Identify More Diverse Cell Types in 10X Genomics Workflows

with the Pala Cell Sorter from Bio-Techne

High dimensional clustering of sequencing reads obtained from Pala sorted nuclei compared to the standard nuclei prep method revealed a greater number of distinct clusters comprising a wider range of cell types.

Abstract

Single-nucleus RNA sequencing (snRNA-seq) is a method used to analyze gene expression in single cells, especially ones where isolation of a single cell is challenging such as specific tumors, heart tissue, and neuronal tissue. Single cell sequencing methods have emerged as powerful tools for identification of heterogeneous cell types within these tissues.

Flow cytometry has been the preferred method for sorting nuclei utilizing FACS instrumentation, however this process can be cumbersome due to long set up times, high instrument costs, and significant training hurdles that make using traditional FACS inaccessible to many labs. Other standard methods of single nuclei clean-up suffer from drawbacks including the inability to remove aggregates or debris and the inability to specifically select intact nuclei.

Bio-Techne's Pala benchtop cell sorter utilizes flow cytometry, liquid dispensing, and microfluidic technology, but with a smaller footprint than a traditional sorter, a simple set up and operation, and significantly less sorting pressure. The Pala can also remove cell aggregates, enrich intact nuclei, and filter out small/large debris making it an ideal choice for pairing with 10x Genomics applications.

In this application note, we compared snRNA-seq data from dissociated nuclei using a standard method of sample clean-up compared to sorting nuclei using Bio-Techne's Pala. In short, sorting cell samples using the Pala system revealed a greater number of distinct clusters, comprising a wider range of cell types, compared to the standard, unsorted sample prep method. In the unsorted sample, only a single large cluster with a few satellite clusters and less diversity in cellular origin were observed.

Introduction

The ability to investigate gene expression on the single cell level using snRNA-seq has propelled significant discoveries in the past decade (Ref 1). With single cell RNA-seq (scRNA-seq) it is now possible to characterize and distinguish each cell at the transcriptome level, which leads to identification of rare and diverse cell populations (Ref 2). However, the processing and homogenization of tissues in order to achieve single cell suspensions is not without its challenges. The process relies on harsh tissue dissociation and enzymatic incubations to lyse cells while the 37°C water bath can induce the expression of stress response genes (Ref 3).

Single nucleus RNA-seq solves several issues and limitations of scRNA-seq sample preparation by minimizing transcriptional stress responses due to its use of low concentrations of detergents to disrupt the cell membrane and release intact nuclei (Ref 3). snRNA-seq has also become extremely useful in diverse tissue types including muscle, heart, lung, kidney, and especially brain tissues, which are difficult to dissociate into intact single cells (Ref 4,5). However, this method presents several

challenges, especially with sample preparation and low cell recovery. Input sample quality is critical for a successful preparation and sequencing run. Dissociation, debris, aggregation, and free-floating RNA are just some of the issues researchers face when running 10X Genomics applications. While droplet-based RNA-seq, such as 10x Genomics, offers a cost-effective, high throughput single cell RNA-seq approach, to achieve optimum RNA-seq results, it's critical to obtain high quality cell or nuclei suspension with minimal aggregates and debris.

Challenges in handling the cells/nuclei are also common. FACS sorters can be extremely harsh with high sorting pressures (psi). Pipetting methods, although gentle, can also prove to be challenging since sample quality can be poor due to contaminants such as free-floating RNA and dying cells. Generating reproducible results can also be difficult due to user variability associated with manual pipetting methods and washing. Bio-Techne's Pala benchtop cell sorter mitigates these common issues with its use of microfluidic technology coupled with gentle sorting pressure less than (<2 psi). The Pala system uses flow cytometry to interrogate and sort positive cells or nuclei, successfully clearing debris and unwanted cells from a suspension. It can also sort out aggregates (doublets) with forward/axial light loss scatter differentiation, and sort with a live/dead stain. The Pala system uses three modes to sort samples from suspension using fluorescence or light scatter: first, it can dispense single cells into 96-well or 384-well plates; second, it can bulk sort a population of cells into a tube; and third, it can enrich very rare cells (<0.1% population) from high density samples based on fluorescence. These characteristics make the Pala system unique in that it is ideally situated to enable both droplet based and plate-based single cell sequencing methods.

