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## Introduction

Recovering highly viable and pure populations of neural cell subtypes from brain tissue has been historically challenging. The high pressure, long fluidics pathway, electrostatic charges, and long processing times of traditional jet-in-air/droplet-based sorters are often not well tolerated by neural cells and can have significant negative effects on the cells' ability to perform in downstream assays. We were able to overcome these challenges by using a proven, automatic

dissociation technique that utilizes mild enzymatic and mechanical mechanisms to dissociate the tissue in combination with a gentle microfluidic cartridge and microchip-based sorting technology to isolate cell subtypes. Here we show the successful isolation of astrocytes, neurons, and microglia with downstream culture, immunofluorescence microscopy, and single-cell RNA sequencing (scRNA-seq) data.

## Methods

### 1 Sample prep

Adult mouse brains from C57BL/6 mice 8–12 weeks old were purchased from Charles River Laboratories and shipped overnight on ice. Tissue was dissociated into a single-cell suspension using a Miltenyi Biotec Adult Brain Dissociation Kit, mouse and rat, and the gentleMACS<sup>®</sup> Octo Dissociator with Heaters. Debris and erythrocytes were subsequently removed to increase the percentage of intact neural cells. In the case of the astrocytes, samples went through a

secondary clean-up step to remove dead and apoptotic cells using a Miltenyi Biotec Dead Cell Removal MicroBead Kit with minor adaptations. Namely, MACSQuant<sup>®</sup> Tyto<sup>®</sup> Running Buffer was used in place of the Annexin Binding Buffer. Prepared cell samples were stained with a Miltenyi Biotec antibody cocktail to label the cell types of interest and then re-suspended in the MACSQuant Tyto Running Buffer for cell sorting (fig. 1).

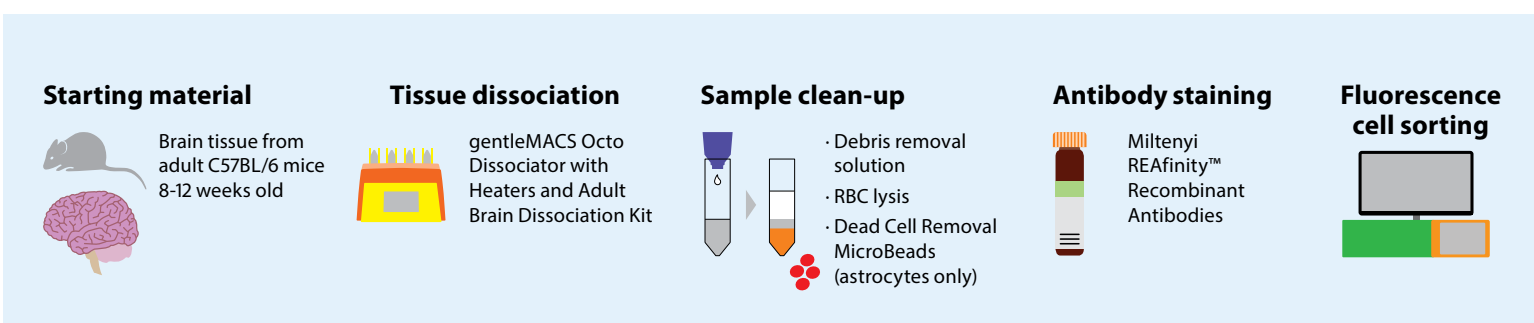


Figure 1. Workflow graphic

Cell subset	Cellular marker identification
Microglia	CD45 <sup>low</sup> , CD11b <sup>+</sup>
Astrocytes	O4 <sup>+</sup> , ACSA2 <sup>+</sup>
Neurons	CD45 <sup>-</sup> , O4 <sup>-</sup> , CD11b <sup>-</sup> , ACSA2 <sup>-</sup> , CD25 <sup>+</sup> , CD81 <sup>+</sup> , CD47 <sup>+</sup>

Table 1. Marker panel for sorting of target cells

### 2 Cell sorting with the MACSQuant Tyto Cell Sorter

Cell subsets were isolated using the MACSQuant Tyto Cell Sorter. The instrument's multi-parametric cell-sorting procedure occurs within the closed environment of the MACSQuant Tyto Cartridge HS. Unlike conventional droplet sorters, cells processed using this automation do not experience high pressures, shear forces, decompression, or electrical charges, ensuring they are minimally perturbed by the sorting procedure (fig. 2). Upon completion of the sorting process, samples of the input and positive and negative fractions were analyzed by flow cytometry on the MACSQuant Analyzer 10 to assess purity, yield, and viability.

