type diversity during processing is increased when using the Singulator Platform.

The Singulator Platform is highly reproducible and precise across biological replicates

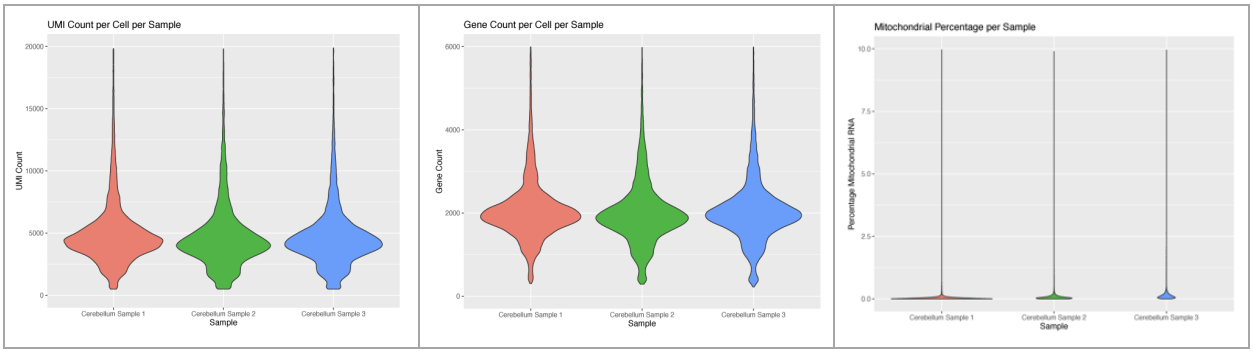

Figure 4: Quality analysis of three biological replicates demonstrate the Singulator Platform isolates high-quality nuclei reproducibly. UMI count per cell and mitochondrial contamination (percent.mt) for down sampled cerebellum samples.

The Singulator Platform is designed to be highly reproducible across biological samples. To demonstrate the reproducibility of the Singulator Platform, we compared the snRNA-seq results from three adult mouse cerebellar hemispheres after down sampling to equivalent read depth (14,150 reads per cell). The three biological replicates exhibit nearly identical gene expression profiles per cell. The median UMI counts per cell were 4,484, 4,215, and 4,303; the median genes per cell were 1,965, 1,907, and 1,967 respectively; and the fraction of mitochondrial reads was exceptionally low across the samples, averaging below 1% for all samples (**Figure 4**). Integrated UMAP of the three biological replicates demonstrates consistent clustering across replicates, indicating consistent cell type recovery and gene expression profiles (**Figure 5**).