In this study, we showed that we were able to successfully sort and clear debris from a notoriously debris-filled sample—human brain tissue. We also show that Pala sorted nuclei provided better sequencing data with greater recovery and demonstrate how the Pala platform provides complimentary advantages for researchers working with 10X Genomics workflows.

Scan the QR Code or Visit:
bio-techne.com/instruments/single-cell-dispensers
Learn more about Pala Cell Sorter and Single Cell Dispenser platform.

Materials

These materials and methods are for all experiments in this study.
The reagents, samples, and instrumentation are listed below (**Table 01**).

TABLE // 01

Materials utilized in this study

Product	Vendor	Part/Reference No.
Namocell Pala 405/488	Bio-Techne	Pala SN:NI007-0015
Namocell Bulk sorting cartridges	Bio-Techne	NC-101
10X Genomics Chromium Controlller X	10X Genomics	N/A
Chromium Next GEM single cell 3' v3.1 kit	10X Genomics	PN-10000268
Chromium Next GEM Chip G single cell kit	10X Genomics	PN-1000120
Single cell 3' Library construction kit	10X Genomics	PN-1000190
Single cell 3' Gel Bead kit v3.1	10X Genomics	PN-1000122
gentleMACS™ Octo Dissociator with heaters	Miltenyi Biotec	Cat. 130-096-427
Multi tissue dissociation kit 2	Miltenyi Biotec	Cat. 130-110-203
Sucrose gradient nuclei isolation kit	Sigma-Aldrich	NUC-201
4200 Tapestation system	Agilent	G2991BA
Qubit 4 Fluorometer	Invitrogen	Q33238
HiSeq 2500 sequencing system	Illumina	SY-401-2501
ASTERAND Human Brain Normal Fresh Frozen	BIOIVT	Cat. 1156118F
Cellaca MX High-Throughput Automated Cell Counter	Nexcelom	N/A

Table 1 Reagents, samples, and instrumentation used in experiments included in this study.

Methods

Tissue Prep and Dissociation

Fresh frozen human brain tissue (BioIVT) was cut into 50mg pieces on dry ice to conserve RNA integrity and loaded onto gentleMACS C-tubes with tissue dissociation cocktail following the multi-tissue dissociation kit protocol provided by Miltenyi. Once dissociated, the nuclei suspension was filtered twice using 70um filters and 40um filters. The samples were kept on ice for the duration of the experiment and sorting.

Debris clearance for the non-sorted brain sample was accomplished using a sucrose gradient method, which proved to be more laborious and required more reagents compared to the Pala sorting system.

The Pala sorted sample (referred to as "Pala sorted") and the sucrose gradient prepped sample (referred to in figures and text as "Standard Method") were subsequently diluted to achieve 10,000 cells and loaded onto the chromium for downstream 10X library preparation and sequencing (**Figure 01**).