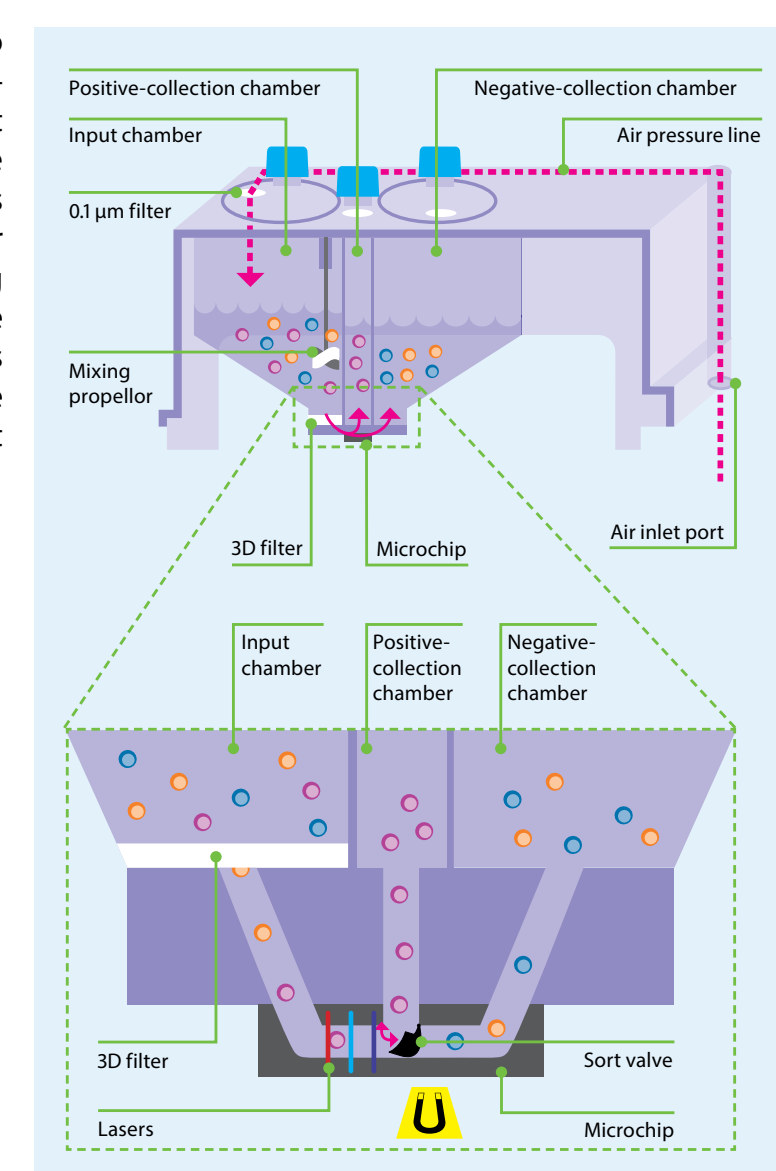


Figure 2. The cartridge and a schematic of how the sorting works with an image of the MACSQuant Tyto Cell Sorter.

### 3 Cell culture

After sorting, isolated astrocytes, microglia, and neurons were plated in a 24-well imaging plate from Miltenyi Biotec that had been coated overnight with Poly-L-lysine followed by an overnight coating of laminin before being washed and dried. For each cell type, 100,000 sorted cells were plated in 50 μL of their respective culture media (table 1). Cells were allowed to attach to the plate for 30 minutes in an incubator before the media was removed to eliminate dead cells and

debris. Each well was then filled with 500 μL of appropriate media and placed in an incubator for culturing. In the case of the neurons, cells were treated with 50 ng/mL of brain-derived neurotrophic factor (BDNF) for the first 3 to 6 hours of culture. The BDNF-containing media was then washed out and replaced with the same media without BDNF. Cultures were maintained by replacing 50% of the media every other day for anywhere from 5 to 7 days.

