APPLICATION NOTE

Gentle Levitation Uncovers Native scRNA-Seq Transcriptomic Signatures

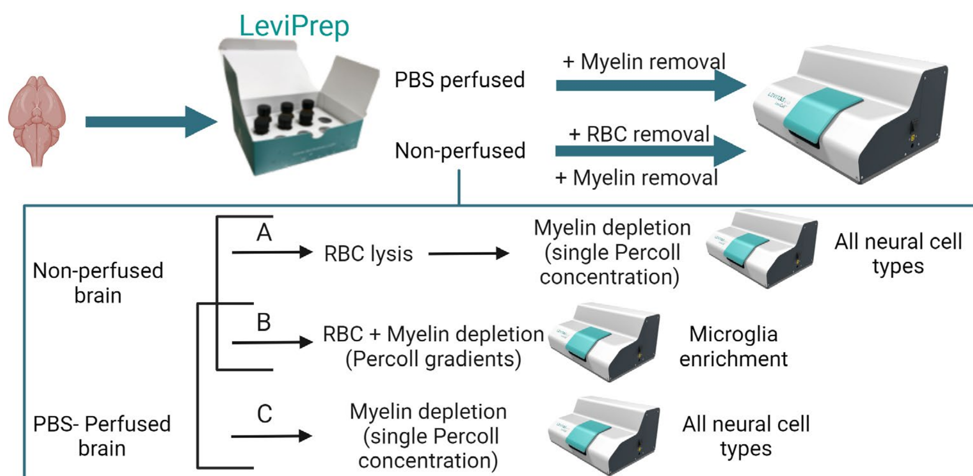
Overview

The brain is a complex organ comprising of different regions and various resident cell types. These different types of cells play their own unique role to maintain proper functioning of the brain. The cell types in the brain are broadly characterized as neurons, microglia, astrocytes, oligodendrocytes and endothelial cells. Neurons are functionally excitable cells which form networks with other neurons to modulate cognition, learning, memory, etc. Their communication relies on the release of neurotransmitters, which can either act to excite or inhibit the following neuron.[10] Neurons are supported by other cell types in the brain known as glial cells. Astrocytes, oligodendrocytes, and immune cells such as microglia make up the glial cells. Astrocytes perform several functions such as maintaining extracellular ion balance and control endothelial cells that form the blood-brain barrier.[11] Oligodendrocytes, on the other hand, support neurons by forming a myelin sheath to provide insulation of neuronal axons.[2] Microglia, the resident immune cell in the brain, act as the first line of defense where they get rid of damaged neurons or other infectious agents.[5]

The ability to isolate and interrogate single cells from fresh brain tissue is crucial for scientists to understand their role in homeostasis, disease conditions and drug treatments. However, isolating these single cells from tissues is problematic as the isolation process needs to be gentle, while providing enough yield and viable cells for further studies. In addition, the extraction process needs to minimize the activation of certain genes that could alter their characteristics and the research study.

The LeviPrep™ Mouse Tissue Dissociation Kit (PN 1005001) provides a pre-optimized protocol for isolating either all brain cell types or microglia specific cell type using PBS-perfused and non-perfused mouse brain samples. The kit also provides specific protocols for myelin removal from adult brain samples that can yield different final neural cell composition. The LeviPrep Mouse Tissue Dissociation Kit protocol is optimized for the LeviCell™ system, whose gentle Levitation Technology enriches and delivers the most viable cells that were isolated.

Figure 1 depicts the LeviPrep-LeviCell™ workflow and different protocols that one can follow to achieve a heterogeneous sample enriched for all neural cell types or a homogeneous sample of microglial cells only. It is important to note that the perfusion state of the starting tissue has no bearing on the ability to isolate all brain resident cell types or only microglial cells.



