Minimizing the \textit{ex vivo} confounds of cell-isolation techniques on transcriptomic and translatomic profiles of purified microglia

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Abstract

Modern molecular and biochemical neuroscience studies require analysis of specific cellular populations derived from brain tissue samples to disambiguate cell type-specific events. This is particularly true in the analysis of minority glial populations in the brain, such as microglia, which may be obscured in whole tissue analyses. Microglia have central functions in development, aging, and neurodegeneration and are a current focus of neuroscience research. A long-standing concern for glial biologists using in vivo models is whether cell isolation from CNS tissue could introduce ex vivo artifacts in microglia, which respond quickly to changes in the environment. Mouse microglia were purified by magnetic-activated cell sorting (MACS), as well as cytometer- and cartridge-based fluorescence-activated cell sorting (FACS) approaches to compare and contrast performance. The Cx3cr1-NuTRAP mouse model was used to provide an endogenous fluorescent microglial marker and a microglial-specific translatome profile as a baseline comparison lacking cell isolation artifacts. All sorting methods performed similarly for microglial purity with main differences being in cell yield and time of isolation. Ex vivo activation signatures occurred principally during the initial tissue dissociation and cell preparation and not the cell sorting. The cell preparation-induced activational phenotype could be minimized by inclusion of transcriptional and translational inhibitors or non-enzymatic dissociation carried out entirely at low temperatures. These data demonstrate that a variety of microglial isolation approaches can be used, depending on experimental needs, and that inhibitor cocktails are effective at reducing cell preparation artifacts.

Significance Statement

Purification of brain microglia from laboratory animal models allows for cell-type specific molecular and biochemical analyses. A long-standing concern of microglial purification is the introduction of artifacts through the isolation process. Comparison of magnetic-activated cell sorting (MACS) and both cytometer- and cartridge-based fluorescence-activated cell sorting (FACS) show equivalent and sufficient cellular yield/purity and similar levels of ex vivo activation which was determined to arise during tissue dissociation. Ex vivo microglial activation in cell suspensions generated by a combination of enzymatic and mechanical dissociation procedures was prevented by supplementation with transcription/translation inhibitors during cell preparation. Alternatively, non-enzymatic dissociation conducted at low temperatures prevented such ex vivo activational signatures. Cell preparation and sort methods can be further optimized depending on experimental needs.
Introduction

Microglia, the brain's resident macrophages, have come to the forefront of neuroimmunology research (Prinz et al., 2019). They serve as surveyors of the central nervous system and exhibit behavior derived from their embryonic precursors, myeloid cells (Cuadros and Navascues, 1998; Rock et al., 2004), with roles in neurodevelopment, sex differences, as well as in health and neurodegenerative diseases (Butovsky and Weiner, 2018; Han et al., 2021; Salter and Stevens, 2017). Microglial activity is governed by local microenvironments and through communication with neighboring cells. Under stress, microglial cells transition to an activated phenotype, classically defined by morphological transformation from ramified to amoeboid, release of pro-inflammatory cytokines, and a shift in global gene expression (Avignone et al., 2015; Rock et al., 2004; Sierra et al., 2013). With the advent of single cell transcriptomic sequencing, the field has undergone a taxonomy reclassification (Dubbelaar et al., 2018; Provenzano et al., 2021). Current evidence suggests microglia exist within a phenotypic gradient, and the transition away from a quiescent state is no longer viewed as binary ‘on’ or ‘off’. Thus, the use of microglial gene expression profiles to infer functional status has bolstered the use of transcriptomic profiling as a powerful technique for microglial classification. To delineate the full heterogeneity of microglial population with aging and disease using single cell techniques, it is necessary to minimize activational effects of cell preparation and isolation.

Traditionally, molecular analyses from specific CNS cell types required the liberation of cells from their native environment and use of fluorescence-activated cell sorting (FACS) or MACS labeling techniques prior to RNA extraction (Cahoy et al., 2008). Cell dissociation primarily consists of enzymatic and/or mechanical dissociation with quality checks for cell viability, debris removal, and absence of cell aggregation (Reichard and Asosingh, 2019). Creation of a single-cell suspension from brain tissue and isolation of microglia is harsh and may alter the phenotypic state of microglia ex vivo (Haimon et al., 2018; Wu et al., 2017). Ex vivo microglial activation has the potential of introducing confounds that may mask endogenously induced activation, such as in a pathological state. To avoid cell-isolation confounds, microglial-specific cre-lines (Cx3cr1-cre/ERT2) have been combined with various floxed ribosomal tagging models: 1) ribosome tagging (RiboTag) (Haimon et al., 2018), 2) translating ribosome affinity purification (TRAP) (Ayata et al., 2018), and 3) nuclear tagging and translating ribosome affinity purification (NuTRAP) (Chucair-Elliott et al., 2020), allowing the immunoprecipitation (IP) of tagged polysomes to isolate microglial-specific translatomes without the need for cell isolation. Although transgenic ribosome IP-approaches overcome many of the potential confounds of ex vivo activation,
activation, experimental endpoints such as proteomics and single cell heterogeneity still require cell isolation of intact microglial cells. Additionally, animal availability, complex breeding strategies, and cost will continue to be deterrents for many investigators to using transgenic microglial labeling. While single-cell sequencing allows for broad and potentially unbiased analysis of various cell types, it too is predicated on the creation of a single-cell suspension. Thus, an understanding of the effects of cell preparation and isolation methods on ex vivo activation while maintaining highly pure microglial enrichment is needed. The advent of ribosome tagging approaches allows generation of a reference microglial signature to which sorted microglial profiles can be compared. The goals of this study were to compare purity and yield of isolated microglia and assess the relative level of ex vivo activation by comparing Cx3cr1-TRAP-isolated RNA to various sorting techniques used for microglia: MACS (Holt and Olsen, 2016; Nikodemova and Watters, 2012), cytometer-based FACS (Hickman et al., 2013), and newly available low-pressure cartridge-based FACS (Roberts et al., 2021) using the TRAP-enrichment profile as a baseline purified microglial translatome for ex vivo activation.

We compared artifacts induced through three cell sorting techniques via transcriptomic profiling of bulk tissue, sorted cells, and immunoprecipitated translatomes and found similar performance with ex vivo activational signatures principally occurring during the enzymatic digestion and mechanical dissociation during initial cell preparation. Inclusion of transcriptional and translational inhibitors during the cell preparation step prevented most of these artifacts. Additionally, non-enzymatic tissue dissociation carried out entirely at 4°C was successful at preventing the ex vivo activational signature. These studies provide critical insight into the sensitivity of microglia and guidance on experimental design to minimize ex vivo confounds of microglial isolation.

**Materials and Methods**

**Animals**

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Oklahoma Medical Research Foundation (OMRF). Parent mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred and housed under SPF conditions in a HEPA barrier environment on a 14/10 light/dark cycle (lights on at 6:00am). Cx3cr1-cre/ERT2+/wt males (stock # 020940) (Yona et al., 2013) were mated with NuTRAP^flox/flox^ females (stock # 029899) (Roh et al., 2017) to generate the desired progeny, Cx3cr1-cre/ERT2^+/wt^; NuTRAP^flox/wt^ (Cx3cr1-
NuTRAP). In the Cx3cr1-NuTRAP mice, following cre recombination in Cx3cr1+ cells, deletion of the floxed stop cassette causes activation of the NuTRAP allele, labeling microglial ribosomes with eGFP and nuclei with biotin and mCherry. For flow cytometry experiments, cre negative controls (Cx3cr1-cre/ERT2wt/wt NuTRAPfloxed/wt) were used to establish eGFP gating. DNA was extracted from mouse ear punch samples for genotyping. Mice were ~3-8 months old at the time of performing experiments. Within each experimental group, both male and female mice were used. The sex of each experimental sample is denoted in Tables 2-1, 4-1, 6-1, and 7-1.

Mice were euthanized by cervical dislocation followed by rapid decapitation. Genotyping primers were obtained from Integrated DNA Technologies (Coralville, IA) and used for PCR detection of: 1) generic cre (Jax protocol 22392, Primers: oIMR1084, oIMR1085, oIMR7338, oIMR7339), 2) Cx3cr1-cre/ERT2 (Jax protocol 27232, Primers: 20669, 21058, 21059), 3) mCherry (Jax protocol 24693, Primers: 9703, 9704, oIMR8744, oIMR8745), and 4) NuTRAP floxed allele (Jax protocol 21509, Primers: 21306, 24493, 32625, 32626) according to protocols published by the Jackson Laboratory, as previously described (Chucair-Elliott et al., 2020).

Tamoxifen (Tam) induction of cre recombinase
At ~3-months-old (mo), mice received a daily intraperitoneal (ip) injection of tamoxifen (Tam) solubilized in 100% sunflower seed oil by sonication (100 mg/kg body weight, 20 mg/mL stock solution, #T5648; MilliporeSigma, St. Louis, MO) for five consecutive days. Based on an average weight of 20 g per mouse, each daily injection of Tam consisted of 100 µL of 20 mg/mL stock solution. Adjustments were made for mice that significantly deviated from the average weight. Tissues were harvested from 5-8 mo mice, allowing at least 2 months following the last Tam injection and turnover of CX3CR1+ circulating cells.

Flow cytometry from blood
Blood was collected from the facial vein of Cx3cr1-NuTRAP (3 and 60 days post-tamoxifen induction) and cre negative (WT) controls (n=3-10/group) using a 5-mm sterile Goldenrod animal lancet (MEDpoint, Mineola, NY) and mixed with 10 µL 0.5 M EDTA, pH 8.0 to avoid coagulation (Chucair-Elliott et al., 2020). Ten volumes of room temperature 1X Red Blood Cell Lysis Solution (#130-094-183, Miltenyi Biotec) were added to one volume of collected blood, vortexed for 5 seconds, and then incubated in the dark at room temperature for 10 minutes. Cells were pelleted at 300 x g for 10 minutes at room temperature and then the supernatant was decanted. The cells were resuspended in 1 mL 0.5% BSA (#130-091-376, Miltenyi Biotec) in 1X D-PBS containing calcium, magnesium, glucose, and pyruvate (#14287-072, Thermo Fisher...
Scientific, Waltham, MA) and then washed by centrifugation at 300 \(\times\) g for 10 minutes at room temperature. The cell pellet was resuspended in 200 \(\mu\)L 0.5% BSA in 1X D-PBS and split into 50 \(\mu\)L for antibody staining. Cell aliquots were then stained with: 1) 1 \(\mu\)L CD11b-APC (M1/70.15.11.5, #130-113-793, Miltenyi Biotec), 2) 1 \(\mu\)L of CD45-VioBlue (REA737, #130-110-802, Miltenyi Biotec), 3) 1 \(\mu\)L CD11b-APC and 1 \(\mu\)L of CD45-VioBlue, or 4) left unstained as a control. Stained cells were gently mixed and then incubated for 10 minutes in the dark at 4ºC. Cells were then washed with 1 mL of 0.5% BSA in 1X D-PBS and then pelleted at 300 \(\times\) g for 10 minutes at room temperature. The cell pellet was resuspended in 250 \(\mu\)L 0.5% BSA in 1X D-PBS prior to flow cytometric analysis on the MACSQuant Analyzer 10 (#130-096-343, Miltenyi Biotec).