Cell type	Media
Microglia	MACS <sup>®</sup> Neuro Medium, 2% MACS <sup>®</sup> Neuro Brew <sup>™</sup> -21, 1% penicillin/streptomycin, 2 mM L-glutamine, and 20 ng/mL M-CSF
Astrocytes	MACS <sup>®</sup> Neuro Medium, 0.2% AstroMACS supplement, 2% MACS <sup>®</sup> NeuroBrew <sup>™</sup> -21, 1% penicillin/streptomycin and 0.5 mM L-glutamine
Neurons	MACS <sup>®</sup> Neuro Medium, 2% MACS <sup>®</sup> Neuro Brew <sup>™</sup> -21, 1% penicillin/streptomycin and 0.5 mM L-glutamine

Table 2. Cell culture media

### 4 Immunofluorescence microscopy

Cells were allowed to grow for 5 to 7 days before they were fixed with 2% PFA for 10 minutes at room temperature (RT) and stained with DAPI to identify nuclei. Cells were then incubated for 10 minutes at RT in staining buffer (MACS FcR Blocking Reagent, Mouse diluted 1:10 with autoMACS Running Buffer) to block background and nonspecific staining. Fixed and blocked cells were stained with unconjugated primary antibodies from Miltenyi Biotec,

washed and then stained with fluorescently conjugated secondary antibodies, also from Miltenyi Biotec. In the case of neurons, cells were permeabilized prior to staining using Triton-X because CD171 is a cytoplasmic marker. Incubation times differed depending on the antibodies used and the markers of interest. Samples were stored in autoMACS Running Buffer prior to imaging.

Cell type	Primary antibody	Secondary antibody
Microglia	Anti-CD11b	Anti-rat IgG2bk FITC
Neurons	Anti-CD171	Anti-rat IgG2a FITC

Table 3. Antibodies utilized in immunofluorescence identification

### 5 Single-cell RNA sequencing via Parse Biosciences Evercode Whole Transcriptome v2 technology

Sorted cells were then fixed using the Parse Biosciences Evercode Cell Fixation kit (fig. 3) and stored frozen at -80 °C until ready to be processed through the Evercode WT Kit and sequenced. The transcriptome of each single cell was appended with a unique combination of barcodes by progressing through four rounds of split-pool combinatorial

barcoding using the Evercode Whole Transcriptome kit which allows for the instrument-free profiling of single-cell whole transcriptomes from a bulk-sorted sample on any next-generation sequencing (NGS) device. In this case, cells were then sequenced on an Illumina NovaSeq.