Enrichment For All Brain Cell Types (LeviPrep-LeviCell Workflow, Protocol A/C)

The LeviPrep-LeviCell workflow enables one to obtain all the different brain resident cell types if desired. It is essential to remove red blood cells (RBCs) and myelin from the single cell suspension prior to a LeviCell run. The high concentration of RBCs can hide the viable cell band during levitation and disrupt the process of viable cell enrichment. Two different protocols (A and C, as indicated in Figure 1) can lead to enrichment of all neural cell types, with the RBC lysis step as the key differentiator. When working with a non-perfused brain sample, protocol A includes a RBC lysis buffer step before myelin removal. In both protocols, the steps for myelin removal are the same.[9] First, the sample is overlaid on top of a 50% Percoll® solution. Next, centrifugation will separate and enable removal of the myelin ring. Finally, the sample is rinsed of any residual Percoll solution (Figure 2).

At this point, the single cell suspension is ready to be resuspended and loaded on the LeviCell system for enrichment of all viable brain cells. In Figure 3, approximately 140,000 neural cells were introduced into the cartridge and after 20 minutes of levitation, the top fraction was collected and measured at 80% viability (dual staining with Acridine Orange and Propidium Iodide was performed).

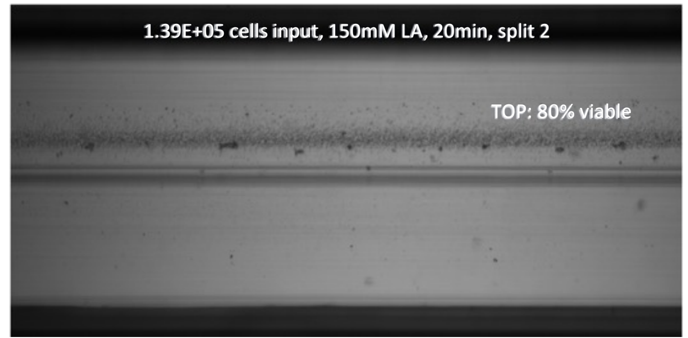


Figure 3. Levitation of cells from a PBS-perfused adult mouse brain digested with LeviPrep kit and enriched for all neural cell types (Protocol C). *Levitation conditions: 150 mM Levitation Agent in 1X PBS + 0.5% BSA.*

This top fraction of neural cell types was then further processed. After single-cell RNA sequencing and profiling of the isolated single cells, we obtained cell clusters corresponding to neurons, microglia, astrocytes, oligodendrocytes and endothelial cells (Figure 4). Cell type specific markers such as *CSF1R* (microglia), *ALDH1A1* (astrocytes), *MBP* (oligodendrocytes), *PCP4* (neurons) and *FIT1* (endothelial cells) were used to annotate and confirm the identify of cell clusters.

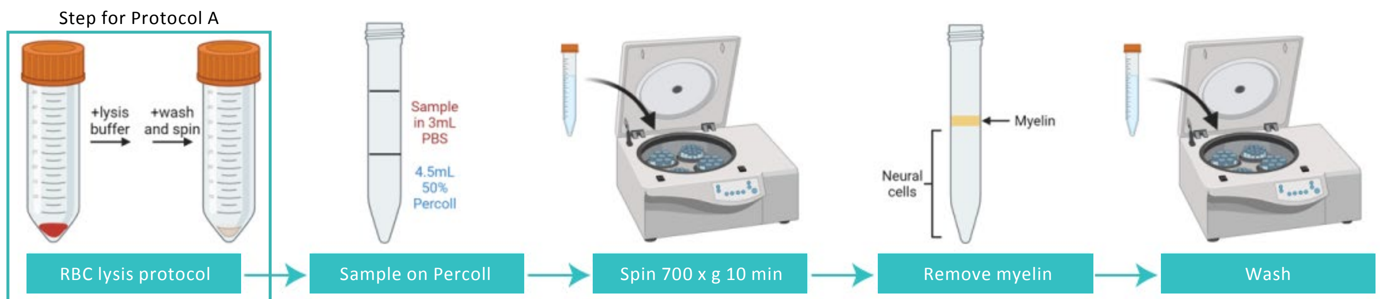


Figure 2. Schematic representation of Protocol A and C. *These steps allow all neural cells to be obtained from the digested brain after the LeviPrep workflow.[9]*