FIGURE // 01

Method workflow comparison

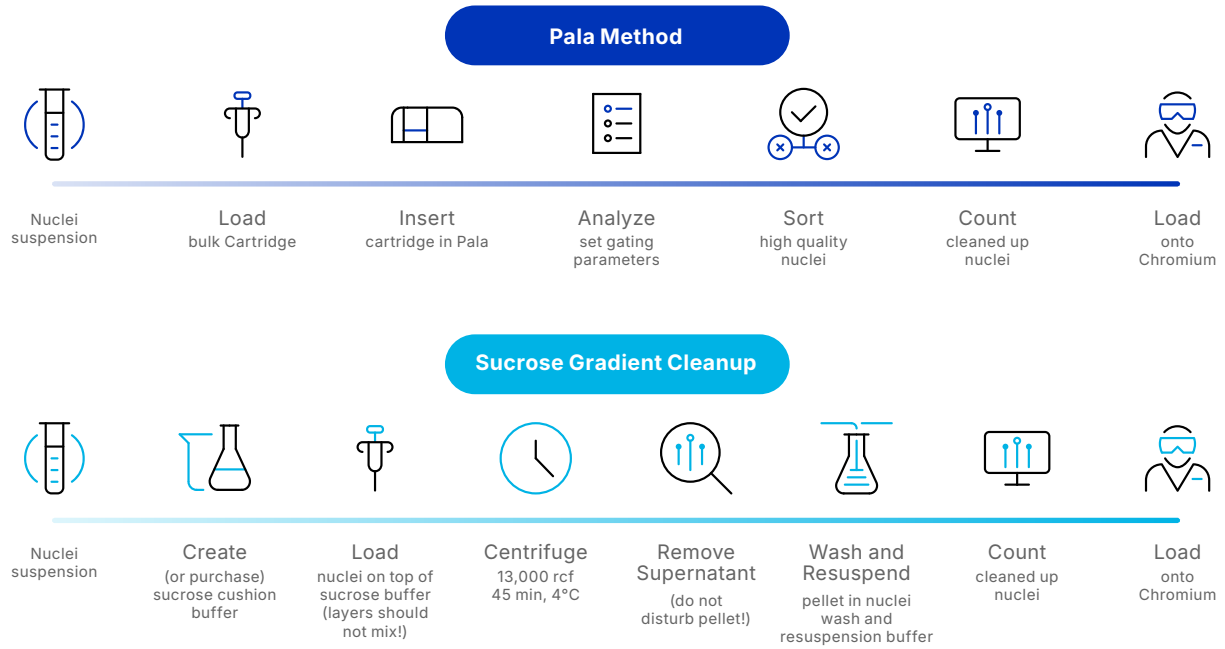


Figure 1: Pala Sorting Method vs. Sucrose Gradient Clean-up method (AKA "standard" method) workflow diagram

Cell Counting and Imaging

Dissociated nuclei were stained with propidium iodine (PI) with a working concentration of 50ug/mL and incubated for 10 minutes in the dark. Following the Nexcelom automated cell counter protocol, nuclei were loaded onto a plate for counting and imaging. After sorting nuclei with the Pala system, nuclei were counted and imaged to determine cell count and debris cleanup efficiency.

Dilution and loading onto the Pala system

Following nuclei counting, nuclei were diluted to achieve a working dilution of 300,000 nuclei/mL, which is ideal for bulk sorting. The suspension (600uL) was then added to a bulk sorting single use cartridge and loaded onto the Pala system. Nuclei were then analyzed and gated on FSC (Forward Scatter)/ALL (Axial Light Loss) and FSC/PI charts to remove doublets and debris, before 50,000 nuclei were dispensed into a 1.5mL Eppendorf tube containing 100ul of resuspension solution.

10X Genomics Chromium Controller Loading

The nuclei suspension was centrifuged at 4°C for 10mins @800xg and excess supernatant was removed, leaving only 50ul of suspension in the tube. Post-sort samples were counted and imaged once more and diluted to achieve a loading concentration of 10,000 nuclei per sample. A Chromium Next-Gem 3′ protocol was followed for the remainder of the loading and library preparation processes.