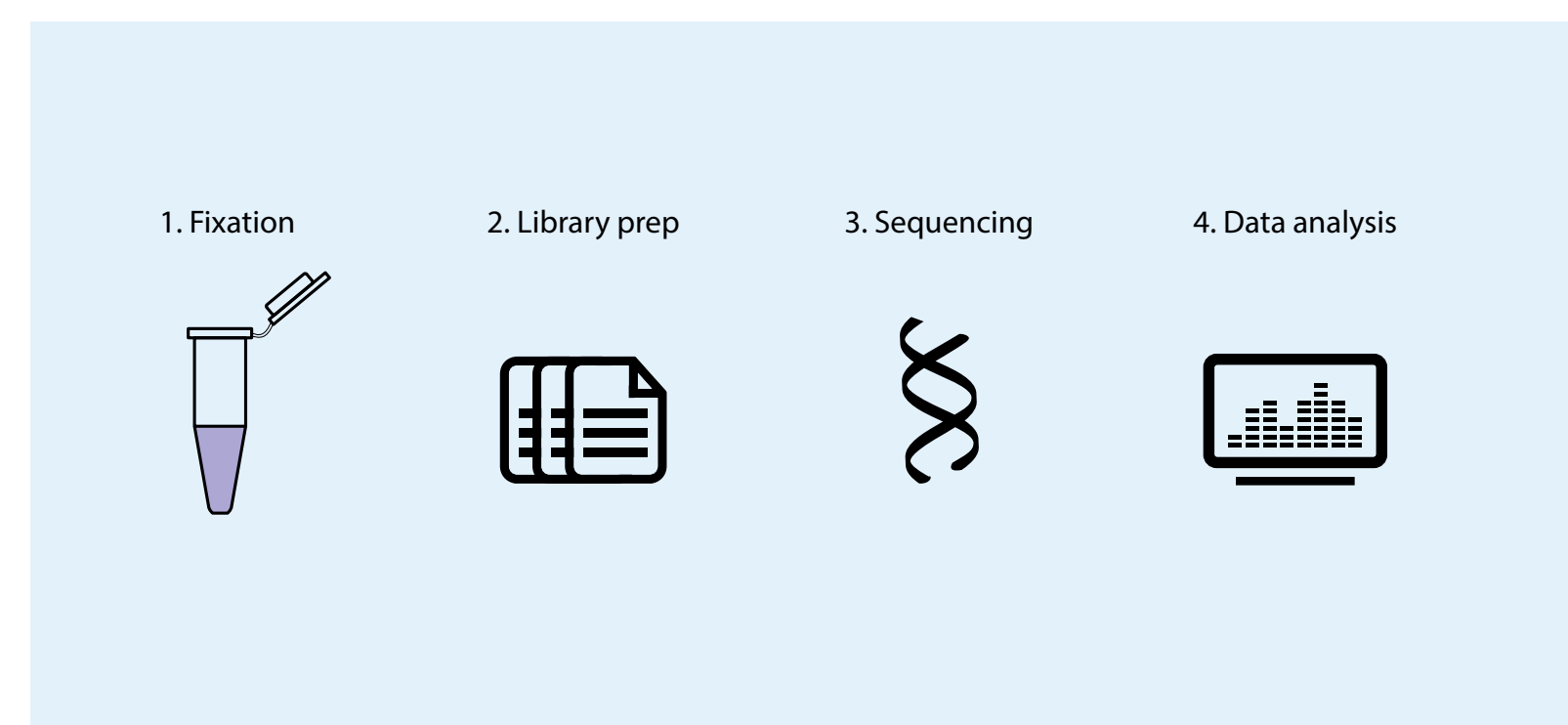


Figure 3. Parse Biosciences Evercode Cell Fixation Kit workflow

## Results

### 1 MACSQuant Tyto Cell Sorter results

All sorts were analyzed for purity, sort efficiency, and viability and assessed by propidium iodide (PI) staining prior to culture or fixation for scRNA-seq. The results are shown in figure 4. Purity is the percentage of target cells in the sort fraction. Sort efficiency is a measurement of yield

and tells us what fraction of the target cells recovered out of the cartridge made it into the sort fraction. Viability tells us what percentage of the sorted cells were negative for staining with PI (fig. 4).

