Minimizing the *ex vivo* confounds of cell-isolation techniques on transcriptomic and translatomic profiles of purified microglia

<https://doi.org/10.1523/ENEURO.0348-21.2022>

Cite as: eNeuro 2022; 10.1523/ENEURO.0348-21.2022

Received: 22 August 2021

Revised: 10 December 2021

Accepted: 25 January 2022

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

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1 **1. Manuscript Title (50 word maximum):**

2 Minimizing the *ex vivo* confounds of cell-isolation techniques on transcriptomic and
3 translatomic profiles of purified microglia

4 **2. Abbreviated Title (50 character maximum):**

5 Microglial activation during cell isolation

6 **3. List all Author Names and Affiliations in order as they would appear in the**
7 **published article**

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16 **4. Author Contributions:** Each author must be identified with at least one of the
17 following: Designed research, Performed research, Contributed unpublished reagents/
18 analytic tools, Analyzed data, Wrote the paper. Example: CS and JS Designed
19 Research; MG and GT Performed Research; JS Wrote the paper

20 S.R.O., A.J.C-E, W.M.F Designed research; S.R.O., K.P., H.E.P., A.M., and A.J.C-E
21 Performed research; S.R.O., K.P., H.E.P., A.M., A.J.C-E, and W.M.F. Analyzed Data;
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29 **6. Number of Figures:** 19

30 **7. Number of Tables:** 8

31 **8. Number of Multimedia:** 0

32 **9. Number of words for Abstract:** 237

33 **10. Number of words for Significance Statement:** 120

34 **11. Number of words for Introduction:** 702

35 **12. Number of words for Discussion:** 1201

36 **13. Acknowledgements**

37 This work was supported by grants from the National Institutes of Health (NIH)
38 P30AG050911, R01AG059430, R56AG067754, T32AG052363, F31AG064861,
39 P30EY021725, Oklahoma Center for Adult Stem Cell Research (OCASCR), a program
40 of the Oklahoma Tobacco Settlement Endowment Trust, BrightFocus Foundation
41 (M2020207), and Presbyterian Health Foundation. This work was also supported in part
42 by the MERIT award I01BX003906 and a Shared Equipment Evaluation Program
43 (ShEEP) award ISIBX004797 from the United States (U.S.) Department of Veterans
44 Affairs, Biomedical Laboratory Research and Development Service. The authors would
45 also like to thank the Clinical Genomics Center (OMRF), Imaging Core Facility (OMRF)
46 and DMEI (OUHSC), and Flow Cytometry and Cell Sorting Core Facility (OMRF) for
47 assistance and instrument usage and Jan Brandel for assistance with illustrations.
48 Some reagents and loan of a cytometer instrument were supplied at no cost by Miltenyi
49 Biotec.

50 **14. Conflict of Interest:** Authors report no conflict of interest.

51 **15. Funding sources**

52 This work was supported by grants from the National Institutes of Health (NIH)
53 P30AG050911, R01AG059430, R56AG067754, T32AG052363, F31AG064861,
54 P30EY021725, Oklahoma Center for Adult Stem Cell Research (OCASCR), a program
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57 by the MERIT award I01BX003906 and a Shared Equipment Evaluation Program
58 (ShEEP) award ISIBX004797 from the United States (U.S.) Department of Veterans
59 Affairs, Biomedical Laboratory Research and Development Service.

60 **Abstract**

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

80 **Significance Statement**

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

92 Introduction

93 Microglia, the brain's resident macrophages, have come to the forefront of neuroimmunology
 94 research (Prinz et al., 2019). They serve as surveyors of the central nervous system and exhibit
 95 behavior derived from their embryonic precursors, myeloid cells (Cuadros and Navascues,
 96 1998; Rock et al., 2004), with roles in neurodevelopment, sex differences, as well as in health
 97 and neurodegenerative diseases (Butovsky and Weiner, 2018; Han et al., 2021; Salter and
 98 Stevens, 2017). Microglial activity is governed by local microenvironments and through
 99 communication with neighboring cells. Under stress, microglial cells transition to an activated
 100 phenotype, classically defined by morphological transformation from ramified to amoeboid,
 101 release of pro-inflammatory cytokines, and a shift in global gene expression (Avignone et al.,
 102 2015; Rock et al., 2004; Sierra et al., 2013). With the advent of single cell transcriptomic
 103 sequencing, the field has undergone a taxonomy reclassification (Dubbelaar et al., 2018;
 104 Provenzano et al., 2021). Current evidence suggests microglia exist within a phenotypic
 105 gradient, and the transition away from a quiescent state is no longer viewed as binary 'on' or
 106 'off'. Thus, the use of microglial gene expression profiles to infer functional status has bolstered
 107 the use of transcriptomic profiling as a powerful technique for microglial classification. To
 108 delineate the full heterogeneity of microglial population with aging and disease using single cell
 109 techniques, it is necessary to minimize activational effects of cell preparation and isolation.