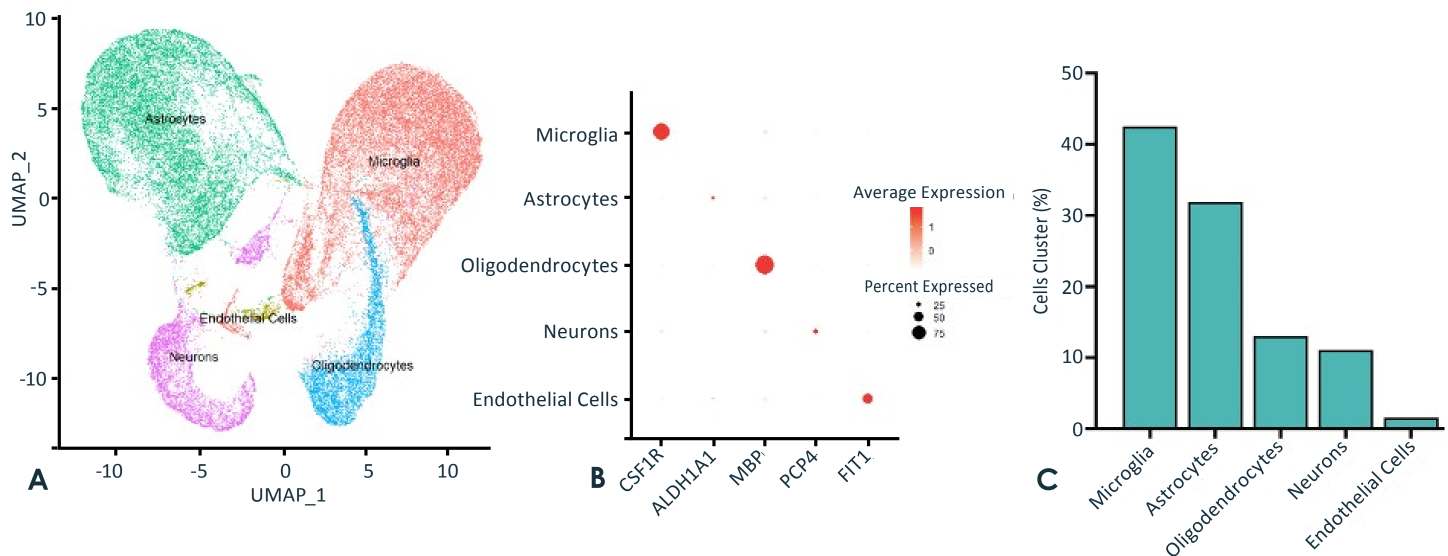


Figure 4. All neural cell types profiled from an adult mouse brain. *(A) Representative UMAP demonstrate clusters of cells corresponding to neurons, astrocytes, microglia, oligodendrocytes and endothelial cells. (B) Representative Dotplot demonstrate the genes used to annotate the clusters based on the expression of cell-type specific genes. (C) Quantification of percentage of cells per cluster obtained after using the LeviPrep-LeviCell workflow.*

The Need to Isolate and Enrich Microglia

As previously described, microglia are brain resident innate immune cells that regulate brain development and play a critical role in the etiology and progression of different brain disorders such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and traumatic brain injury.[8]

As interesting as they are, microglia are also highly sensitive cells that are easily activated with routine handling and in particular, excessive mechanical stress. The aim of microglial-targeted therapy is to maintain the homeostatic state of microglia and restrict the induction of an inflammatory/activated state, which can alter their transcriptome profile. This enables researchers to more accurately study the effect of stress/toxins/drugs on their ability to activate microglia. However, maintaining microglia in their native homeostatic state is difficult to achieve when the enrichment step involves the use of FACS or other high-cell-stress sorting methods. The stress these cells go through while sorting further leads to their activation which is not ideal when studying dysregulated molecular pathways and designing targeted therapies.

The LeviCell system, a label-free solution for sample processing, addresses all of these challenges through fast, simple, and extremely gentle microglia/macrophage enrichment without altering transcriptome signatures.