Preparation of single cell suspension from mouse brain using a combination of enzymatic and mechanical dissociation

Halves of Cx3cr1-NuTRAP mouse brains were rinsed in ice-cold D-PBS containing calcium, magnesium, glucose, and pyruvate (#14287-072, Thermo Fisher Scientific), sliced into 4 sagittal sections on a chilled, metal block and placed into ice-cold gentleMACS C-tubes (#130-093-237, Miltenyi Biotec, San Diego, CA), containing 1,950 \(\mu\)L of papain-based, Enzyme Mix 1. For each reaction, Enzyme Mix 1 was created by combining 50 \(\mu\)L of Enzyme P with 1900 \(\mu\)L of Buffer Z, while Enzyme Mix 2 was created by combining 10 \(\mu\)L of Enzyme A with 20 \(\mu\)L of Buffer Y per reaction, with all reagents included in the Adult Brain Dissociation Kit (#130-107-677, Miltenyi Biotec). Each sample then had 30 \(\mu\)L of Enzyme Mix 2 added prior to being mechanically dissociated for 30 minutes at 37ºC on the gentleMACS Octo Dissociator with Heaters (#130-096-427, Miltenyi Biotec) using the 37C_ABDK_01 program. Following enzymatic and mechanical dissociation, the C-tubes were quickly spun in a chilled (4ºC) Allegra-30R centrifuge (#B08708, Beckman Coulter, Indianapolis, IN) with an SX4400 swinging bucket rotor to collect the samples in the bottom of the tubes. Next, samples were resuspended and passed through a pre-wet 70 \(\mu\)m MACS SmartStrainer (#130-110-916, Miltenyi Biotec) and collected in a 50 mL conical tube (#21008-178, VWR International, Radnor, PA). The C-tubes were washed with 10 mL of ice-cold D-PBS and the washed volume was passed through the 70 \(\mu\)m MACS SmartStrainer. The cells were then pelleted by centrifugation at 300 \(\times\) g for 10 minutes at 4ºC. Following centrifugation, the supernatant was aspirated and debris was removed using the Debris Removal Solution (#130-109-398, Miltenyi Biotec) provided in the Adult Brain Dissociation Kit (#130-107-677, Miltenyi Biotec). Briefly, cells were resuspended in 3.1 mL of ice-cold D-PBS and passed to a 15 mL conical tube (#21008-214, VWR International) and 900
µL of cold Debris Removal Solution was mixed into the cell suspensions. Next, 4 mL of D-PBS was gently overlaid on top of the cell suspension, ensuring the layers did not mix. Centrifugation at 3,000 x g for 10 minutes at 4ºC separated the suspension into three phases, of which the top two phases were aspirated. The cell pellet was gently resuspended in 15 mL ice-cold D-PBS before centrifugation at 1,000 x g for 10 minutes at 4ºC. After aspirating the supernatant completely, the cells were resuspended in 1 mL 0.5% BSA (#130-091-376, Miltenyi Biotec) in D-PBS and filtered through a 35 µm filter (#352235, Fisher Scientific). An aliquot of 100 µL cells was retained as “Cell-Input” for flow cytometric and RNA-Seq analyses.

**Preparation of single cell suspension from mouse brain using mechanical dissociation**

As a comparator to the enzymatic and mechanical dissociation, which requires a 30-minute incubation at elevated temperatures (37ºC), single cell suspensions were also created using mechanical dissociation alone with all steps carried out at 4ºC or on ice. The protocol for this cell preparation was modified from a previously published study (Hammond et al., 2019). Briefly, halves of Cx3cr1-NuTRAP mouse brains were rinsed in ice-cold 1X HBSS with no calcium, no magnesium, and no phenol red (diluted 1:10 from #14185-052, ThermoFisher), sliced into 4 sagittal sections and placed into a 7 mL Kimble Dounce homogenizer (#D9063, MilliporeSigma) containing 5 mL pre-chilled 1X HBSS. The brain tissue was homogenized using 15 strokes of the loose and tight pestles while simultaneously rotating the pestle and then passed through a pre-wet 70 µm cell strainer (#130-095-823, Miltenyi Biotec) placed onto a prechilled 15 mL conical tube. The glass homogenizer was washed twice with 5 mL of 1X HBSS and the washed volume was transferred to the 15 mL conical tube containing the brain homogenate. Next, cells were pelleted at 300 x g for 5 minutes at 4ºC. Following centrifugation, the supernatant was aspirated and the cells were resuspended in 10 mL 40% ice-cold Percoll (#17-0891-02, MilliporeSigma) in 1X HBSS (4 mL Percoll, 1 mL 10X HBSS, 5 mL RNase-free water). The cell mixture was spun at 500 x g for 30 minutes at 4ºC using full acceleration and braking. The myelin and Percoll were carefully aspirated, leaving the microglia-containing cell pellet undisturbed. The cell pellet was then washed in 10 mL of ice-cold HBSS and spun again at 300 x g for 5 minutes at 4ºC. After aspirating the supernatant completely, the cells were resuspended in 1 mL 0.5% BSA (#130-091-376, Miltenyi Biotec) in D-PBS and filtered through a 35 µm filter (#352235, Fisher Scientific). An aliquot of 200 µL cells was retained as “Dounce-Input” for flow cytometry. The remaining cells were pelleted at 300 x g for 10 minutes at 4ºC to process for Translating Ribosome Affinity Purification (TRAP) and RNA-Seq library preparation.

**Cell Counting from brain tissue**
Filtered cells were diluted 1:10 in 0.5% BSA (#130-091-376, Miltenyi Biotec) in D-PBS prior to cell counting on a MACSQuant Analyzer 10 (#130-096-343, Miltenyi Biotec). 50 µL of diluted cells were analyzed to determine absolute cell count. Cells were gated on FSC-A/SSC-A to determine cell count and FSC-A/FSC-H to determine singlet count. Absolute cell counts were used to determine antibody staining ratios.

**CD11b Magnetic Labeling and Separation from brain tissue**

Cells from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were pelleted at 300 x g for 10 minutes at 4°C and resuspended in 90 µL of 0.5% BSA in D-PBS with 10 µL of CD11b (Microglia) MicroBeads (#130-093-636, Miltenyi Biotec) per 10^7 total cells. After gently pipetting to mix, cells were incubated for 15 minutes at 2-8°C protected from light. Cells were washed with 1 mL of 0.5% BSA in D-PBS and pelleted at 300 x g for 10 minutes at 4°C. The cell pellet was resuspended in 500 µL of 0.5% BSA in D-PBS. After priming the autoMACS Pro Separator (#130-092-545, Miltenyi Biotec), sample and collection tubes were placed in a cold MACS Chill 5 Rack (#130-092-951, Miltenyi Biotec) with both positive and negative fractions being collected. The double-positive selection (Posseld) program (ie., positive fraction cells are then run over a second magnetic column for higher purity) was used to elute highly pure CD11b+ cells in 500 µL of autoMACS Running Buffer (#130-091-221, Miltenyi Biotec). Following separation, the positive fraction was reserved for further applications and analysis.

**Antibody Labeling for FACS from brain tissue**

Cell suspensions from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were pelleted at 300 x g for 10 minutes at 4°C and resuspended in 96 µL of 0.5% BSA in D-PBS, 2 µL of CD11b-APC antibody (M1/70.15.11.5, #130-113-793, Miltenyi Biotec), and 2 µL of CD45-VioBlue antibody (REA737, #130-110-802, Miltenyi Biotec). After mixing well, cells were incubated for 10 minutes in the refrigerator (2-8°C) protected from light. Cells were washed with 1 mL of 0.5% BSA in D-PBS and pelleted at 300 x g for 10 minutes. Cell suspensions from half brains were processed in parallel for cartridge-based FACS (MACSQuant Tyto) and cytometer-based FACS (FACSria).

**Cartridge-based FACS (MACSQuant Tyto) from brain tissue**

Stained cell pellets from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were resuspended in 10 mL of 0.5% BSA in D-PBS. A MACSQuant Tyto Cartridge (#130-106-088, Miltenyi Biotec) was primed using 1 mL of 0.5% BSA in D-PBS. The cell suspension was then...
filtered through 20 µm Pre-Separation Filters (#130-101-812, Miltenyi Biotec). An aliquot of 500 µL of filtered cell suspension was saved as the Tyto-Input fraction for analysis. The remaining cell suspension was then loaded into the input chamber of a MACSQuant Tyto cartridge. After loading labeled cells into the input chamber, the cartridge was scanned into the MACSQuant Tyto Cell Sorter system (#130-103-931, Miltenyi Biotec) and sorting parameters were selected. The MACSQuant Tyto cartridge is a sterile, closed, single-use system that relies on accurate activation of the sort valve to pass cells of interest (in this case microglia) into the positive sort chamber. Cell speed (or time-of-flight) was determined by the time it took a cell to pass between two adjacent PMT lasers. In this study, the V1 filter (450/50 nm) of the violet (405 nm) laser was used as a cell trigger - the first PMT channel used to measure cell speed, to detect CD45-VioBlue positive cells at a threshold signal of 20. The B1 filter (525/550) of the blue (488 nm) laser was used as the cell speed channel to detect eGFP+ cells at a signal threshold of 4. A blue (488 nm) laser with B1 (525/50 nm) and B2 (585/40 nm) filter combinations was used to gate on eGFP+ cells without auto-fluorescence interference. Subsequent gating based on CD11b-APC fluorescence used a red (638 nm) laser and R1 (655-730 nm) filter. The gating strategy was set to CD11b+CD45+eGFP+ for isolation of microglia (Figure 1-5). After completion of the sort, the cells from the positive fraction chamber were collected in 0.5% BSA in D-PBS. The positive fraction chamber was washed twice using 450 µL of 0.5% BSA in D-PBS per wash and combined with the initial positive fraction collection. After sorting was completed, an aliquot of (10%) of the positive fraction was kept for analysis on the MACSQuant Analyzer 10 Flow Cytometer.

**Cytometer-based FACS (FACSAria) from brain tissue**

Following staining, cells from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were pelleted and then resuspended in 2 mL of 0.5% BSA in D-PBS for cytometer-based sorting (FACSAria Illu cell sorter, BD Biosciences). An aliquot of 200 µL stained cells was saved as FACS-Input for analysis. A violet (405 nm) laser was used to gate CD45-VioBlue positive cells using a 450/40 nm filter. A blue (488 nm) laser with 530/30 nm and yellow-green (561 nm) laser with 610/20 nm filter combinations was used to gate on eGFP+ cells without auto-fluorescence interference. A red (640 nm) laser was used to detect CD11b-APC fluorescence with a 660/20 nm filter. The gating strategy was set to CD11b+CD45+eGFP+ for isolation of microglia (Figure 1-6). Cells were sorted into 500 µL of 0.5% BSA in D-PBS. After sorting was completed, an aliquot (10%) of the positive fraction was analyzed on the MACSQuant Analyzer 10 Flow Cytometer.
Addition of Inhibitors

Transcription and translation inhibitors were included during cell preparation, as previously described (Marsh et al., 2020) with some minor modifications. Actinomycin D (#A1410, MilliporeSigma) was reconstituted in DMSO to a concentration of 5 mg/mL before being aliquoted and stored at -20°C protected from light. Triptolide (#T3652, MilliporeSigma) was reconstituted in DMSO to a concentration of 10 mM before being aliquoted and stored at -20°C protected from light. Anisomycin (#A9789, MilliporeSigma) was reconstituted in DMSO to a concentration of 10 mg/mL before being aliquoted and stored at 4°C protected from light. For the samples to be treated with inhibitors, 2 µL each of Actinomycin D, Triptolide, and Anisomycin stocks were added to the initial Enzyme Mix 1 prior to dissociation for a final concentration of 5 µg/mL, 10 µM, and 10 µg/mL, respectively. The transcription and translation inhibitors were only included during the enzymatic and mechanical dissociation step for 30 minutes at 37°C on the gentleMACS Octo Dissociator with Heaters (#130-096-427, Miltenyi Biotec) using the 37C_ABDK_01 program. The transcription and translation inhibitors were not present in the dissection or sorting buffers, as the cell preparation was kept at 4°C for all other steps.