110 Traditionally, molecular analyses from specific CNS cell types required the liberation of cells
 111 from their native environment and use of fluorescence-activated cell sorting (FACS) or MACS
 112 labeling techniques prior to RNA extraction (Cahoy et al., 2008). Cell dissociation primarily
 113 consists of enzymatic and/or mechanical dissociation with quality checks for cell viability, debris
 114 removal, and absence of cell aggregation (Reichard and Asosingh, 2019). Creation of a single-
 115 cell suspension from brain tissue and isolation of microglia is harsh and may alter the
 116 phenotypic state of microglia *ex vivo* (Haimon et al., 2018; Wu et al., 2017). *Ex vivo* microglial
 117 activation has the potential of introducing confounds that may mask endogenously induced
 118 activation, such as in a pathological state. To avoid cell-isolation confounds, microglial-specific
 119 cre-lines (Cx3cr1-cre/ERT2) have been combined with various floxed ribosomal tagging models:
 120 1) ribosome tagging (RiboTag) (Haimon et al., 2018), 2) translating ribosome affinity purification
 121 (TRAP) (Ayata et al., 2018), and 3) nuclear tagging and translating ribosome affinity purification
 122 (NuTRAP) (Chucair-Elliott et al., 2020), allowing the immunoprecipitation (IP) of tagged
 123 polysomes to isolate microglial-specific translatoemes without the need for cell isolation. Although
 124 transgenic ribosome IP-approaches overcome many of the potential confounds of *ex vivo*

125 activation, experimental endpoints such as proteomics and single cell heterogeneity still require
 126 cell isolation of intact microglial cells. Additionally, animal availability, complex breeding
 127 strategies, and cost will continue to be deterrents for many investigators to using transgenic
 128 microglial labeling. While single-cell sequencing allows for broad and potentially unbiased
 129 analysis of various cell types, it too is predicated on the creation of a single-cell suspension.
 130 Thus, an understanding of the effects of cell preparation and isolation methods on *ex vivo*
 131 activation while maintaining highly pure microglial enrichment is needed. The advent of
 132 ribosome tagging approaches allows generation of a reference microglial signature to which
 133 sorted microglial profiles can be compared. The goals of this study were to compare purity and
 134 yield of isolated microglia and assess the relative level of *ex vivo* activation by comparing
 135 Cx3cr1-TRAP-isolated RNA to various sorting techniques used for microglia: MACS (Holt and
 136 Olsen, 2016; Nikodemova and Watters, 2012), cytometer-based FACS (Hickman et al., 2013),
 137 and newly available low-pressure cartridge-based FACS (Roberts et al., 2021) using the TRAP-
 138 enrichment profile as a baseline purified microglial transcriptome for *ex vivo* activation.

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

148

149 **Materials and Methods**

150 **Animals**

151 All animal procedures were approved by the Institutional Animal Care and Use Committee at the
 152 Oklahoma Medical Research Foundation (OMRF). Parent mice were purchased from the
 153 Jackson Laboratory (Bar Harbor, ME) and bred and housed under SPF conditions in a HEPA
 154 barrier environment on a 14/10 light/dark cycle (lights on at 6:00am). Cx3cr1-cre/ERT2^{+/+} males
 155 (stock # 020940) (Yona et al., 2013) were mated with NuTRAP^{flox/flox} females (stock # 029899)
 156 (Roh et al., 2017) to generate the desired progeny, Cx3cr1-cre/ERT2^{+/wt}; NuTRAP^{flox/wt} (Cx3cr1-