Library Prep and Sequencing

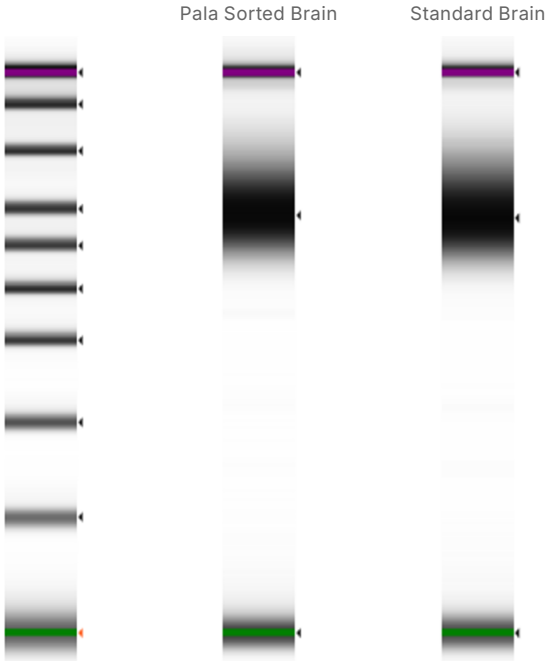
Both sorted and standard prepped samples were library prepared using the Chromium Next GEM single cell 3′ v3.1 kit and loaded onto the Chromium instrument. The samples underwent library preparation and QC using Azenta's standard operating procedure for determining sample quality, and both samples passed initial and final library prep QC. The Pala sorted brain sample provided comparable Tapestation QC results (**Figure 02 A**) with consistent cDNA concentrations (**Figure 02 B**)

as the “standard” sample. cDNA were then prepared and loaded onto a HiSEQ 2500 to sequence a total of 3,000 nuclei per sample and 50,000 reads per sample for a total of 150 million reads per sample.

FIGURE // 02

Tapestation QC data, cDNA concentrations, and consistent base pair range

A



B

Sample name	Average Base Pairs	Concentration
Pala Sorted Brain	518	31.4
Standard Brain	510	34.5

Figure 02. (A) Tapestation QC data from final libraries of sorted samples compared to samples that weren’t sorted.(B) Table showing cDNA concentrations, as well as consistent base pair range for samples sorted.

Sequencing/Bioinformatics

Prepared samples were indexed and loaded onto an illumina HiSEQ for sequencing following Illumina protocols. Bioinformatics analysis was performed utilizing Cell Ranger systems as well as methods created by our ExosomeDx Bioinformatics team.

Results

Nuclei Sorting and Debris Cleanup

Fresh frozen tissue samples of human brain were initially cut into 50mg pieces on dry ice and dissociated using a gentleMACS tissue dissociator. The samples were handled and dissociated at the same time to maintain consistent results. After two rounds of filtration, the samples were split into two separate Eppendorf tubes, one for Pala sorting and the other to be used in the standard method.

Samples were stained with Propidium Iodide (PI), which showed that there were abundant amounts of positively stained nuclei in the suspension and both samples showed high quantities of debris and aggregation (**Figure 02**, top panel). The samples displayed considerable debris, consisting of excess myelin and cell bodies prior to Pala sorting or sucrose gradient centrifugation, which is consistent with other findings (ref 6). Highly efficient debris removal was observed in both Pala sorted and standard samples (**FIgure 03**, middle & bottom panel).

FIGURE // 03

Images of nuclei from dissociated brain

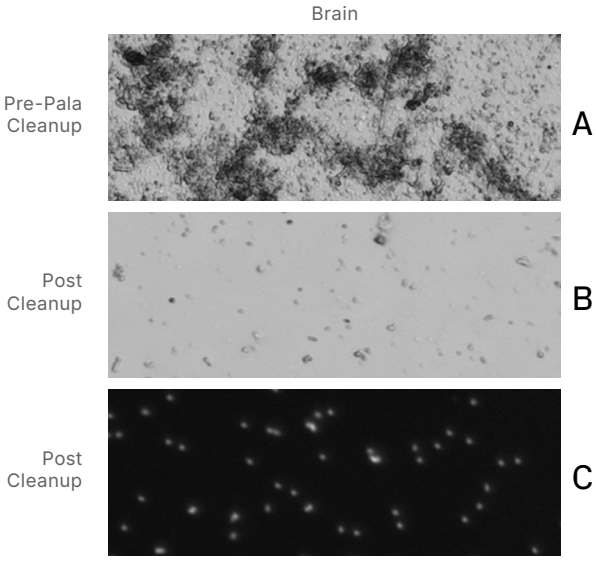


Figure 03. (A) Brightfield images of tissue samples prior to sorting, high amounts of aggregation and debris are observed. (B) Brightfield images of tissue samples after sorting on Pala and successfully clearing debris. (C) Sorted nuclei are stained with DAPI to show relative abundance in each sample.