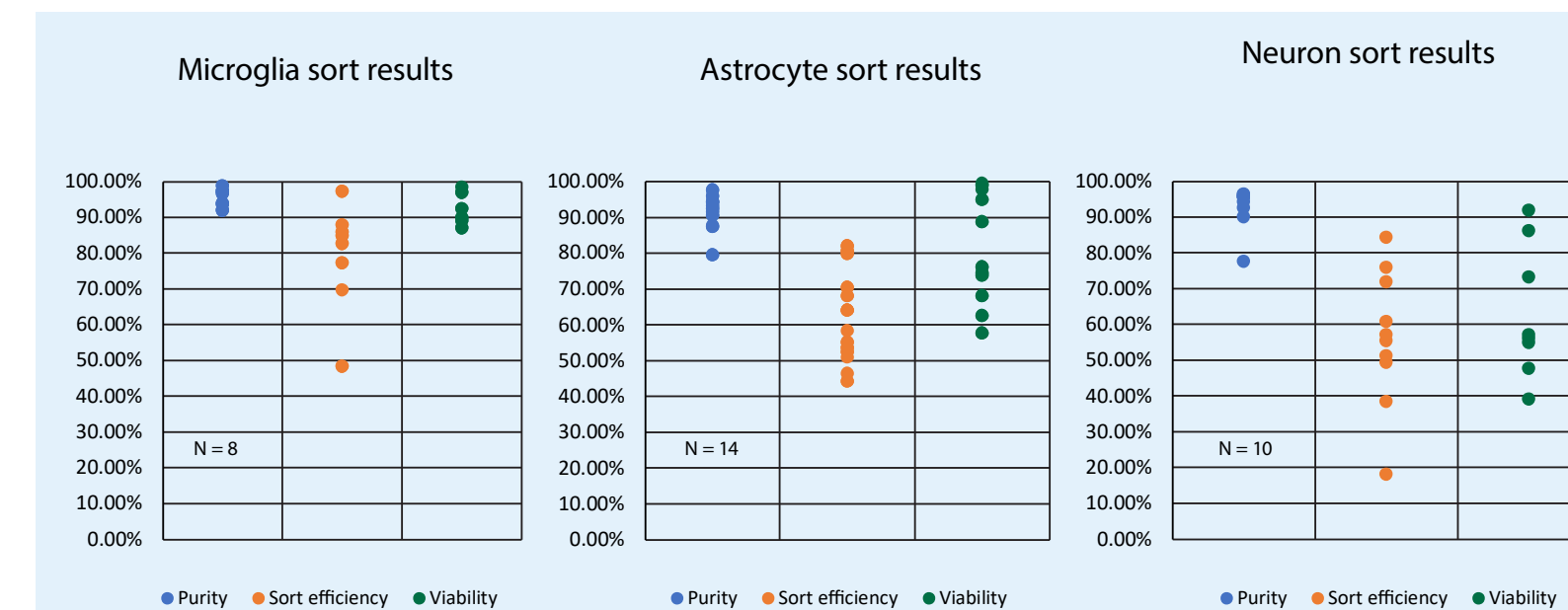


Figure 4. Purity, sort efficiency, and viability of sorted cells

### 2 Culture and imaging

After plating, the cells were imaged at two time points using brightfield microscopy to confirm that they exhibited morphology characteristic of the sorted subtype and were

ready for further processing (fig. 5). Cell type was further confirmed through immunofluorescent microscopy (fig. 6).