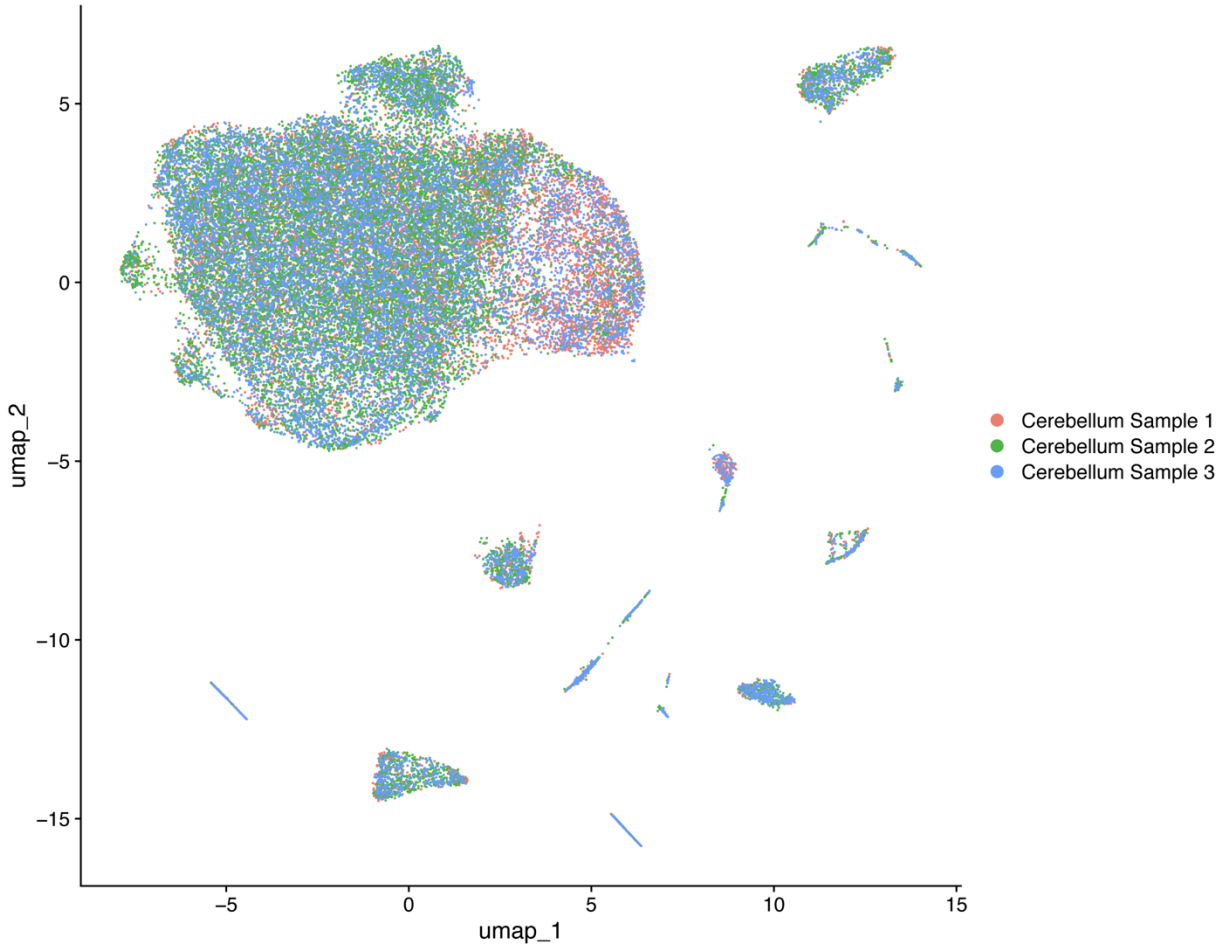

Figure 5: Integrated UMAP of biological replicates demonstrates the Singulator Platform is reproducible and precise across samples. Nuclei from each sample were integrated, projected as a UMAP, and clustered. Each biological replicate was assigned a different color.

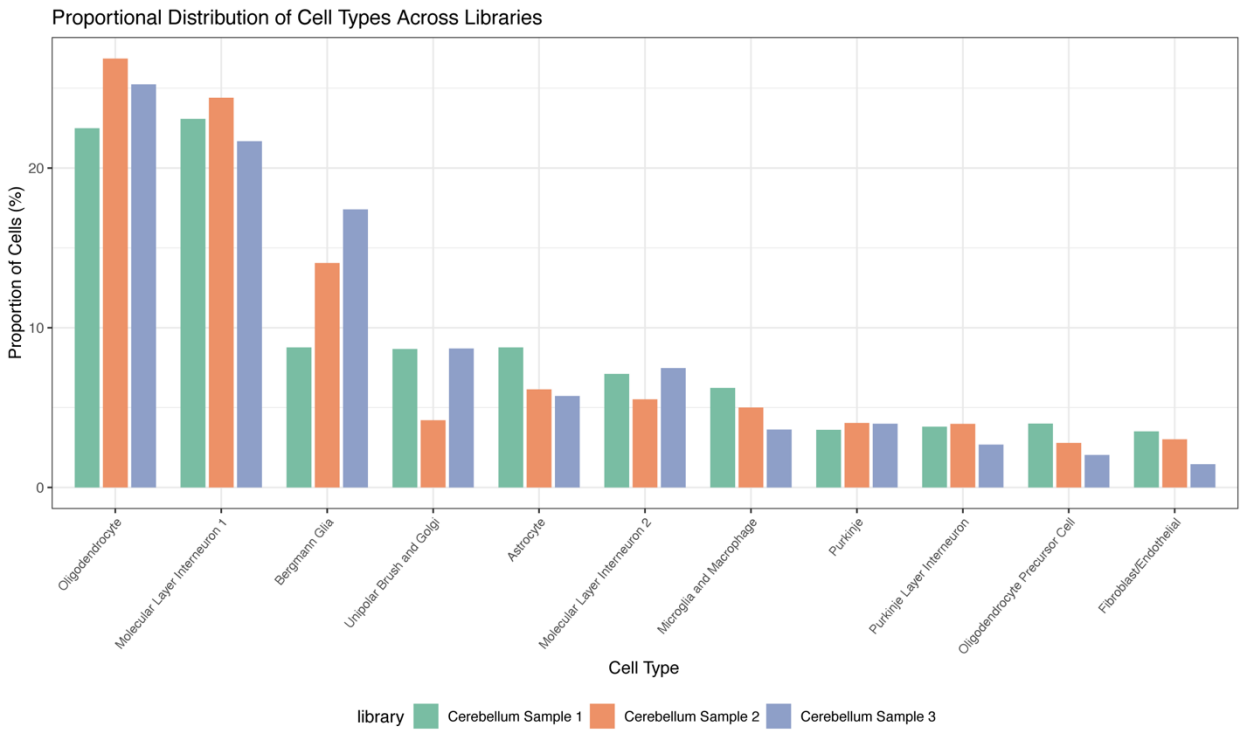

Figure 6: QC metrics including genes per cell (nFeature_RNA), UMI per cell (nCount_RNA), and mitochondrial contamination (percent.MT) for down-sampled normal lung samples for patients 1,2 and 3.