Sequencing and Bioinformatics data

A comprehensive analysis was performed to compare the Pala sorted and standard nuclei using the sequencing data from the above workflow and programs including Cell Ranger, Loupe browser, R and Python.

First looking at recovery, the Pala sorted sample produced 10% more cells compared to the standard sample, indicating that more debris was sequenced in the standard method (**Figure 04 A**).

FIGURE // 04

Pala Sorted nuclei vs. standard clean up

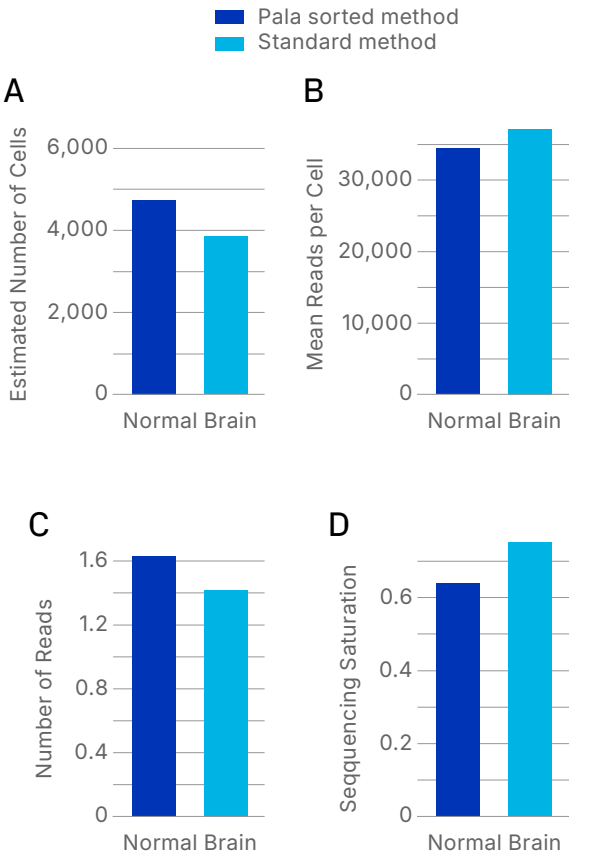


Figure 04. Bar graphs comparing Pala sorted sample vs. Standard sample statistics generated with cell ranger. Pala Sorted compared to the standard sample show (A) greater number of estimated cells, (B) lower mean reads/cell, (C) greater total reads per sample, and (D) lower sequencing saturation.

Interestingly, the mean reads per cell were lightly lower in the Pala sorted sample compared to the standard sample (**Figure 04 B**). This is likely because more nuclei were recovered in the Pala sorted sample, thus spreading out the sequencing reads across more cells (**Figure 04 C**).

The overall QC metrics passed acceptability thresholds including fraction of reads per sample, sequencing saturation, and were all very comparable between the Pala sorted sample and the standard sample. However, significantly lower sequencing saturation was observed in the Pala sorted sample indicating the Pala method produced a more complex library with more diverse reads (**Figure 04 D**).

It is worth noting that although unsorted samples produced higher median reads per cell, these reads may include unhealthy and apoptotic cells, thereby reducing transcriptome quality.

Barcode rank plot for the Pala sorted sample (**Figure 05**, dark blue line) showed an expected, characteristic distribution, compared to the standard method (**Figure 05**, light blue line). This information can help researchers determine both the quality and heterogeneity of the cells recovered in the data set.