Isolation and Enrichment For Microglia (LeviPrep-LeviCell Workflow, Protocol B)

The simple LeviPrep-LeviCell workflow can be used

for microglia enrichment and is illustrated in Figure 1, starting with tissue digestion through cell enrichment with the LeviCell system. The LeviPrep protocol begins with a 30 minute cold enzymatic digestion of the minced brain tissue. The enzyme is then inactivated and the sample is washed. If working with a non-perfused brain, the resulting single cell suspension will contain myelin and RBCs. Enrichment of microglia cells will require use of a Percoll gradient for simultaneous removal of myelin and RBCs.[7]

As indicated in Figure 1, protocol B can be used with PBS-perfused or non-perfused mouse brain to isolate

microglia cells. The sample is first resuspended in 37% Percoll solution. A 70% Percoll solution is then underlaid together with an overlay of a 30% Percoll solution and PBS. After centrifugation, the myelin ring can be removed and the fraction of cells collected and washed (Figure 5). At this point, the single cell suspension is ready to be resuspended and loaded on the LeviCell system for enrichment of all viable microglial cells. Our recommended standard levitation conditions of 150 mM of Levitation Agent and 20 minutes are sufficient to obtain a clean and enriched microglia fraction (Figure 6).

Efficient Microglia Enrichment Post LeviPrep-LeviCell Workflow

To effectively study the role of microglia in diseases, researchers have long tried to isolate, enrich and culture these cells, thereby modeling the disease *in vitro*.

The LeviPrep workflow and Percoll gradient method was used to isolate cells (Figure 5) followed by label-free LeviCell enrichment (Figure 6). Single cell RNA sequencing was performed on the collected cells. The LeviCell data was compared with the previously published FACS dataset[6] and downstream data analysis was performed. The analysis revealed that the LeviPrep-LeviCell workflow successfully enriches a higher percentage of microglia compared to FACS (Figure 7).

Figure 5. Schematic representation of Protocol B. *A Percoll gradient and centrifugation step removes myelin and RBCs in order for microglia cells to be isolated from the digested brain after the LeviPrep workflow.*

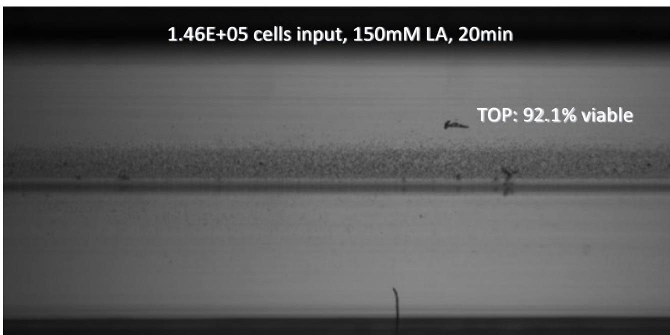


Figure 6. Levitation of cells from an adult mouse brain digested with LeviPrep kit and enriched for microglia cells (Protocol B). *Approximately 150,000 cells were introduced into the cartridge and after 20 minutes of levitation, the top fraction was collected and measured at 92% viability (dual stained with Acridine Orange and Propidium Iodide). Levitation conditions: 150 mM Levitation Agent in 1X PBS + 0.5% BSA.*