Flow Cytometry Analysis from brain tissue

For analysis of cell sorting, aliquots of input and positive fractions from each of the sort methods (autoMACS, autoMACS to MACSQuant Tyto, MACSQuant Tyto, FACSaria) were taken for analysis on the MACSQuant Analyzer 10 Flow Cytometer. autoMACS input and positive fractions were stained with CD11b-APC (M1/70, #130-113-793, Miltenyi Biotec) and CD45-VioBlue (REA737, #130-110-802, Miltenyi Biotec) after completion of the sort, according to manufacturer’s instructions. autoMACS to MACSQuant Tyto, MACSQuant Tyto, and FACSSSaria input and positive fractions were stained prior to cell sorting. Following staining, cells were resuspended in 500 µL of 0.5% BSA/D-PBS and run on the MACSQuant Analyzer 10 Flow Cytometer. Post-sort purity was assessed by: 1) percent eGFP+ singlets and 2) percent CD11b+CD45 singlets (Figure 1-7) using MACSQuantify v2.13.0 software.

To test the effect of cell preparation method on the relative abundance of cell types, aliquots of cells were stained with: 1) Microglial (CD11b-APC (M1/70, #130-113-793, Miltenyi Biotec) / CD45-VioBlue (REA737, #130-110-802, Miltenyi Biotec)), 2) Neuronal (CD24-VioBlue (REA743, #130-110-831, Miltenyi Biotec)), 3) Astrocytic (ACSA2-APC (REA969, #130-116-245, Miltenyi Biotec)), or 4) Oligodendrocytic (O4-APC (REA576, #130-119-982, Miltenyi Biotec))
fluorophore-conjugated antibodies, according to manufacturer’s instructions. Cells were washed and resuspended in 500 µL and run on the MACSQuant Analyzer 10 Flow Cytometer. Relative cell proportions with and without transcription/translation inhibitors were assessed (Figure 6-3) using MACSQuantify v2.13.0 software.

To test the effect of cell preparation method on the viability of single cell suspensions, aliquots of cells were stained with Viobility 405/520 Fixable Dye (#130-110-206, Miltenyi Biotec), according to manufacturer’s instructions. Briefly, 100 µL of cells in 1X D-PBS were incubated with 1 µL of Viobility 405/520 Fixable Dye for 15 minutes in the dark at room temperature. After washing with 1 mL of 0.5% BSA/D-PBS, the cells were resuspended in 250 µL of 0.5% BSA/D-PBS and run on the MACSQuant Analyzer 10 Flow Cytometer. The percent viable singlets and eGFP+ singlets were assessed (Figure 6-4) using MACSQuantify v2.13.0 software.

Translating Ribosome Affinity Purification (TRAP) and RNA extraction

For TRAP from whole tissue, a hemisected half-brain was minced into small pieces and homogenized in 1.5 mL ice-cold complete homogenization buffer (50 mM Tris, pH 7.4; 12 mM MgCl2; 100 mM KCl; 1% NP-40; 1 mg/mL sodium heparin; 1 mM DTT; 100 µg/mL cycloheximide (#C4859-1ML, MilliporeSigma); 200 units/mL RNaseOUT Recombinant Ribonuclease Inhibitor (#10777019; Thermo Fisher Scientific); 0.5 mM Spermidine (#S2626, MilliporeSigma), 1X complete EDTA-free Protease Inhibitor Cocktail (#11836170001; MilliporeSigma) with a glass Dounce tissue grinder set (#D8938; MilliporeSigma), as previously (Chucair-Elliott et al., 2020). For TRAP from cells, a single hemisphere of a Cx3cr1-NuTRAP mouse brain was used to generate a cell suspension prior to sorting. After pelleting cells at 1,000 x g for 10 min at 4°C, cells were resuspended in 400 µL of ice-cold complete homogenization buffer, transferred to a glass Dounce tissue grinder set, and homogenized 15 times with pestle A. Volume was brought up to 1.5 mL with complete homogenization buffer. Homogenates (from tissue or cells) were transferred to 2 mL round-bottom tubes and centrifuged at 12,000 x g for 10 minutes at 4°C. After centrifugation, 100 µL of the supernatant was saved as TRAP "Input". The remaining supernatant was transferred to a 2 mL round-bottom tube and incubated with 5 µg/µL of anti-GFP antibody (ab290; Abcam) at 4°C with end-over-end rotation for one hour. Dynabeads Protein G for Immunoprecipitation (#10003D; Thermo Fisher Scientific) were washed three times in 1 mL ice-cold low-salt wash buffer (50 mM Tris, pH 7.5; 12 mM MgCl2; 100 mM KCl; 1% NP-40; 100 µg/mL cycloheximide; 1 mM DTT). After removal of the last wash, the homogenate/antibody mixture was transferred to the 2 mL round-bottom tube containing the washed Protein-G Dynabeads and incubated at 4°C with end-over-end rotation.
overnight. Magnetic beads were collected using a DynaMag-2 magnet and the unbound-ribosomes and associated RNA discarded. Beads and GFP-bound polysomes were then washed three times with 0.5 mL of high-salt wash buffer (50 mM Tris, pH 7.5; 12 mM MgCl2; 300 mM KCl; 1% NP-40; 100 μg/mL cycloheximide; 2 mM DTT). Following the last wash, 350 μL of Buffer RLT (Qiagen, Germantown, MD) supplemented with 3.5 μL 2-β mercaptoethanol (#44203, MilliporeSigma) was added directly to the beads and incubated with mixing on a ThermoMixer (Eppendorf) for 10 minutes at room temperature. The beads were magnetically separated and the supernatant containing the target bead-bound polysomes and associated RNA was transferred to a new tube. 350 µL of 100% ethanol was added to the tube (“TRAP” fraction: enriched in transcriptome associated to eGFP-tagged ribosomes) and then loaded onto a RNeasy MinElute column (Qiagen). RNA was isolated using RNeasy Mini Kit (#74104, Qiagen), according to manufacturer’s instructions. RNA was quantified with a Nanodrop One c-spectrophotometer (#ND-ONEC-W, Thermo Fisher Scientific) and its quality assessed by HSRNA ScreenTape (#5067-5579, Agilent Technologies) with a 4150 Tapestation analyzer (#G2992AA, Agilent Technologies).

Library construction and RNA sequencing (RNA-seq)

Directional RNA-Seq libraries were made according to the manufacture’s protocol from 2-100 ng RNA. TRAP input and positive fraction RNA from all samples were used to create individual RNA-Seq libraries (no pooling of samples was performed). Briefly, poly-adenylated RNA was captured using NEBNext Poly(A) mRNA Magnetic Isolation Module (#NEBE7490L; New England Biolabs, Ipswich, MA). Following mRNA capture, mRNA was eluted from the oligo-dT beads and fragmented by incubating with the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) from the NEBNext Ultra II Directional Library Prep Kit for Illumina (#NEB7760L; New England Biolabs) for 15 minutes at 94°C. First and second strand cDNA synthesis were performed sequentially, as instructed by the manufacturer’s guidelines. After purification of double-stranded cDNA with 1.8X SPRISelect Beads (#B23318, Beckman Coulter), purified cDNA was eluted in 50 µL 0.1X TE Buffer and subjected to end prep. The NEBNext Adaptor was diluted 1:100 in Adaptor Dilution Buffer (provided) before ligating the adaptor to the cDNA. After purifying the ligation reaction with 0.9X SPRISelect Beads (#B23318, Beckman Coulter), cDNA was eluted in 15 µL of 0.1X TE (provided). Next, cDNA libraries were amplified with 16 cycles of PCR using the NEBNext Ultra II Q5 Master Mix (provided) and unique index primer mixes from NEBNext Multiplex Oligos for Illumina Library (#E6609L, New England Biolabs). Libraries were purified with 0.9X SPRISelect Beads (#B23318, Beckman
Coulter) and then sized with HSD1000 ScreenTapes (#5067-5584; Agilent Technologies). Libraries had an average peak size of 316 bp. Libraries were quantified by Qubit 1X dsDNA HS Assay kit (#Q33230, Thermo Fisher Scientific). The libraries for each sample were pooled at 4 nM concentration and sequenced using an Illumina NovaSeq 6000 system (SP PE50bp, S4 PE150). The entirety of the sequencing data is available for download in FASTQ format from NCBI Gene Expression Omnibus (to be included on acceptance).

**RNA-Seq Data Analysis**

Following sequencing, reads were trimmed and aligned prior to differential expression statistics and correlation analyses in Strand NGS software package (v4.0) (Strand Life Sciences). Reads were aligned against the full mm10 genome build (2014.11.26). Alignment and filtering criteria included: adapter trimming, fixed 2bp trim from 5’ and 2bp from 3’ ends, a maximum number of one novel splice allowed per read, a minimum of 90% identity with the reference sequence, a maximum 5% gap, and trimming of 3’ end with Q<30. Alignment was performed directionally with Read 1 aligned in reverse and Read 2 in forward orientation. All duplicate reads were then removed. Normalization was performed with the DESeq2 algorithm. Transcripts with an average read count value >5 in at least 100% of the samples in at least one group were considered expressed at a level sufficient for quantitation per tissue and those transcripts below this level were considered not detected/not expressed and excluded, as these low levels of reads are close to background and are highly variable. For statistical analysis of differential expression, a one-way ANOVA or two-way ANOVA with the factors of TRAP fraction and treatment and a Benjamini-Hochberg Multiple Testing Correction followed by Student-Newman Keuls post hoc test were used (FDR<0.1). For those transcripts meeting this statistical criterion, a fold change >2 cutoff was used to eliminate those genes which were statistically significant but unlikely to be biologically significant and orthogonally confirmable due to their very small magnitude of change. Visualizations of hierarchical clustering and principal component analyses were performed in Strand NGS (Version 4.0, Bangalore, India). Cell type specific marker gene lists were generated from the re-analysis published by McKenzie et al. (McKenzie et al., 2018) of immunopurified and high throughput single cell data from mice. Over-representation analysis (ORA) was conducted using WEB-based Gene SeT AnaLysis Toolkit (WebGestalt, www.webgestalt.org) (Liao et al., 2019). Top over-represented biological processes were selected from gene ontology functional database with no redundant option selected (Hypergeometric test, BHMTC, FDR<0.05) and background reference gene list of all expressed genes (raw count > 5 in all samples from at least one group). Top over-represented transcription...
factor targets were selected from network functional database with all expressed genes as the reference gene list (Hypergeometric test, BHMC, FDR<0.05). Heatmaps of over-represented biological processes and gene lists were created using Morpheus (https://software.broadinstitute.org/morpheus). Upset plots were created using UpSetR v1.4.0 package in RStudio v1.4.1106 with R v4.0.5. Previously published microglial ex vivo activational lists were compared (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020) and genes included in at least two of the three previous studies were considered “ex vivo activational transcripts”. Gene set enrichment analysis (GSEA) was run using GSEA v4.1.0 (Mootha et al., 2003; Subramanian et al., 2005).