FIGURE // 05

Pala sorted sample vs. standard sample

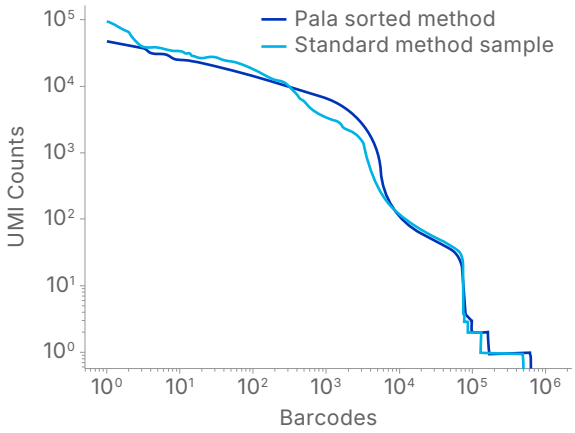


Figure 05. Barcode rank plots taken from cell ranger showing cells that received a barcode compared to background noise. Standard sample (light blue) vs. Pala sorted sample (dark blue).

The plots in **Figure 06** reveal the percentage of reads for a given GEM (cell) that are highly enriched for mitochondrial or top 100 genes and is indicative of GEMs containing intact nuclei vs. fragmented nuclei or debris. These plots allow one to understand the quality of each sample, and the percent of mitochondrial genes present, which describes the state of the sample and stress response due to the sample prep. All sample data were processed to filter out cells with more than 10% reads belonging to mitochondria, a hallmark of dying or apoptotic cells and cells with more than 50% of reads belonging to top 100 highly expressed genes, which is indicative of low-quality cells or cell fragments. Adopting these thresholds to filter out low quality cells, the Pala sorted sample necessitated only 8.6% of cells to be filtered while the standard method required 9.6% of cells to be filtered (**Figure 06**). This further shows the improved sample quality when utilizing the Pala to sort nuclei prior to 10x library prep and sequencing.

FIGURE // 06

Sample purity comparison

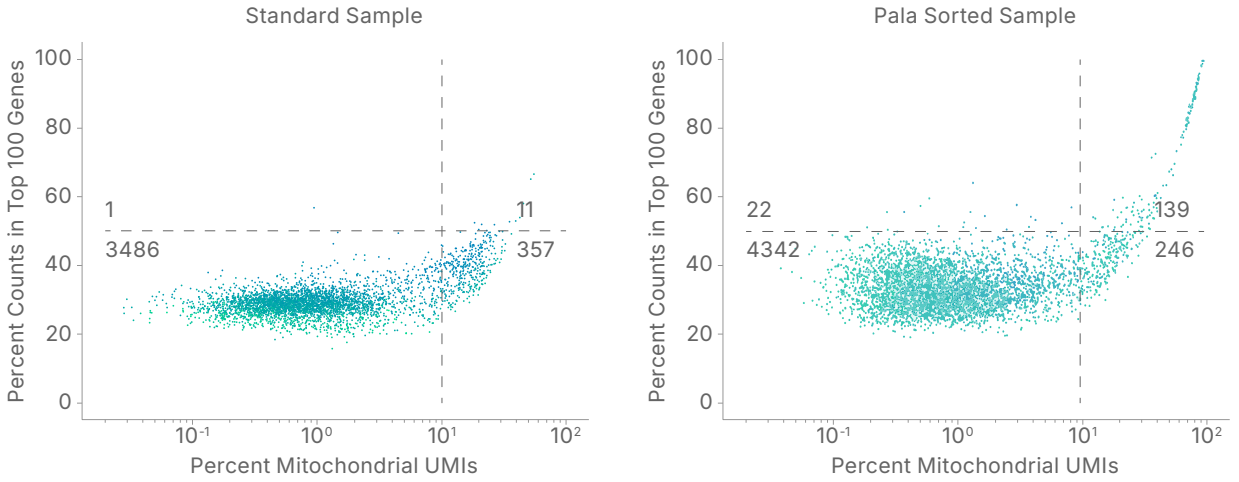


Figure 06. Scatter plots denoting the purity of each sample ran, data was filtered to remove any cells that attributed more than 10% reads to mitochondria or cells that attributed more than 50% to top 100 expressed genes. Pala sorted samples showed 1% less filtered cells compared to the standard method.

Sequenced nuclear reads from the Pala sorted and standard sample were plotted using UMAP clustering to group similar transcriptional signatures between cells and provide indications of cell type, sample diversity, and rare cell identification.