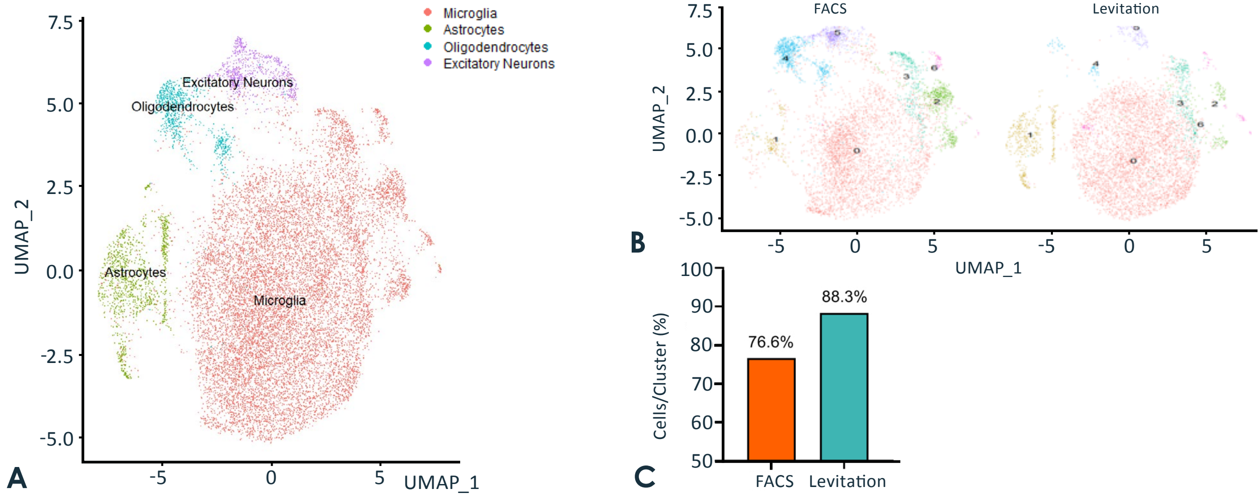


Figure 7. Efficient microglia enrichment post LeviPrep-LeviCell workflow. *(A) Representative UMAP demonstrating different cell types profiled by single-cell sequencing after either FACS or levitation. (B) Representative split UMAP demonstrating the contribution of either method to the distinct cell types profiled. (C) Quantification of percentage of microglial cells per cluster obtained after FACS (76.6%) or LeviPrep-LeviCell workflow (88.3%).*

Enrichment of Homeostatic Microglia versus Proliferative and Inflammatory Microglia

The most common way of monitoring microglial cell activation is the determination of changes in extracellular levels of cytokines, chemokines, or nitric oxide (NO). Excitotoxins induce activation and proliferation of microglial cells and thereby enhance the release of microglial toxins, including IL-1β and NO, resulting in increased neuronal death.[4]

Upon subsetting the microglial cluster from the dataset and re-analyzing the cells, we were able to distinguish microglia subtypes based on their cell state, namely: homeostatic, proliferative, inflammatory microglia and infiltrating macrophages. As seen in Figure 8, the LeviPrep-LeviCell workflow enriches homeostatic microglia as demonstrated by the expression of homeostatic microglial marker, *CSF1R*. On the other hand, FACS led to a higher proportion of activated microglia, demonstrated by increased expression of markers corresponding to proliferation (*TOP2A*) and inflammation (interferon response - *IFIT3*) as compared to the LeviCell processed samples.