Immunohistochemistry and imaging

Brain samples were fixed for 4h in 4% PFA, cryoprotected by sequential incubations in PBS containing 15% and 30% sucrose, and then frozen in Optimal Cutting Temperature medium (#4583, Tissue-Tek). Twelve µm-thick sagittal sections were cryotome-cut (Cryostar NX70, Thermo Fisher Scientific). Tissue sections were rinsed with PBS containing 1% Triton X-100, blocked for 1h in PBS containing 10% normal donkey serum, and processed for fluorescence immunostaining and downstream analysis. The primary antibodies included rabbit anti-GFP (#ab290, 1:100, Abcam, Cambridge, MA), rat anti-CD11b (#C227, Clone M1/70, 1:100, Leinco Technologies, St. Louis, MO), and rat anti-CD45 (#550539, Clone 30-F111, 1:100, BD Biosciences). Sequential imaging was performed on an Olympus FluoView confocal laser-scanning microscope (FV1200; Olympus; Center Valley, PA) at the Dean McGee Eye Institute imaging core facility at OUHSC. Microscope and FLUOVIEW FV1000 Ver. 1.2.6.0 software (Olympus) settings were identical for samples using the same staining-antibody combination and at same magnification. The experimental format files were oib. The Z-stack generated was achieved at 1.26 µm step size with a total of 8 optical slices at 20X magnification (2X zoom).

Results

The goal of this study was to compare microglial sorting techniques and determine the relative levels of ex vivo activation induced during cell preparation and microglial isolation. A schematic of the experimental design is represented in Figure 1A. In the Cx3cr1-NuTRAP mice, following cre recombination in Cx3cr1+ cells, deletion of the floxed stop cassette causes activation of the NuTRAP allele, labeling microglial ribosomes with eGFP and nuclei with biotin and mCherry. For the first part of the present study, we used eGFP as a sorting criterion and in the evaluation of post-sort microglial purity, along with CD11b and CD45 co-expression. Colocalization of
eGFP with microglial markers CD11b and CD45 in Cx3cr1-NuTRAP brains was verified by immunohistochemistry (Figure 1-2). Enzymatic and mechanical dissociation of Cx3cr1-NuTRAP brains was performed to generate single-cell suspensions. For each sort method, both male and female samples were used.

Flow cytometric analysis of sort fractions from various microglial sorting techniques.

After reserving an aliquot as input, cells were subjected to one of four isolation techniques: 1) CD11b⁺ magnetic-bead based isolation (autoMACS), 2) Cartridge-based FACS on CD11b⁺CD45⁺eGFP⁺ (MACSQuant Tyto), 3) Cytometer-based FACS on CD11b⁺CD45⁺eGFP⁺ (FACSAria), and 4) autoMACS debulking of cells prior to cartridge-based FACS (autoMACS to MACSQuant Tyto) (Figure 1A).

Aliquots of cell input and positive sort fractions from each of the four sort methods were analyzed by flow cytometry. All sort methods showed enriched populations of eGFP⁺ and CD11b⁺CD45⁺ singlets in their positive fractions as compared to Cell-Input (Figure 1B). A leftward shift in CD11-APC was evident in autoMACS samples, likely from competition from the CD11b microbeads used in that isolation. The positive fractions of all sort methods were enriched in eGFP⁺ singlets as compared to the input fraction (Figure 1C, Figure 1-1; Two-way ANOVA, main effect of sort fraction, ***p<0.001). The autoMACS sort resulted in lower overall percentage of eGFP⁺ singlets compared to all other sort methods and the FACSAria sort resulted in a higher overall percentage of eGFP⁺ singlets, though all approaches demonstrated a high level of enrichment (Figure 1C, Figure 1-1; Two-way ANOVA, Tukey’s post-hoc, *p<0.05). The positive fractions of all sort methods were enriched in CD11b⁺CD45⁺ singlets as compared to the input fraction (Figure 1D, Figure 1-1; Two-way ANOVA, Main effect of sort fraction, p<0.001). FACSAria sort resulted the highest overall percentage of CD11b⁺CD45⁺ singlets (Figure 1D, Figure 1-1; Two-way ANOVA, Tukey’s post-hoc, *p<0.05). Although FACSAria had higher microglial purity than other sort methods, it showed a significantly lower yield than the MACSQuant Tyto sort (Figure 1E, Figure 1-1; One-way ANOVA, Tukey’s post-hoc, #p<0.05).

Comparison of transcriptomic profiles of microglia isolated from various sort methods.

Following cell preparation and isolation using methods displayed in Figure 1A, RNA was isolated from cells for preparation of stranded RNA-Seq libraries. RNA yield and quality by sort method, in addition to basic sequencing metrics are given in Figure 2-1. Gene body coverage plots by experimental group are shown in Figure 2-2. All sort methods showed similar coverage
We first examined enrichment/depletion of previously published microglial, astrocytic, oligodendrocytic, neuronal, and endothelial markers in the transcriptomic profiles (Chucair-Elliott et al., 2020; McKenzie et al., 2018) (Figure 2-1). Each of the four sort methods showed similar levels of enrichment of microglial marker genes (Figure 2A) and depletion of astrocytic, oligodendrocytic, neuronal, and endothelial marker genes (Figure 2B-E) when compared to Cell-Input. In combination with the flow cytometric data presented in Figure 1, this gives confidence that each of the sort methods are effective in isolating highly pure populations of microglia.

Next, we examined the transcriptomic data in an unbiased manner. Principal component analysis (PCA) of all expressed genes (>5 counts in all samples from at least one group) showed clear separation of Cell-Input from all sort methods in the first component with 81% of the explained variance (Figure 2F). Differentially expressed genes were called by One-way ANOVA with Benjamini-Hochberg multiple testing correction (BHMT) followed by Student-Newman Keuls post hoc (FDR<0.1, |FC|>2; Figure 2-1). Hierarchical clustering of the 7,378 DEGs shows separation of Cell-Input from all sort methods with similar patterning of enrichment and depletion of DEGs across all sort methods scaled to Cell-Input (Figure 2G). The majority of pairwise DEGs (sort method v Cell-Input) were in common between all sort methods (7,084/7,378 = 96%) (Figure 2H), suggesting a high degree of similarity between each of the sort methods. In addition, 5322 DEGs (72%) that were down and 1,759 DEGs (24%) were up in all sort methods compared to Cell-Input. There were only 297 discordant DEGs (4%) between the different sort methods as compared to Cell-Input (Figure 2I).

Over-representation analysis of gene ontology (ORA GO) of the 1,759 genes that were up across all sort methods identified 177 over-represented biological processes pathways (BHMT, FDR<0.05; Figure 2-1). The “Enrichment ratio” is the number of observed genes divided by the number of expected genes from the given gene list. Examination of the top 10 over-represented biological processes reveals several pathways involved in microglial function, including: cytokine-mediated signaling, immune response activation, and adaptive immune response, among others (Figure 2J). Running a similar ORA GO on the 5,322 genes down across all sort methods (compared to Cell-Input) revealed 252 over-represented biological processes (BHMT, FDR<0.05; Figure 2-1). The top 10 processes include many neuron-focused pathways, such as: neurotransmitter transport, neurotransmitter level regulation, and membrane potential regulation (Figure 2K), indicating depletion of these genes in the sorted cells.
Next, we examined the common 1,759 up-regulated and 5,322 down-regulated genes across
the four sort methods for over-representation of transcription factor targets. Network ORA on the
1,759 genes enriched in the positive fraction of all sort methods identified 21 over-represented
transcription factor targets, including the top five hits: ELF1, ETS2, IRF, PU.1, and NFKB
(Figure 2L, Figure 2-1). Three of the top five transcription factor targets (ELF1, ETS2, PU.1)
are part of the ETS family of transcription factors that assist in regulating immunity (Gallant and
Gilkeson, 2006), with PU.1 being a “master regulator” of microglial identity and function (Yeh
and Ikezu, 2019). The other two transcription factors (IRF and NFKB) are also critical regulators
of inflammation and antiviral response (Iwanaszko and Kimmel, 2015), an important function of
microglia. The IRF_Q6 transcription factor motif contains IRF1-regulated genes with 3'UTRs
containing the motif BNCRSTTCANTTYY.

Network ORA of the 5,322 genes down in all sort methods compared to input identified 468
over-represented transcription factor targets, including top five hits: AP-1, TATA, LEF1, PAX4,
and AP-4. LEF1 is an endothelium-specific transcription factor (Hupe et al., 2017). AP-4 is an
adaptor protein complex that is involved in vesicular trafficking of membrane proteins. Lack of
AP-4 has been shown to cause accumulation of axonal autophagosomes containing AMPA
receptor components in hippocampal neurons and cerebellar Purkinje cells (Matsuda et al.,
2008). Overall, the top transcription factor targets of the genes depleted in the sort fractions
(compared to Cell-Input) modulate important non-microglial cellular functions and, as such, are
depleted in purified microglia.

In combination, the flow cytometric data, distribution of marker gene enrichments/depletion, and
analysis of differentially expressed genes (including pathway and transcription factor analysis)
suggest that each of the sort methods are producing highly pure populations of microglia with
very similar transcriptomic profiles.

Flow cytometric analysis of blood from Cx3cr1-NuTRAP mice following tamoxifen-
mediated cre recombination

In addition to microglia, the Cx3cr1-cre/ERT2 also targets circulating and peripheral CX3CR1+
cells for tamoxifen-mediated cre recombination (Yona et al., 2013) and concomitant expression
of the NuTRAP allele. Without perfusion, there is a concern that contamination of circulating
CX3CR1+ cells may be present in eGFP sorted cells and in TRAP-isolated RNA from Cx3cr1-
NuTRAP brain tissue. However, the half-life of circulating CX3CR1+ cells in mice is less than 3
days (Yona et al., 2013) and, accordingly, loss of eGFP-labelling in blood samples from Cx3cr1-
NuTRAP mice is evident as CX3CR1+ cells are rapidly turned over following tamoxifen induction. To demonstrate the loss of eGFP+ in circulating CX3CR1+ cells, we collected blood from Cx3cr1-NuTRAP mice 3- and 60- days post-cessation of tamoxifen administration and compared the levels of CD11b+CD45+ cells expressing eGFP to that of cre negative (WT) controls. There was a marked increase in CD11b+CD45+eGFP+ cells 3 days post-tamoxifen induction, which decreased to levels indistinguishable from WT controls after 60 days (Figure 1-3). As such, brains were collected from Cx3cr1-NuTRAP mice at least 60 days following tamoxifen treatment. In studies without transgenic labeling, brains can be perfused with ice-cold physiological saline to eliminate contaminating cells from circulation.

Assessment of relative levels of non-microglial macrophages within different sort fractions from Cx3cr1-NuTRAP brains.

Perivascular, choroid plexus, and meningeal macrophages also express Cx3cr1 (Faraco et al., 2017), and thus are labeled by the NuTRAP allele (including eGFP-labelled polysomes) upon tamoxifen-mediated cre recombination. CD45 expression levels are often used as a FACS sort criteria to separate microglia (CD45^{Mid}) from non-microglial macrophages (CD45^{High}) (Haimon et al., 2018; Marschallinger et al., 2020; Pan and Wan, 2020). The relative levels of non-microglial, macrophage contamination was first assessed by flow cytometry. Cells from each group shown in Figure 1A were stained with CD11b and CD45 fluorescent antibodies and analyzed on a MACSQuant Analyzer 10. The percent CD45^{Mid} and CD45^{High} of CD11b+CD45+ singlets were assessed according to the gating strategy displayed in Figure 1-4A. The Cell-Input fraction had the highest (16%) CD45^{High} population of CD11b+CD45+ cells, as compared to the autoMACS (8%), autoMACS to Tyto (5%), Tyto (4%), and FACSaria (6%) (Figure 1-4B). There was not a significant difference in the proportion of CD45^{Mid} or CD45^{High} (out of CD11b+CD45+) cells by sort method. The vast majority (>95%) of CD45^{Mid} cell populations co-expressed eGFP regardless of sort method. In contrast, only ~74% of CD45^{High} cells from the Cell-Input and autoMACS sort fraction co-expressed eGFP. The remaining 26% eGFP CD45^{High} cells are likely contaminating cells from blood circulation. Since the FACS-based methods used eGFP as a sorting criteria, the CD45^{High} cells co-expressing eGFP in the cartridge-based Tyto (96%) and cytometer-based FACSaria (99%) were higher than the Cell-Input (Figure 1-4C).