Further analysis revealed the abundance of the rare cell types isolated with the Singulator Platform in each sample is highly reproducible (**Figure 6**). Cell proportions were calculated and compared from each cerebellum sample excluding the granule cells. Nearly identical cell type compositions were observed across biological replicates, with only minor expected variations. Taken together, these data indicate the Singulator Platform reproducibly preserves cell type diversity during nuclei isolation, including the isolation of rare and fragile cell types.

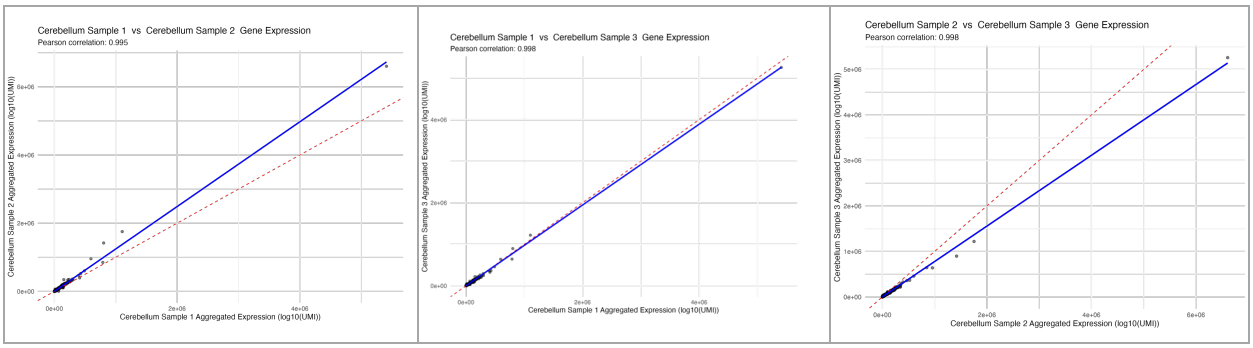

Figure 7: High concordance between nuclei isolations performed on the Singulator Platform by Pearson correlation. Average expression graphs with Pearson correlations demonstrate reproducibility of the nuclei isolations between three down sampled cerebellum samples.

Finally, we generated Pearson correlation coefficients from pseudo-bulk average gene expression for all comparisons between the three samples. All three comparisons generate a Pearson correlation equal to or above 0.955 for pseudo-bulk average gene expression, demonstrating a high concordance between nuclei isolations performed on the Singulator Platform (**Figure 7**). These metrics collectively indicate that the three cerebellum samples produce reproducible and reliable data, ensuring consistency and comparability across experiments.

Conclusion

The Singulator™ Platform provides a reproducible and precise method for isolating nuclei from complex tissues suitable for single-nuclei RNA sequencing. Our results demonstrate consistent nuclei yield and high-quality sequencing metrics across biological replicates, making it a reliable tool for neuroscientific research. The ability to produce high-quality nuclei with minimal variability enhances the accuracy of downstream snRNA-seq analyses, facilitating deeper insights into normal biology and disease. Finally, the ability to identify rare cell types underscores the efficacy of the Singulator Platform in comprehensive cellular analysis, providing a detailed representation of the cellular landscape.

References

1. CG000731_ChromiumGEM-X_SingleCell3_ReagentKits_v4_UserGuide_RevA.pdf
2. Young MD, Behjati S (2020). "SoupX removes ambient RNA contamination from droplet-based single-cell RNA sequencing data." GigaScience. doi: https://doi.org/10.1093/gigascience/giaa151
3. McGinnis CS, Murrow LM, Gartner ZJ. DoubletFinder: Doublet Detection in Single-Cell RNA Sequencing Data Using Artificial Nearest Neighbors. Cell Syst. 2019 Apr 24;8(4):329-337.e4. doi: https://doi.org/10.1016/j.cels.2019.03.003. Epub 2019 Apr 3. PMID: 30954475; PMCID: PMC6853612.
4. Hao, Y., Stuart, T., Kowalski, M.H. et al. Dictionary learning for integrative, multimodal and scalable single-cell analysis. Nat Biotechnol 42, 293–304 (2024). https://doi.org/10.1038/s41587-023-01767-y
5. Korsunsky, I., Millard, N., Fan, J. et al. Fast, sensitive and accurate integration of single-cell data with Harmony. Nat Methods 16, 1289–1296 (2019). doi: https://doi.org/10.1038/s41592-019-0619-0
6. Kozareva, V., Martin, C., Osorno, T. et al. A transcriptomic atlas of mouse cerebellar cortex comprehensively defines cell types. Nature 598, 214–219 (2021). https://doi.org/10.1038/s41586-021-03220-z
7. Sepp, M., Leiss, K., Murat, F. et al. Cellular development and evolution of the mammalian cerebellum. Nature 625, 788–796 (2023). https://doi.org/10.1038/s41586-023-06884-x