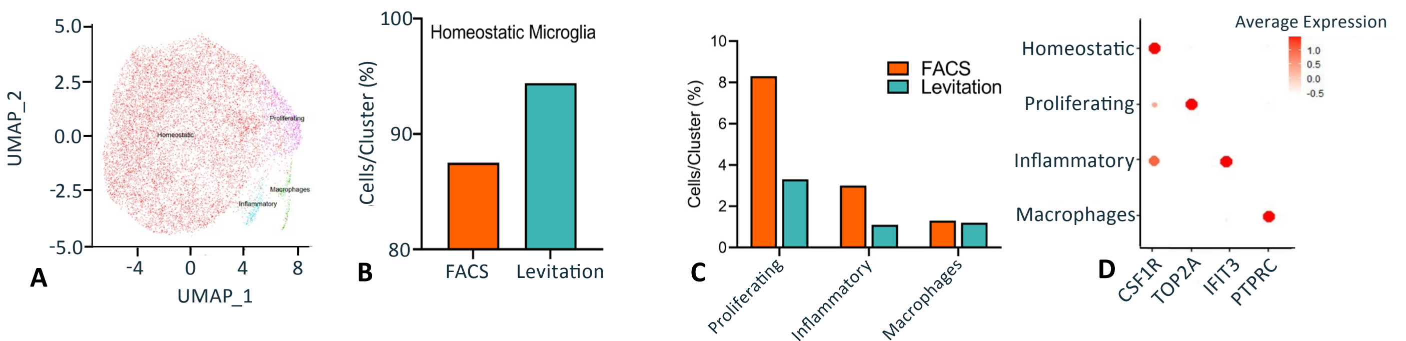


Figure 8. LeviPrep-LeviCell workflow enriches homeostatic microglia. *(A) Representative UMAP demonstrating different microglia types profiled using single cell sequencing after FACS or LeviCell. (B-C) Quantification of percentage of homeostatic microglia (B), proliferating, inflammatory microglia and macrophages (C) per cluster obtained after FACS or LeviCell. (D) Representative genes (CSF1R, TOP2A, IFIT3 and PTPRC) used for annotating the clusters.*

Maintenance of Transcriptomic Signature Through Gentle Levitation

As previously demonstrated in Figure 7 and 8, the LeviPrep-LeviCell workflow enriches microglial cells, specifically homeostatic microglia. When we looked at expression of different markers, we concluded that the LeviPrep-LeviCell workflow enables enrichment of microglia with less activation-inducing stress and altering transcriptional signature. Figure 9 shows the average expression of known stress genes expressed in microglial cells isolated from the adult brain after levitation or FACS. Along with significant improvement in cell viability, the LeviCell system isolated these cells in a stress-free environment and successfully preserved their transcriptomic profile. This is depicted by lower expression levels of activated gene markers of various known stress pathways such as the Lysosomal pathway genes (*CD68, LYZ2*), antigen presenting gene (*CD74*), Complement pathway (*C5AR1*) and Interferon response genes (*IFIT3, IRF7*) as compared to FACS processed microglia.