Many recent studies have examined differences in the transcriptomic signatures of microglia and non-microglial brain resident macrophages. To further refine our microglial marker list constructed based off a meta-analysis of cell sorting studies (McKenzie et al., 2018), we compared previously published microglial marker lists from cell sorting (Chucari-Elliott et al.,
ribosomal tagging (Chucair-Elliott et al., 2020), and a meta-analysis distinguishing microglia from peripheral monocytes and macrophages (Haage et al., 2019). There were 113 genes that were identified as microglial markers in at least two of the three gene lists (highlighted purple) and were used for further analysis (Figure 3A, Figure 3-1). A list of macrophage marker genes was constructed by taking the union of three previously published studies from infiltrating macrophages (DePaula-Silva et al., 2019), peripheral macrophages and monocytes (Haage et al., 2019), and CNS-associated macrophages (CAMs) (Faraco et al., 2017) to identify 174 macrophage-specific genes (highlighted green) (Figure 3B, Figure 3-1). It is of note that there is no overlap between the 113 microglial-specific genes identified in Figure 3A and the 174 macrophage marker genes identified in Figure 3B.

To increase the specificity of microglial transgenic labeling approaches, recent models such as the Hexb-cre/ERT2 (Takahiro Masuda et al., 2020), Tmem119-cre/ERT2 (Kaiser and Feng, 2019), and P2ry12-cre/ERT2 (McKinsey et al., 2020) are more specific to microglia and do not label other CNS-associated macrophages (CAMs). Siglec-H has also been demonstrated as a discriminating mark on microglia that is absent in CAMs (Konishi et al., 2017). All of these microglial-specific marker genes (Hexb, Tmem119, P2ry12, and Siglech) were enriched in the sort fractions when compared to Cell-Input (Figure 3C, Figure 3-1); however, there were no differences in expression between the various sort methods. Next, we examined four marker genes of CAMs (Lyve1, Ly6c1, CD163, and Mrc1) (Faraco et al., 2017) that are not expressed by microglia. Lyve1 was enriched in the autoMACS to Tyto sort fraction, CD163 was enriched in the autoMACS to Tyto and Tyto sort fractions, and Mrc1 was enriched in all sort fractions as compared to Cell-Input, whereas Ly6c1 was depleted across all sort methods compared to Cell-Input. Based on this analysis, the sort fractions contain primarily microglia with a small proportion of CAMs present. Separation of microglia and CAMs can be achieved by adding more specific markers to the cell sorting criteria. For the purpose of this study, we will refer to the sort fraction as “microglia”. Overall, markers of infiltrating macrophages (Emp1, Scarf1, and Emb) (DePaula-Silva et al., 2019) and peripheral macrophages/monocytes (Emilin2, F5, Fn1, Gda) were depleted in the sort fractions when compared to the Cell-Input (Figure 3D, Figure 3-1).

Gene set enrichment analysis of the 113 microglial-specific genes identified in Figure 3A and the 174 macrophage marker genes identified in Figure 3B revealed overall enrichment of microglial marker genes (Figure 3E) and depletion of macrophage marker genes (Figure 3F) when comparing the combined sort fractions from all methods to the Cell-Input. GSEA
enrichment scores were similar between the different sort methods (Figure 3G, Figure 3-1). Based on the flow cytometric and transcriptomic data, all sort methods have high levels of microglial purity and low levels of CAM and monocyte contamination.

Comparison of TRAP-isolated microglial translatome from tissue homogenate, cell suspension, and various microglial sort methods.

A schematic of the experimental design is represented in Figure 4A. Cx3cr1-NuTRAP brains were hemisected and processed in halves for whole-tissue homogenization or enzymatic and mechanical dissociation to create single cell suspensions. Single cell suspensions were then sorted using one of four methods: 1) CD11b magnetic-bead based isolation (autoMACS), 2) Cartridge-based FACS on CD11b’CD45’eGFP’ (MACSQuant Tyto), 3) Cytometer-based FACS on CD11b’CD45’eGFP’ (FACS(Aria), or 4) autoMACS debulking of cells prior to cartridge-based FACS (autoMACS to MACSQuant Tyto), as before. Tissue homogenate (Tissue-TRAP), mixed-cell suspension (Cell-TRAP), and sorted microglia (Sort-TRAP) were then subjected to TRAP pull-down of microglial-specific translating RNA for creation of RNA-Seq libraries. RNA yield and quality by sort method, in addition to basic sequencing metrics are given in Figure 4-1. Gene body coverage plots by experimental group are shown in Figure 4-2. All sort methods showed similar coverage across the gene body.

PCA of all expressed genes showed separation of Tissue-TRAP from all other groups in the first component with 79% of the explained variance and separation of Cell-TRAP from all other groups with 11% of the explained variance (Figure 4B). The autoMACS-TRAP group separated from the other groups in the third component with 7% of the explained variance (Figure 4C). Based on this unbiased dimensionality reduction, it appears that the MACS- and FACS-based sorted cells have similar translatome profiles. Each of the four Sort-TRAP methods showed similar levels of enrichment of microglial marker genes (Figure 4D, Figure 4-1). Depletion of astrocytic, oligodendrocytic, neuronal, and endothelial marker genes (Figure 4E-H, Figure 4-1) was greater in Sort-TRAP methods as compared to Cell-TRAP (One-way ANOVA, Tukey’s post-hoc, **p<0.001). This shows that the extra enrichment step of sorting microglia followed by TRAP-isolation of translating microglial RNA, leads to more pure microglial RNA than Tissue-TRAP or Cell-TRAP alone.

In recent years, several studies have suggested that cell-isolation methods cause ex vivo activational effects in microglia (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020).
this section, our goal was to determine if different sorting techniques result in different levels of ex vivo activation. We used Tissue-TRAP as an “unactivated” microglial reference group, since the Tissue-TRAP method does not rely on the creation of a cell suspension or cell sorting techniques. There were 8076 DEGs between the translatomes when Cell- and Sort-TRAP methods were compared to the Tissue-TRAP reference (One-way ANOVA, BHMTC, SNK FDR<0.1, |FC|>2). Upset plot of the 8076 DEGs shows the majority of the DEGs (7800/8076=97%) are in common between all groups (Cell-/Sort-TRAP v. Tissue-TRAP) (Figure 5A, Figure 5-1). Hierarchical clustering of all 8076 DEGs shows distinct clustering of Tissue-TRAP from all other groups. Cell-TRAP also clusters separately from the Sort-TRAP v. Tissue-TRAP, revealed 5337 common DEGs (67%) that were depleted and 2329 common DEGs (29%) that were enriched in all groups (Cell- and Sort-TRAP) compared to Tissue-TRAP. There were only 352 discordant DEGs (4%) between the different sort methods as compared to Cell-Input (Figure 5C). These data, together with cell-type enrichments from Figure 4D-H, suggests that the act of creating a cell suspension is the largest contributor to differences seen between Tissue-TRAP and Sort-TRAP methods.

Next, we identified over-represented pathways among the up-regulated genes compared to Tissue-TRAP. The top 10 biological processes of the 2329 genes up-regulated in comparison to Tissue-TRAP were microglial-related pathways, including: myeloid leukocyte activation, cell activation involved in immune response, and leukocyte-mediated immunity (Figure 5D; Figure 5-1). Comparing the biological processes identified in the transcriptomic analysis (Figure 2J; Figure 2-1) and the translatomic analysis (Figure 5D; Figure 5-1) revealed 55 biological processes that were only up-regulated in the translatome (Figure 5E). Several of the biological processes uniquely upregulated in the translatome comparisons were involved in microglial activation pathways, including: response to type I interferon (IFN), response to transforming growth factor beta (TGFβ), response to interleukin-6 (IL-6), and NIK/NF-kappaB signaling (NFkB) (Figure 5F). IL-6 is a pro-inflammatory cytokine extensively studied in brain aging and disease (Borovcanin et al., 2017; Singh-Manoux et al., 2014; Ye and Johnson, 1999). Microglia have higher IL-6 receptor (IL-6R) expression than any other cell type. As such, microglia are highly responsive to IL-6 and transition into a “primed” state when exposed to high levels of IL-6. Hierarchical clustering of the “Response to IL-6” pathway genes showed overall higher levels of expression in Cell- and Sort-TRAP groups (Figure 5G; Figure 5-1). The Cell-TRAP did not
cluster separately from the Sort-TRAP groups, providing further evidence that the ex vivo activational signature is a function of cell preparation.

Next, we looked at gene expression of common cytokines (Figure 5H; Figure 5-1) and chemokines (Figure 5I; Figure 5-1) across Tissue-, Cell-, and Sort-TRAP groups. We observed higher levels of cytokine and chemokine transcripts across all Cell- and Sort-TRAP groups when compared to Tissue-TRAP (One-way ANOVA, BHMT, SNK FDR<0.1, |FC|>2). In an effort to cross-validate our finding with previous studies, we intersected ex vivo microglial activational gene lists from three previous studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020) and identified 21 ex vivo activational transcripts represented in at least two of the studies (Figure 5J; Figure 5-1). PCA of the TRAP data from the present study on the 21 ex vivo activational genes shows clear separation of Tissue-TRAP from all other groups in the first component (92.8% explained variance) (Figure 5K). Again, suggesting that the ex vivo signature is a function of cell preparation. Hierarchical clustering of the 21 activational genes, shows similar patterning as in the “Response to IL-6” pathway with higher levels of expression across Cell- and Sort-TRAP groups compared to Tissue TRAP (One-Way ANOVA, Tukey’s posthoc, ***p<0.001) (Figure 5L; Figure 5-1).

Zfp36 was one of the ex vivo activational genes identified in all three previous studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020). Zinc finger protein 36 (Zfp36) encodes for the protein Tristetraprolin (TTP) which is involved in regulating immune responses through mRNA destabilizing and alternative splicing (Tu et al., 2019). Zfp36 is enriched in Cell- and Sort-TRAP compared to Tissue TRAP (One-Way ANOVA, Tukey’s posthoc, ***p<0.001) (Figure 5M; Figure 5-1).

In summary, these data suggest that ex vivo microglial activation is primarily occurring during cell preparation and is sustained through microglial isolation by various sort methods but there were no major differences between the different sort methods.

Comparison of cellularity and microglial yield between different cell preparation methods.