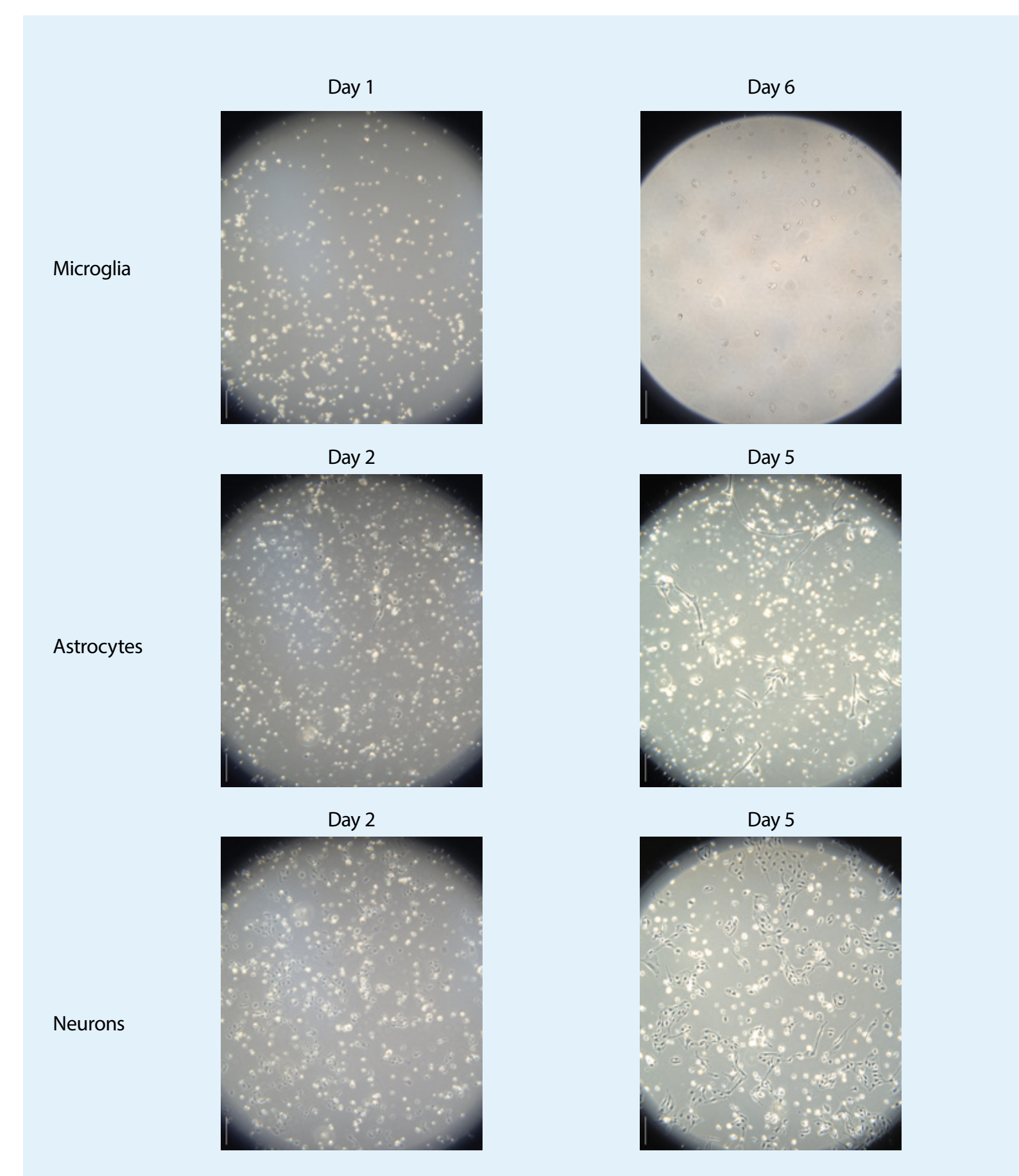


Figure 5: 40x magnification; scale bar 50 μm

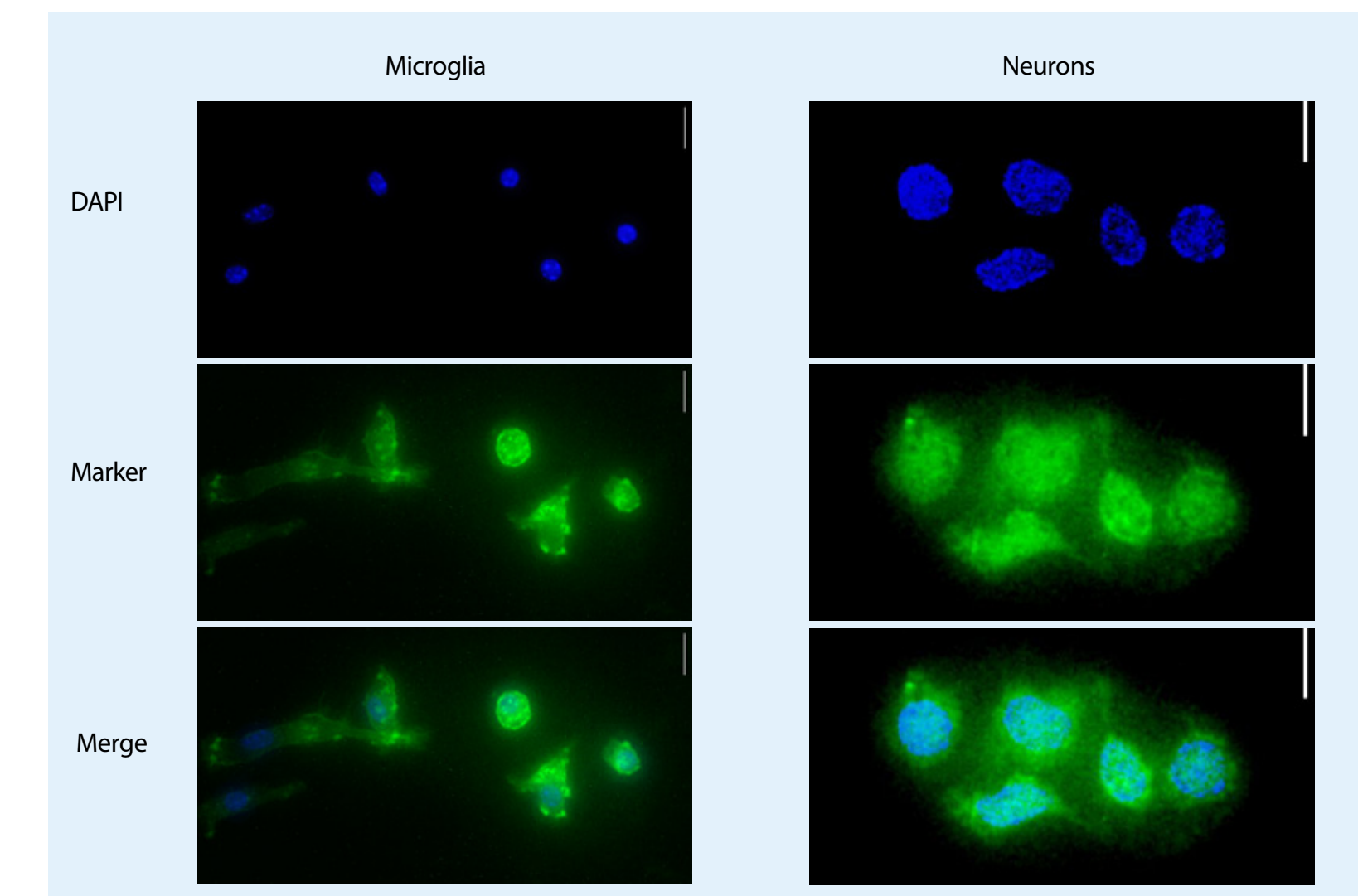


Figure 6. Immunofluorescence imaging: Images taken with 60x objective, scale bar 20 μm

### 3 Parse Biosciences Evercode WT v2 single-cell RNA sequencing analysis

The median number of genes and transcripts per cell were more abundant in the sorted samples versus the unsorted control. The median number of genes per cell was between 1,661 and 2,198 for the sorted cells and 1,485.5 for the unsorted control, and the median number of transcripts per cell was between 3,879 and 5,656 for the sorted cells and 3,079 for the unsorted control.

populations for each subtype at the gene level. The identity of the cell subtypes can be confirmed by looking at the gene expression levels of specific genes known to be differentially expressed in the cell type of interest (fig. 7). A significant discovery is that we saw no evidence of sorting-induced cell stress. The percentage of mapped reads attributable to mitochondrial RNA was very low. We saw 1.83% for the unsorted control and between 0.41% and 1.65% for the sorted cells.