The data show that although the samples come from the same starting material, they cluster differently between the two methods used to prepare nuclei (**Figure 07**). Comparing UMAP plots between the Pala sorted and standard samples show the improved quality of the sorting method. For example, significantly more distinct clusters composed of more diverse cell types were observed, including more cells represented in each cluster, when the sample was sorted (**Figure 07 B**) compared to the standard sample where a single large cluster with a few satellite clusters are seen (**Figure 07 A**).

These data highlight the value of sorting samples with the Bio-Techne Pala system by improving the quality of the input sample.

Conclusion

Here we demonstrate how the Pala single cell dispenser can effectively clear debris and aggregated nuclei from dissociated brain samples upstream of 10X Genomic workflows. It was found that after running a successful 3′ single cell library prep and

FIGURE // 07

Standard method compared to Pala sorted method

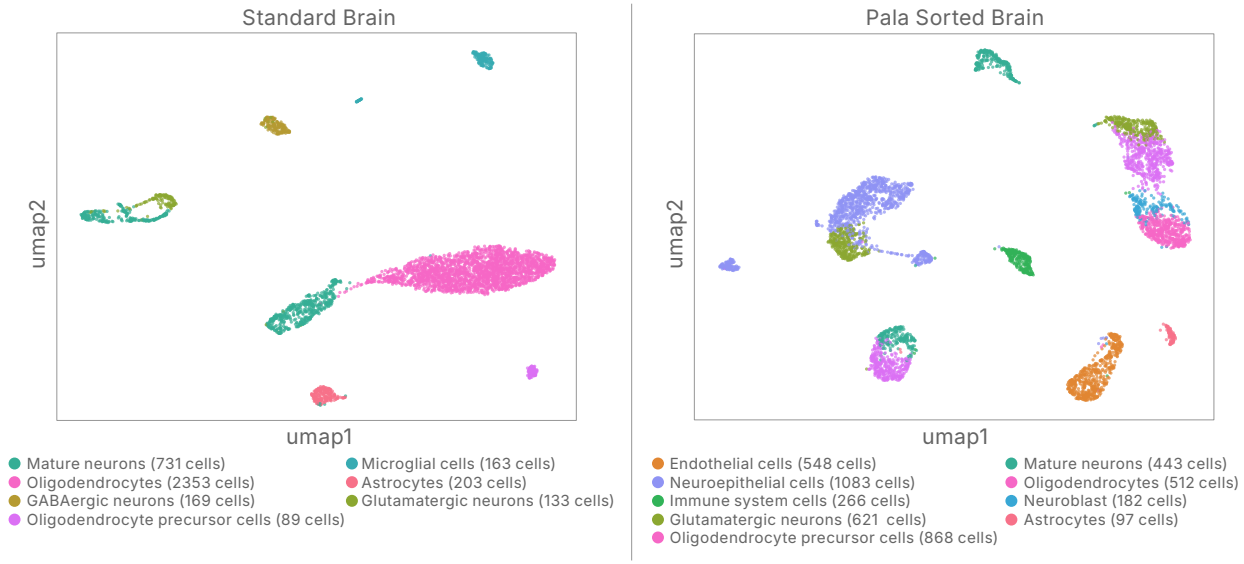


Figure 07. UMAP plots comparing data from the standard method vs. the Pala sorted method. Oligodendrocytes dominate the standard sample while with the Pala sorted sample shows more clusters and a more even distribution between recovered cell types.

sequencing that more nuclei are recovered compared to the standard sample isolation method.

The sequencing data suggest that sorting the nuclei improved QC metrics, and improved quality of sample sequenced with less ambient RNA. It was also found that these improvements in sample quality led to more diverse cell types, more representation of diverse cell types, and more rare cell types recovered within the dataset. In contrast, the standard sample exhibited a single large cluster with a few satellite clusters and a reduced diversity in cellular origin.

We can conclude that the Pala system is able to identify and sort positive nuclei for downstream 10X Genomics workflows, and that utilization of this instrument can be a very powerful tool for researchers who would like to improve their genomics workflows.

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