Since ex vivo activation appears to be an artifact of cell preparation, three different methods were compared for changes in cellularity within the cell preparation. The experimental design is displayed in Figure 6A. Tissue-Input, generated by immediately Dounce homogenizing tissue in TRAP lysis buffer, was used as a comparator to the three methods of cell preparation. The first cell preparation method was a mechanical dissociation carried out entirely at 4ºC followed by a
40% Percoll gradient to removed undissociated tissue and myelin debris (Dounce-Input). The second cell preparation method used a papain-based enzyme mixture which requires a brief (30 minute) incubation at 37°C followed by myelin debris removal (Cell-Input (-) Inhibitors) which was the method of cell preparation used from Figure 1 to Figure 5. The third cell preparation method used the same papain-based enzyme mixture, this time supplemented with transcriptional and translation inhibitors (Marsh et al., 2020; Wu et al., 2017) with incubation at 37°C followed by myelin debris removal (Cell-Input (+) Inhibitors). Cells from each of the three methods were pelleted and lysed with TRAP lysis buffer prior to RNA extraction and assessment of the transcriptome. RNA yield and quality by sort method, in addition to basic sequencing metrics are given in Figure 6-1. The average RNA yield was higher in the Tissue-Input (5.8 µg/half brain) than the Cell-Input (+/-) Inhibitors (360 ng/half brain) and Dounce-Input (35 ng/half brain) (One-way ANOVA, Tukey’s post hoc, p<0.05). The average RNA Integrity Number (RIN) was lowest in the Dounce-Input (3.4) when compared to the Tissue-Input (7.8) and Cell-Input (+/-) Inhibitors (7.3). In addition, use of an entire Cx3cr1-NuTRAP brain was needed for RNA isolation from the Dounce-Input group, whereas half a brain was used for all other methods. Gene body coverage plots by experimental group are shown in Figure 6-2. The Tissue-Input and Dounce-Input groups showed more 3' bias in their gene body coverage than the Cell-Input (+/-) Inhibitor groups.

To assess the relative cellularity between each of the cell preparation methods, flow cytometry was conducted on each of the cell preparations, using markers for microglia (CD11b+CD45+ or eGFP+), astrocytes (ACSA2+), oligodendrocytes (O4+), and neurons (CD24+). After gating on cells and singlets (as in Figure 6-3) the percent singlets positive for each of the cell markers were compared between methods. There was a higher percentage of neurons and lower percentage of microglia and astrocytes in the Dounce-Input samples as compared to the Cell-Input (+/-) Inhibitors (Figure 6B). There was no overall difference in cell viability between the three cell preparation methods examined. However, there was higher viability among eGFP+ singlets in the Cell-Input (+/-) Inhibitors as compared to the Dounce-Input. Inclusion of transcription and translation inhibitors during enzymatic and mechanical dissociation did not alter cell viability (Figure 6-4).

Stranded RNA-Seq libraries were constructed from each of the groups displayed in Figure 6A. Heatmaps of microglial, astrocyte, oligodendrocytic, neuronal, and endothelial cell marker lists for each of the cell preparation methods were normalized to Tissue-Input. All three cell preparation methods appear to be enriched for microglial transcripts as compared to Tissue-
Input, with the strongest enrichment in the Cell-Input (+/-) Inhibitors. The Cell-Input (+/-) Inhibitors also appear to have greater enrichment of astrocytic and endothelial markers and depletion of neuronal transcripts when compared to Tissue- and Dounce-Input (Figure 6C, Figure 6-1). CIBERSORTx, or digital cytometry, estimates cell-type abundance from bulk transcriptomics. Using publicly available cell-type specific data from mouse brain (Zhang et al., 2014) (GSE52564) as a digital cytometry reference matrix, we estimated the cellularity from each of the four groups (Zhang et al., 2014). Based on this analysis, there was no difference in the cellularity between the Tissue-Input and Dounce-Input or between the Cell-Input (+) Inhibitors and the Cell-Input (+/-) Inhibitors. However, there were more astrocytic, oligodendrocytic, microglial, and endothelial transcripts in the Cell-Input (+/-) Inhibitors than the Tissue- and Dounce-Input. Further, there were less neuronal transcripts in the Cell-Input (+/-) Inhibitors than the Tissue- and Dounce-Input (Two-Way ANOVA, Tukey’s post hoc) (Figure 6D, Figure 6-1). These CIBERSORTx results are consistent with the flow cytometric data displayed in Figure 6B and suggest that the enzymatic and mechanical dissociation carried out at partially elevated temperatures depletes neurons from the cell preparation.

Lastly, we compared the cell yield per half brain between the various cell preparation techniques. The average total cell count was slightly higher in the Dounce-Input (~3.7x10^6 cells/half brain) when compared to Cell-Input (+/-) Inhibitors (~1.7x10^6 cells/half brain) (Figure 6-1). However, due to the increased proportion of microglia in the Cell-Input (+/-) Inhibitors compared to the Dounce-Input, there were ~20 times more eGFP+ microglia in the Cell-Input (+/-) Inhibitors compared to the Dounce-Input (Figure 6E, Figure 6-1). Thus, the enzymatic and mechanical dissociation carried out at partially elevated temperatures will minimize the need for pooling samples when using small tissue sections for microglial sorting applications.

Effect of different cell preparation techniques on ex vivo activational profiles following cell preparation.

Recent studies have suggested that inclusion of transcriptional and/or translational inhibitors during cell preparation can prevent ex vivo activational confounds in microglia (Marsh et al., 2020) and other neuronal cell types (Wu et al., 2017). Additionally, ex vivo artifacts can be prevented by mechanical dissociation carried out entirely at low temperatures (Marsh et al., 2020). To compare the relative levels of ex vivo activation between various cell preparation methods, we TRAP-isolated RNA from Tissue, Dounce, and Cell (+/-) Inhibitor cell preparations as shown in Figure 7A. Stranded RNA-Seq libraries were constructed from TRAP-isolated translating RNA from each of the four groups. RNA yield and quality by sort method, in addition
to basic sequencing metrics, are given in Figure 7-1. Gene body coverage plots by experimental group are shown in Figure 7-2. The Tissue-TRAP and Dounce-TRAP groups showed more 3' bias in their gene body coverage than the Cell-TRAP (+/-) Inhibitor groups.

There were 4,475 differentially expressed genes between Tissue-TRAP, Dounce-TRAP, Cell-TRAP (-) Inhibitors, and Cell-TRAP (+) Inhibitors (One-way ANOVA, |FC|>2, BHMTC, FDR<0.1) (Figure 7-3, Figure 7-1). Many of these transcripts differ between all cell preparation methods and the Tissue-TRAP, suggesting the differences may result from slight differences in the purity of TRAP-isolated RNA. There were 85 DEGs that had higher expression in the Cell-TRAP (-) Inhibitors compared to Tissue-TRAP and lower expression in Cell-TRAP (+) Inhibitors compared to Cell-TRAP (-) Inhibitors. These 85 transcripts are ex vivo activational transcripts that are prevented by the inclusion of transcriptional and translational inhibitors during enzymatic and mechanical dissociation at partially elevated temperatures. A heatmap of these 85 prevented ex vivo activational transcripts showed both the Dounce-TRAP and Cell-TRAP (+) Inhibitors were successful at preventing the induction of ex vivo artifacts (Figure 7B, Figure 7-1). The top 10 GO biological processes over-represented in the 85 prevented ex vivo transcripts include: ERK1 and ERK2 cascade, cytokine-mediated signaling, cellular response to biotic stimulus, positive regulation of response to external stimulus, response to mechanical stimulus, regulation of inflammatory response, response to tumor necrosis factor, regulation of hemopoiesis, response to chemokine, and response to molecule of bacterial origin (Figure 7C, Figure 7-1). In addition, CREB, CEBP, NFKB, and SRF are over-represented transcription factors in the 85 prevented ex vivo transcripts (Figure 7D, Figure 7-1). Overall, pathway analyses of the 85 prevented ex vivo transcripts suggest that mechanical dissociation carried out entirely at chilled temperatures or inclusion of transcription and translation inhibitors during enzymatic and mechanical dissociation at elevated temperatures are successful methods at preventing upregulation of ex vivo responsive pathways during cell preparation.

In order to study in vivo pathophysiological microglial responses, it is necessary to reduce the ex vivo confounds of cell preparation. For example, with aging and disease microglia increase production of inflammatory cytokines, chemokines, and reactive oxygen species (Von Bernhardi et al., 2015). Response to oxidative stress and reactive oxygen species were also identified as pathways significantly over-represented in the 85 prevented ex vivo activational transcripts. Induction of the response to oxidative stress pathway (Figure 7E, Figure 7-1) and generation of common chemokines (Figure 7F, Figure 7-1) were prevented in the Dounce-TRAP and Cell-TRAP (+) Inhibitors as compared to the Cell-TRAP (-) Inhibitors group.
Another active area of research in glial biology is that of cellular senescence, characterized by cell-cycle arrest and secretion of pro-inflammatory cytokines and chemokines (Hou et al., 2021; Hu et al., 2021). Cell preparation method altered expression of cellular senescence-related genes identified from a previously published study (Aird et al., 2016). Inclusion of transcription and translation inhibitors during cell preparation reduced the induction of these senescence-associated genes (Figure 7G, Figure 7-1).

Heatmap of the 21 ex vivo activational transcripts identified in at least two of the three examined previous studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020) from Figure 5J shows prevention of ex vivo activation with the addition of transcriptional and translational inhibitors or mechanical dissociation carried out entirely at low temperatures (Figure 7H, Figure 7-1). PCA of the 21 ex vivo activational transcripts identified shows strong separation of Cell-TRAP (-) Inhibitors from all other groups in the first component (86.1% explained variance) and clustering of Dounce-TRAP and Cell-TRAP (+) Inhibitors together (Figure 7I).

In summary, the addition of transcriptional and translational inhibitors during enzymatic and mechanical dissociation of brain tissue or mechanical dissociation carried out entirely at low temperatures can prevent ex vivo activational artifacts in microglia.

Discussion

Microglia have emerged as key players in brain disease, including age-related neuroinflammation and neurodegeneration (Colonna and Butovsky, 2017; Lana et al., 2021). As a minority cell population in the brain, in depth microglial molecular and biochemical analyses benefit from enrichment strategies to provide cell-specific data (Okaty et al., 2011). While in vitro cell culture models are useful for mechanistic studies, they fail to recapitulate the complexity of the nervous system milieu. As such, models and methods to ‘debulk’ microglia from brain tissue have become a focus in the field (Chucair-Elliott et al., 2020; McKinsey et al., 2020). The most common methods for isolating microglia include enzymatic and/or mechanical dissociation of brain tissue followed by immunolabeling with magnetic beads for MACS (Bordt et al., 2020) or fluorescent-conjugated antibodies for FACS-based enrichment (Bohlen et al., 2019). As these methods continue to evolve, quantitative comparisons of cell preparation and sort strategies are needed to aid decision making of what approaches to use in specific studies. As well, there are legitimate concerns that these isolation approaches introduce artifacts, especially in glial cell populations which by nature are sensitive to changes in their microenvironment (Wu et al., 2017). Determining the degree of ex vivo activational artifacts, and how they may vary between isolation approaches, has been challenging because the field lacked a resting cell type-specific
reference (absent of ex vivo activational confounds related to enzymatic/mechanical dissociation) as a comparator.

To address this barrier to progress, we used a ribosomal-tagging model (NuTRAP) in combination with a microglial-specific cre (Cx3cr1-cre/ERT2) to generate a microglial signature without the confounds of cell isolation. We then compared relative microglial enrichment and ex vivo activational artifacts between multiple MACS- and FACS-based cell isolation techniques. All sort methods were successful in isolating highly pure microglia, as evidenced by flow cytometric and transcriptomic analyses. Our MACS - FACS comparison is in line with previous findings on yield and speed (Pan and Wan, 2020). Magnetic bead-based isolation produced nearly as pure of a microglial population as FACS-based approaches. The advantages of the magnetic bead isolation include the rapid isolation (<1hr), multiplexing 6 samples at a time, and least amount of instrumentation. The limitation of magnetic bead-based approaches is the single dimension of labeling as compared to FACS.