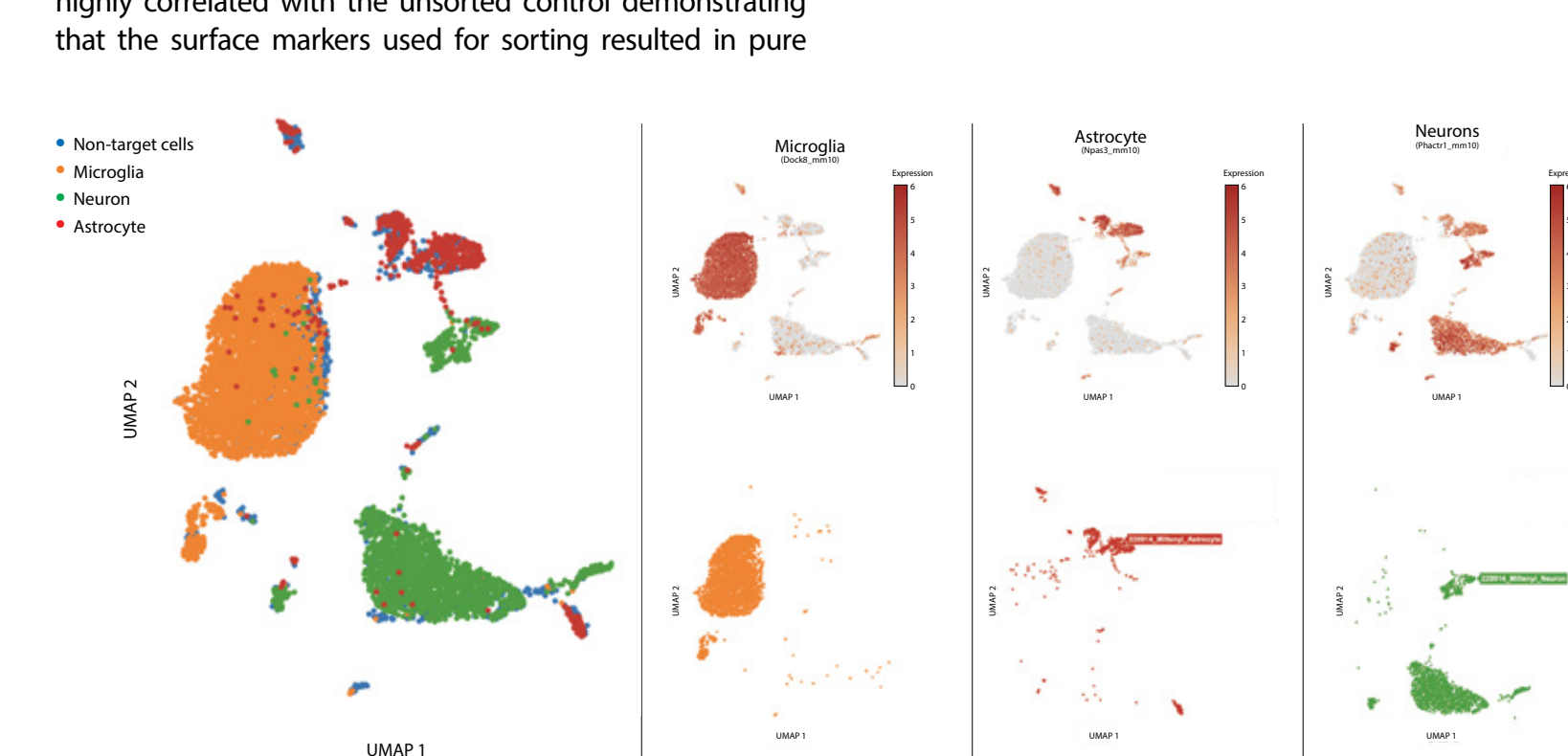


Figure 7. Single cell analysis of microglia, astrocytes, and neurons

## Conclusion

- When the gentleMACS Octo Dissociator with Heaters and the MACSQuant Tyto Cell Sorter are used together, the two instruments provide a gentle, fast, and complete workflow for the isolation of specific cell subsets from adult mouse brain.
- Sorted cells can be used for a variety of downstream assays including culture, immunofluorescent imaging, and scRNA-seq.
- The scRNA-seq data shows high-quality samples in terms of the number of gene reads and tight clustering of cell populations through unbiased principal component analysis. We can also confirm the identity of the sorted population by looking at the gene expression of genes associated with each isolated subtype.

### References:

- Miltenyi Biotec GmbH [application protocol] (2017). Isolation and cultivation of astrocytes from adult mouse brain. <https://www.miltenyibiotec.com/US-en/applications/all-protocols/isolation-and-cultivation-of-astrocytes-from-adult-mouse-brain.html#lgref>
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