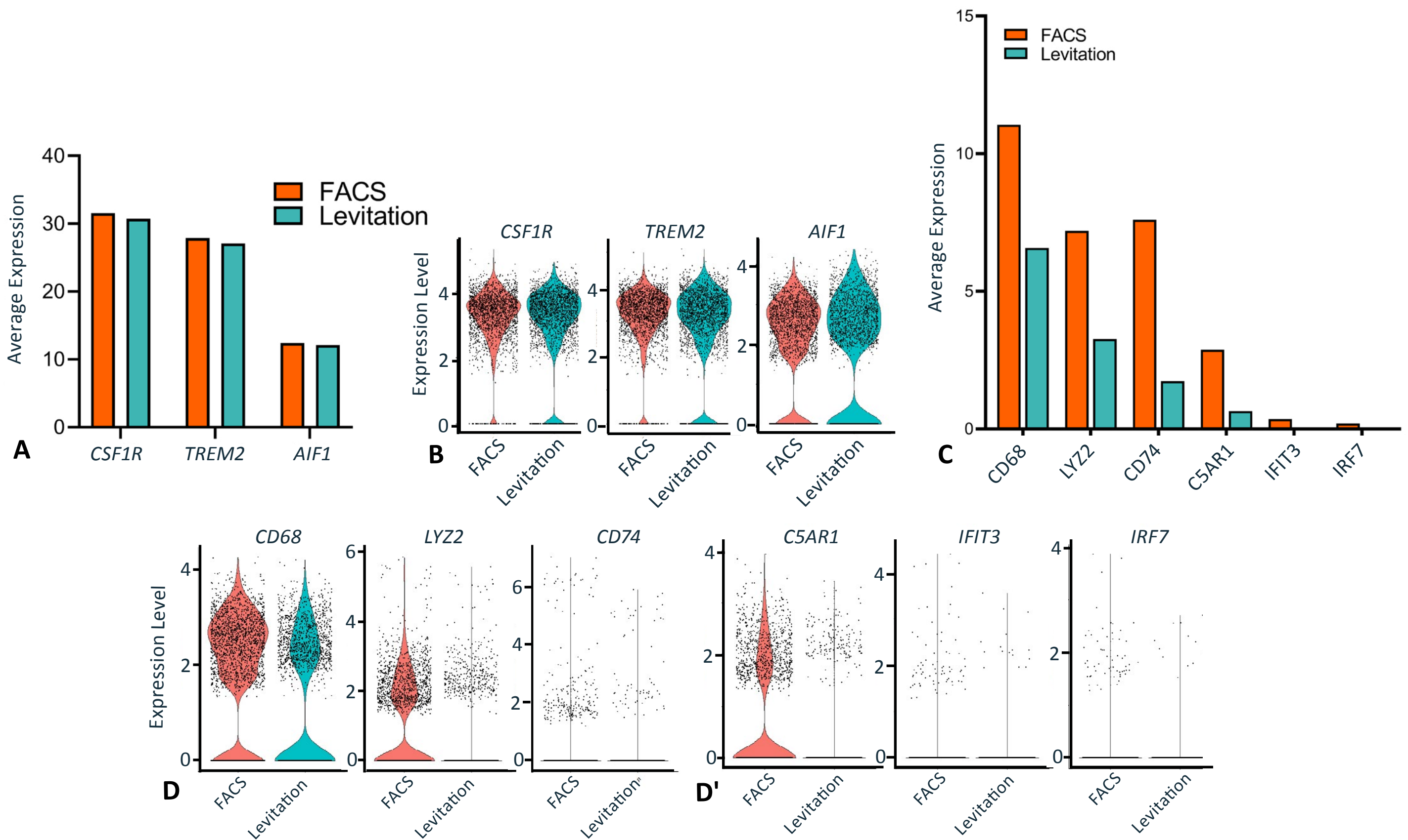


Figure 9. LeviPrep-LeviCell workflow maintains the transcriptomic signatures of profiled microglia. *(A-B) Quantification of average expression (A) and representative violin plots (B) of three general microglia markers (CSF1R, TREM2 and AIF1) does not show any difference in the LeviCell system as compared to FACS. (C-D) Quantification of average expression (C) and representative violin plots (D-D') of six markers for activated microglia (CD68, LYZ2, CD74, C5AR1, IFIT3 and IRF7) demonstrating a reduced expression of activation markers in LeviCell processed microglia as compared to FACS sorted microglia.*

Stress-Free Environment Leads To 2-6 Fold Lower Expression of Activation Markers

As demonstrated in Figure 9, the microglia enriched using LeviPrep-LeviCell workflow demonstrated a lower average expression of microglia activation markers, without altering the expression of other microglial markers such as *CSF1R*, *TREM2*, and *AIF1*. Upon quantification of the differences in average expression of these markers, we observed a 2-6 fold lower expression of microglia activation markers in cells subjected to enrichment via LeviCell as compared to the enrichment performed using FACS (Figure 10).

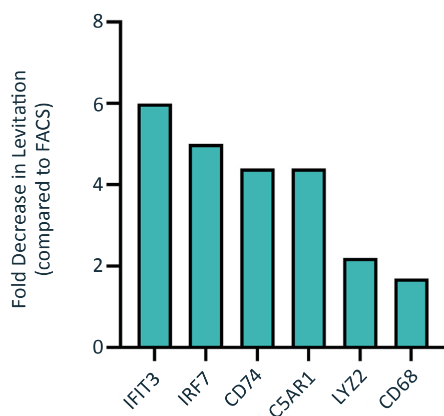


Figure 10. LeviPrep-LeviCell system maintains microglia in a stress-free environment. *Quantification demonstrates a 2-6 fold decrease in microglia activation markers when processed via the LeviPrep-LeviCell workflow compared to the FACS sorted method.*

Conclusion

In conclusion, therapeutic targets against disease-associated-microglial induction mechanisms might serve as a way to ameliorate neurodegeneration.[3] However, it is imperative to isolate and enrich microglia in their native homeostatic state to elucidate their involvement and/or response in different neurodegenerative disorders. Throughout this application note, we have demonstrated how isolation and enrichment of homeostatic microglia with the use of the gentle, label-free LeviPrep-LeviCell workflow uncovers native scRNA-seq transriptomic signatures.

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