Cartridge-based FACS is a new iteration of FACS and produced nearly equivalent cell purities as traditional cytometry-based FACS but with greater cell yield. Given the higher cell yield, cartridge-based FACS may be beneficial for applications requiring large amounts of starting material, including ChIP-seq, HiC-based techniques, proteomics, and primary microglial cultures. Another advantage of this cartridge-based approach is the self-contained nature of the system that does not produce aerosols thus not requiring biosafety containment (Roberts et al., 2021). Cytometry-based FACS produced the purest cell population and has the highest capabilities for dimensions of labeling. However, this approach is also the slowest and had the lowest cellular yield. A strict comparison of FACS methods relies on details of the gating strategies used and these can likely be tuned to emphasize highest purity or cell yield. Cytometer-based FACS easily incorporates various staining controls, which allows for detailed refinement of gating without additional cost. For cartridge-based FACS, separate cartridges are needed for staining controls, increasing the cost. Additionally, the cartridge-FACS does not have traditional forward scatter detection, but instead utilizes back scatter, making it difficult to gate cell populations from debris. Validation of cartridge-FACS sorted cells should be performed on a traditional cytometer as a control.

Combining MACS with cartridge-based FACS, to initially de-bulk microglia and then further purify, did not result in higher purity of microglia and the presence of the magnetic beads shifted signals in the FACS. Taken together, these data demonstrate that all of these approaches are
valid for microglial isolation from brain and return highly pure cell populations that are suitable for molecular and biochemical analyses.

An unexpected finding of the analyses was the shift in cellularity caused by the cell suspension preparation. Neuronal cells and transcriptomic signals were depleted during cell preparation with enzymatic methods. While this has the effect of aiding microglial isolation by diminishing the majority neuronal cell population, this has profound effects on neuronal cell isolation studies. Mechanical dissociation at low temperature retained a much higher proportion of neuronal cells. Outside of these studies, others have also observed that alternative cell preparation methods cause less neuronal cell loss (Saxena et al., 2012). We did not test alternate cell preparation approaches such as these for their differential effects on microglial ex vivo activation and it remains possible that these different methods, through causing less cell death, could lead to less microglial activation. The large neuronal loss during cell preparation may contribute to microglia activation ex vivo given that low temperature mechanical dissociation demonstrated lower activational signal, though this is only an association.

Using TRAP isolation of the microglial translatome as a baseline measure of microglia, induction of ex vivo activational pathways occurred during enzymatic and mechanical cell preparation and was sustained during microglial isolation, independent of sort method. Compared to previous studies of these activational artifacts (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020) we found a common set of activational markers centered on immediate early genes. This unified set of markers can be used in future studies as markers of artifactual microglial cell activation. More broadly the use of ribosomal tagging methodologies to provide a comparator for optimizing cell preparation could be applied to any relevant organ.

The use of transcriptional and translational inhibitors (Marsh et al., 2020; Wu et al., 2017) during the cell preparation was investigated in the context of acutely isolating cells for immediate use in downstream molecular and biochemical analyses. Addition of transcriptional and translational inhibitors blocked much of the ex vivo activational artifacts without otherwise changing the cell phenotype. Whether this is a valid approach for cells that will subsequently be cultured was not assessed. Nonetheless, as studies also delve into microglial heterogeneity at the single cell level (T. Masuda et al., 2020; Stratoulias et al., 2019) inclusion of inhibitors in the preparation and enrichment of microglia can reduce artifacts in these single cell studies as well (Marsh et al., 2020). Application of transcriptional and translational inhibitors in the context of specific CNS pathologies should be validated on a case-by-case basis, potentially using the same approaches presented here. As an alternative to enzymatic and mechanical dissociation,
mechanical dissociation carried out entirely at 4°C also avoids *ex vivo* activation of microglia. However, the cell/RNA yields and RNA integrity associated with mechanical dissociation was much lower than that obtained with enzymatic and mechanical dissociation. Collectively, our data demonstrate that a variety of microglial isolation methods can be used with equivalent results and tuned to the needs of the specific study. In addition, activational artifacts occur during cell preparation and can be prevented by inclusion of specific inhibitors early in the cell preparation protocol or by avoiding high temperatures. The different cell sorting methods did not show additional activational effects or differences between methods, indicating that concerns over artifacts should not drive isolation method selection. Addition of transcriptional and translational inhibitors during cell preparation reduces *ex vivo* artifacts and is an easily implementable approach to avoid potential confounds.
References


Figure 1. Comparison of purity and yield among microglial cell isolation techniques. A) Schematic of the experimental design. Cx3cr1-NuTRAP brains were enzymatically and mechanically dissociated to create a single cell suspension. Different microglial sorting techniques were compared to Cell-Input for purity, yield, and transcriptomic signatures. B) Representative flow cytometry plots of immunostained single-cell suspensions from input and after each of the sorting strategies shows a distinct population of: eGFP+ cells (identified as Cx3cr1+ microglia) and CD11b+CD45+ cells (identified as microglia per traditional cell surface markers, as shown by IHC in Figure 1-2). Assessment of the loss of eGFP in circulating CD11b+CD45+ cells 60-days post-tamoxifen induction (Figure 1-3) and the relative levels of CD45mid and CD45high cell populations from the CD11b+CD45+ each sort method and cell input from brain (Figure 1-4) were also performed to further characterize the sort fractions. Representative plots of the gating strategies used for the Tyto sort (Figure 1-5), FACSaria sort...
(Figure 1-6), and flow cytometry (Figure 1-7) are given as supplements. C-D) All sort positive fractions were enriched for: (C) eGFP+ singlets and (D) CD11b+CD45+ singlets (as compared to input) (Two-Way ANOVA, Main effect of TRAP Fraction, ***p<0.001)(Figure 1-1). When comparing positive fractions, the autoMACS positive fraction had lower % eGFP+ singlets as compared to all other sort methods. FACSARia had higher percentage of eGFP+ singlets than all other sort methods. FACSARia had higher percentage of CD11b+CD45+ singlets as compared to all other sort methods positive fractions (Two-way ANOVA, Tukey’s post-hoc, *p<0.05). E) Microglial yield was significantly higher in the MACSQuant Tyto positive fraction as compared to the autoMACS to MACSQuant Tyto and FACSARia positive fractions (One-Way ANOVA, Tukey’s posthoc, #p<0.01). Created with BioRender.com.
Figure 2. Comparison of transcriptomic profiles of microglia isolated from various cell sorting methods. RNA-Seq libraries were made from each of the groups represented in Figure 1A to compare the transcriptomic profiles of microglia isolated via four different cell sorting strategies. Basic sequencing metrics and all source data for Figure 2 are provided in Figure 2-1. Gene body coverage plots showed similar coverage across the gene bodies in each group (Figure 2-2). Each of the strategies had similar levels of (A) enrichment of microglial transcripts.
and depletion of: (B) astrocytic, (C) oligodendrocytic, (D) neuronal, and (E) endothelial
transcripts when compared to Cell-Input. F) Principal component analysis of all expressed
genes shows clear separation of Cell-Input from all other sort methods in the first component
with 81% of explained variance. G) Hierarchical clustering of differentially expressed genes
(DEGs) (One-Way ANOVA, BHMTC, SNK FDR<0.1, |FC|>2) shows separation of Cell-Input and
sort methods. Each of the sort methods show very similar patterning of expression across
DEGs. H) Comparison of SNK post-hocs from each of the sort methods v. Cell-Input, showed
the majority of enrichments/depletions (ie. DEGs) (7084/7378 = 96%) were in common between
all sort methods. I) There were 5322 DEGs (72%) that were depleted and 1759 DEGs (24%)
that were enriched in all sort methods compared to Cell-Input. There were only 297 discordant
DEGs (4%) between the different sort methods as compared to Cell-Input. J) Top 10 biological
processes over-represented in the 1759 genes upregulated in all sort methods compared to
Cell-Input (Gene Ontology Over-Representation Analysis, BHMTC FDR <0.05). K) Top 10
biological processes over-represented in the 5322 genes downregulated in all sort methods
compared to Cell-Input (Gene Ontology Over-Representation Analysis, BHMTC FDR <0.05). L)
Top 5 transcription factor targets over-represented in the 1759 genes upregulated in all sort
methods compared to Cell-Input (Transcription factor target network over-representation
analysis, BHMTC FDR<0.05). M) Top 5 transcription factor targets over-represented in the 5322
genes downregulated in all sort methods compared to Cell-Input (Transcription factor target
network over-representation analysis, BHMTC FDR<0.05).
Figure 3. Assessment of expression of non-microglial macrophage markers in sorted cells from Cx3cr1-NuTRAP brains. Gene marker lists and source data for Figure 3 are provided in Figure 3-1. A) Previously curated microglial marker gene lists from cell sorting (Chucair-Elliott et al., 2020; Haage et al., 2019) and ribosomal tagging (Chucair-Elliott et al., 2020) approaches were compared for overlap. Microglial marker genes observed in at least two of the studies (113 genes, purple overlap) were used for further analysis (Figure 3-1). B) Previously curated non-microglial macrophage marker gene lists from peripheral monocytes/macrophages (Haage et al., 2019), non-microglia brain resident macrophages (Faraco et al., 2017), and infiltrating macrophages (DePaula-Silva et al., 2019) were compared.
The union of all macrophage markers (174 genes, green) was used for further analysis (Figure 3-1). C) RNA-Seq gene expression of brain-resident macrophage marker genes from: 1) microglia (Hexb, Tmem119, P2ry12, Siglech) and 2) non-microglia macrophages (Lyve1, Ly6c1, CD163, Mrc1) from each sorting method (AutoMACS, AutoMACS to Tyto, Tyto, and FACSaria) were compared to Cell-Input. D) RNA-Seq gene expression of peripheral macrophage marker genes from: 1) infiltrating macrophages (Emp1, Scarf1, Emb, Clec4e) and 2) peripheral macrophages/monocytes (Emilin1, F5, Fn1, Gda) from each sorting method (AutoMACS, AutoMACS to Tyto, Tyto, and FACSaria) were compared to Cell-Input (One-Way ANOVA, Tukey’s post hoc, *p<0.05). All bars represent the mean normalized gene expression ± SEM. E) Gene set enrichment analysis (GSEA) of the 113 microglial marker genes identified in Figure 3A comparing all sort positive fractions (AutoMACS, AutoMACS to Tyto, Tyto, and FACSaria) to Cell-Input. F) Gene set enrichment analysis (GSEA) of the 174 macrophage marker genes identified in Figure 3B comparing all sort positive fractions (AutoMACS, AutoMACS to Tyto, Tyto, and FACSaria) to Cell-Input. G) GSEA enrichment scores compared to Cell-Input for each of the sorting methods for the microglial marker genes identified in Figure 3A and macrophage marker genes identified in Figure 3B.
Figure 4. Comparison of TRAP-isolated microglial translatome from tissue homogenate, cell suspension, and various microglial cell sorting methods. A) Schematic of the experimental design. Cx3cr1-NuTRAP brains were hemisected and processed in halves for whole-tissue homogenization or enzymatic and mechanical dissociation to create a single cell suspension. Single cell suspensions were then sorted using MACS- and/or FACS-based isolation of microglia. Tissue homogenate, mixed-cell suspension, and microglia sorted by each of the four depicted methods were subjected to TRAP to isolate microglial-specific ribosomally-bound RNA for creation of RNA-Seq libraries. Gene body coverage plots showed no 3' bias in any groups (Figure 4-2). B) PCA of all expressed genes (>5 read counts in all samples from at
least one group) separates Tissue-TRAP from all other groups in the first component (79% explained variance) and Cell Suspension-TRAP from all other groups in the second component (10.9% explained variance). C) Third component of PCA on all expressed genes separated autoMACS-TRAP from all other groups (7.2% explained variance). Each of the sort strategies had similar levels of (D) enrichment of microglial transcripts and depletion of: (E) astrocytic, (F) oligodendrocytic, (G) neuronal, and (H) endothelial transcripts when compared to Tissue-TRAP. All of the sort methods showed stronger depletion of: (E) astrocytic, (F) oligodendrocytic, (G) neuronal, and (H) endothelial transcripts when compared to Cell-TRAP (One-way ANOVA, Tukey’s post-hoc, ***p<0.001) (Figure 4-1). Created with BioRender.com.
Figure 5. Cell isolation and sorting techniques alter TRAP-isolated microglial translatome compared to whole-tissue TRAP. RNA-Seq libraries were made from each of the groups represented in Figure 3A to compare the TRAP-isolated microglial translatomes between whole-tissue-TRAP and each of the cell isolation and sorting methods. All source data for Figure 5 are provided in Figure 5-1. A) Upset plot of DEGs for all groups v. Tissue-TRAP (One-Way ANOVA, BHMTMC, SNK FDR<0.1, |FC|>2). B) Hierarchical clustering of DEGs shows separation of tissue TRAP from all other groups. Cell-TRAP also clusters separately from all Sort-TRAP groups. C) Comparison of DEGs from each group (Cell- and Sort-TRAP) v. Tissue-TRAP, revealed 5337 common DEGs (67%) that were depleted and 2329 common DEGs (29%) that were enriched in all groups (Cell- and Sort-TRAP) compared to Tissue-TRAP. There were only 352 discordant DEGs (4%) between the different sort methods as compared to Cell-Input.
D) Top 10 biological processes over-represented in the 2329 genes upregulated in Cell-TRAP and Sort-TRAP compared to Tissue-TRAP (Gene Ontology Over-Representation Analysis, Hypergeometric test, BHMTCH FDR <0.05). E) Comparison of upregulated transcriptomic pathways (Figure 2J; Figure 2-1) and upregulated translatome pathways (Figure 5D, Figure 5-1) reveal 55 biological processes that are upregulated in the translatome only. F) Selection of 10 biological processes that are uniquely upregulated in the translatome (from the 55 identified in Figure 4E). G) Heatmap of genes involved in “Response to Interleukin-6 (GO:0070741)” biological process. H) Cytokines (I/1a, I/1b, I/6, I/10, I/16, I/27) that are upregulated in the Cell- and Sort-TRAP groups compared to Tissue-TRAP (One-Way ANOVA, BHMTCH, SNK FDR<0.1, |FC|>2). I) Chemokines (Cxx1, Cxx2, Cxx10, Ccl2, Ccl3, Ccl4, Ccl7, Ccl12) that are upregulated in the Cell- and Sort-TRAP groups compared to Tissue-TRAP (One-Way ANOVA, BHMTCH, SNK FDR<0.1, |FC|>2). J) Intersection of activational genes identified in three previous studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020) identified 21 ex vivo activational transcripts represented in at least two of the studies. L) PCA of 21 ex vivo activational genes shows clear separation of Tissue-TRAP from all other groups in the first component (92.8% explained variance). J) Heatmap of 21 activational genes shows high levels of ex vivo activational transcripts across Cell- and Sort-TRAP methods compared to Tissue-TRAP. K) Zfp36 is enriched in Cell-TRAP and Sort-TRAP compared to Tissue TRAP (One-Way ANOVA, Tukey’s posthoc, ***p<0.001).
Figure 6. Changes in cellularity based on cell preparation method. Cx3cr1-NuTRAP brains were hemisected and single cell suspensions were generated by three different methods. Cell preparations were compared via flow cytometry and transcriptomic analyses. The three cell preparation methods were also compared to whole tissue using transcriptomic analysis. Basic sequencing metrics and all source data for Figure 6 are provided in Figure 6-1. Gene body coverage plots showed greater 3’ bias in the Tissue- and Dounce-Input groups than the Cell-Input (+/−) Inhibitors groups (Figure 6-2). A) Schematic of experimental design presented in this figure. B) Flow cytometry on cell type-specific markers for microglia (CD11b+CD45−), astrocytes (ACSA2*), oligodendrocytes (O4+) and neurons (CD24+) for each of the cell preparation methods (One-Way ANOVA, Tukey’s post hoc, ***p < 0.001). Representative gating strategies for flow cytometry are provided in Figure 6-3. C) Heatmap of cell type-specific markers for microglia, astrocytes, oligodendrocytes, neurons, and endothelial cells. D) CIBERSORTx cellularity estimates based on whole-transcriptome RNA-Seq from whole brain tissue and each of the cell preparation techniques. E) Comparison of the number of eGFP+ singlets in each half brain for each of the cell preparation methods (One-Way ANOVA, Tukey’s post hoc, ***p < 0.001). There was no overall difference in cell viability between different cell preparation methods. eGFP+ cells had slightly higher viability in the Cell-Input (+/−) Inhibitors than the Dounce-Input method (Figure 6-4). Boxplots represent median, Q1, Q3, minimum, and maximum of each data set. Created with BioRender.com.
Figure 7. Ex vivo activational profiles by cell preparation method. Basic sequencing metrics and all source data for Figure 7 are provided in Figure 7-1. Gene body coverage plots showed greater 3’ bias in the Tissue- and Dounce-TRAP groups than the Cell-TRAP (+/- Inhibitors) groups (Figure 7-2). A) Schematic of experimental design presented in this figure. B) Differentially expressed genes were called between Tissue-TRAP, Dounce-TRAP, and Cell-TRAP (+/- Inhibitors) groups (Figure 7-1, Figure 7-3). A subset of genes was activated with cell preparation (Up in Cell-TRAP (-) Inhibitors v Tissue-TRAP) and were decreased with the addition of inhibitors (Down in Cell-TRAP (+) Inhibitors v. Cell-TRAP (-) Inhibitors) (One-Way ANOVA, BHMT, SNK FDR<0.1, |FC|>2). These genes were classified as ex vivo activational
transcripts prevented by the addition of inhibitors and are given in the heatmap. C) Top 10 biological processes over-representation in the ex vivo activational genes prevented by the addition of inhibitors (hypergeometric test, BHMT, FDR<0.05). D) Transcription factors over-represented in the ex vivo activational transcripts prevented by the addition of inhibitors (hypergeometric test, BHMT, FDR<0.05). E) Heatmap of select response to oxidative stress pathway genes. F) Heatmap of common chemokines. G) Violin plots of senescent cell marker expression (One-way ANOVA, Tukey’s post-hoc, *p<0.05). H) Heatmap of 21 ex vivo activational genes identified in at least two previous studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020). I) PCA of 21 ex vivo activational genes identified in at least two previous studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020).
Figure 1-2. Validation of microglial identity of recombined cells in the Cx3cr1-NuTRAP brain. Two months after Tam treatment, brains were harvested from Cx3cr1-NuTRAP and cre negative NuTRAP⁺ (control) mice for immunohistochemistry (IHC). A. Representative confocal fluorescent microscopy images of sagittal brain sections captured in the cortex show eGFP expression (green signal) in cells that co-expressed CD11b (red signal, a-a' and b-b') and CD45 (red signal, c-c' and d-d') in Cx3cr1-NuTRAP brains but not in the cre negative counterparts (n=2/group). B. Representative confocal fluorescent microscopy images captured in the hippocampus show eGFP expression (green signal) in cells that co-expressed CD11b (a-a') and CD45 (b-b') in Cx3cr1-NuTRAP brains. DAPI: nuclear counterstain. Scale bar: 50 µm.
Figure 1-3. Flow cytometry of blood from cre negative (WT) and Cx3cr1-NuTRAP blood following tamoxifen-mediated cre-recombination. A-B) Gating strategy to assess the level of eGFP expressing CD11b^+CD45^+ singlets (A) 3- and (B) 60- days following cessation of tamoxifen induction. C) Three days after tamoxifen induction there is significant expression of the NuTRAP allele in circulating CD11b^+CD45^+ cells, which is not evident after 60 days (One-way ANOVA, Tukey’s post-hoc, ***p<0.001).
Figure 1-4. Flow cytometric analysis of CD45\textsuperscript{Mid} and CD45\textsuperscript{High} cell populations from Cx3cr1-NuTRAP brain Cell-Input and sorted microglia. A) Gating strategy to assess the percent CD45\textsuperscript{Mid} and CD45\textsuperscript{High} of CD11b\textsuperscript{+}CD45\textsuperscript{+} singlets. CD45\textsuperscript{Mid} and CD45\textsuperscript{High} populations were then hierarchical gated to assess the percent eGFP\textsuperscript{+} singlets from the CD45\textsuperscript{Mid} and CD45\textsuperscript{High} populations. B) Quantitation of the proportion of CD11b\textsuperscript{+}CD45\textsuperscript{+} singlets that were CD45\textsuperscript{Mid} and CD45\textsuperscript{High} from Cell-Input and each of the microglial sorting methods (AM, AM + Tyto, Tyto, FACS/Aria). C) Quantitation of the proportion of eGFP\textsuperscript{+} singlets within the CD45\textsuperscript{Mid} (left) and CD45\textsuperscript{High} (right) populations (One-way ANOVA, Tukey’s post-hoc, ***p<0.001). Boxplots represent median, Q1, Q3, minimum, and maximum of each data set.
Figure 1-5. Cartidge-based FACS gating strategy for microglial sorting on Miltenyi Biotec MACSQuant Tyto.
Figure 1-6. Cytometer-based FACS gating strategy for microglial sorting on FACS Aria IIIu. A) Unstained cells from a Cx3cr1-cre/ERT2<sup>−/−</sup>;NuTRAP<sup>+</sup> (WT) brain were used to establish...
gating for CD45^eGFP^CD11b^ microglia. B) Gating strategy for Cx3cr1-NuTRAP brain cells stained with CD45-VioBlue and CD11b-APC to sort CD45^eGFP^CD11b^ microglia.
Figure 1-7 Gating strategy for assessment of microglial purity by different sort methods.
Figure 2-2. Gene body coverage plots by sort fraction for sequencing libraries described in Figure 2.
Figure 4-2. Gene body coverage plots by sort fraction for sequencing libraries described in Figure 4.
Figure 6-2. Gene body coverage plots by sort fraction for sequencing libraries described in Figure 6.
Figure 6-3. Gating strategy for assessment of cellularity.
Figure 6. Comparison of cell viability between different cell preparation methods. Following creation of a single cell suspension, cell viability was assessed by flow cytometry. (A-B) Gating strategy to determine viability of all single cells and eGFP+ cells. (C) There was no difference in overall cell viability between the different cell preparation methods. (D) Mechanical dissociation carried out entirely at 4°C (“Dounce”) had slightly lower viability among eGFP+ singlets than the enzymatic and mechanical dissociation (“Cell (+/-) Inhib”). Inclusion of transcription and translation inhibitors did not alter cell viability (One-way ANOVA, Tukey’s post-hoc, **p<0.01). Boxplots represent median, Q1, Q3, minimum, and maximum of each data set.
Figure 7-2. Gene body coverage plots by sort fraction for sequencing libraries described in Figure 7.
Figure 7-3. Hierarchical clustering of differentially expressed genes between Tissue-TRAP, Dounce-TRAP, Cell-TRAP (-) Inhibitors, and Cell-TRAP (+) Inhibitors.
1340 Extended data tables

1341 Figure 1-1. Figure 1 Source Data.

1342 Figure 2-1. Figure 2 Sequencing Metrics and Source Data.

1343 Figure 3-1. Figure 3 Source Data.

1344 Figure 4-1. Figure 4 Sequencing Metrics and Source Data.

1345 Figure 5-1. Figure 5 Source Data.

1346 Figure 6-1. Figure 6 Sequencing Metrics and Source Data.

1347 Figure 7-1. Figure 7 Sequencing Metrics and Source Data.