157 NuTRAP). In the Cx3cr1-NuTRAP mice, following cre recombination in *Cx3cr1*⁺ cells, deletion of
158 the floxed stop cassette causes activation of the NuTRAP allele, labeling microglial ribosomes
159 with eGFP and nuclei with biotin and mCherry. For flow cytometry experiments, cre negative
160 controls (Cx3cr1-cre/ERT2^{wt/wt} NuTRAP^{flox/wt}) were used to establish eGFP gating. DNA was
161 extracted from mouse ear punch samples for genotyping. Mice were ~3-8 months old at the
162 time of performing experiments. Within each experimental group, both male and female mice
163 were used. The sex of each experimental sample is denoted in **Tables 2-1, 4-1, 6-1, and 7-1**.
164 Mice were euthanized by cervical dislocation followed by rapid decapitation. Genotyping primers
165 were obtained from Integrated DNA Technologies (Coralville, IA) and used for PCR detection of:
166 1) generic cre (Jax protocol 22392, Primers: oIMR1084, oIMR1085, oIMR7338, oIMR7339), 2)
167 Cx3cr1-cre/ERT2 (Jax protocol 27232, Primers: 20669, 21058, 21059), 3) mCherry (Jax
168 protocol 24693, Primers: 9703, 9704, oIMR8744, oIMR8745), and 4) NuTRAP floxed allele (Jax
169 protocol 21509, Primers: 21306, 24493, 32625, 32626) according to protocols published by the
170 Jackson Laboratory, as previously described (Chucair-Elliott et al., 2020).

171 **Tamoxifen (Tam) induction of cre recombinase**

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

179 **Flow cytometry from blood**

180 Blood was collected from the facial vein of Cx3cr1-NuTRAP (3 and 60 days post-tamoxifen
181 induction) and cre negative (WT) controls (n=3-10/group) using a 5-mm sterile Goldenrod
182 animal lancet (MEDpoint, Mineola, NY) and mixed with 10 μ L 0.5 M EDTA, pH 8.0 to avoid
183 coagulation (Chucair-Elliott et al., 2020). Ten volumes of room temperature 1X Red Blood Cell
184 Lysis Solution (#130-094-183, Miltenyi Biotec) were added to one volume of collected blood,
185 vortexed for 5 seconds, and then incubated in the dark at room temperature for 10 minutes.
186 Cells were pelleted at 300 x g for 10 minutes at room temperature and then the supernatant was
187 decanted. The cells were resuspended in 1 mL 0.5% BSA (#130-091-376, Miltenyi Biotec) in 1X
188 D-PBS containing calcium, magnesium, glucose, and pyruvate (#14287-072, Thermo Fisher

Scientific, Waltham, MA) and then washed by centrifugation at 300 x g for 10 minutes at room temperature. The cell pellet was resuspended in 200 μ L 0.5% BSA in 1X D-PBS and split into 50 μ L for antibody staining. Cell aliquots were then stained with: 1) 1 μ L CD11b-APC (M1/70.15.11.5, #130-113-793, Miltenyi Biotec), 2) 1 μ L of CD45-VioBlue (REA737, #130-110-802, Miltenyi Biotec), 3) 1 μ L CD11b-APC and 1 μ 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 4°C. Cells were then washed with 1 mL of 0.5% BSA in 1X D-PBS and then pelleted at 300 x g for 10 minutes at room temperature. The cell pellet was resuspended in 250 μ 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 μ L of papain-based, Enzyme Mix 1. For each reaction, Enzyme Mix 1 was created by combining 50 μ L of Enzyme P with 1900 μ L of Buffer Z, while Enzyme Mix 2 was created by combining 10 μ L of Enzyme A with 20 μ 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 μ L of Enzyme Mix 2 added prior to being mechanically dissociated for 30 minutes at 37°C 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 (4°C) 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 μ 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 μ m MACS SmartStrainer. The cells were then pelleted by centrifugation at 300 x g for 10 minutes at 4°C. 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

222 μ L of cold Debris Removal Solution was mixed into the cell suspensions. Next, 4 mL of D-PBS
 223 was gently overlaid on top of the cell suspension, ensuring the layers did not mix. Centrifugation
 224 at 3,000 x g for 10 minutes at 4°C separated the suspension into three phases, of which the top
 225 two phases were aspirated. The cell pellet was gently resuspended in 15 mL ice-cold D-PBS
 226 before centrifugation at 1,000 x g for 10 minutes at 4°C. After aspirating the supernatant
 227 completely, the cells were resuspended in 1 mL 0.5% BSA (#130-091-376, Miltenyi Biotec) in D-
 228 PBS and filtered through a 35 μ m filter (#352235, Fisher Scientific). An aliquot of 100 μ L cells
 229 was retained as “Cell-Input” for flow cytometric and RNA-Seq analyses.

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

231 As a comparator to the enzymatic and mechanical dissociation, which requires a 30-minute
 232 incubation at elevated temperatures (37°C), single cell suspensions were also created using
 233 mechanical dissociation alone with all steps carried out at 4°C or on ice. The protocol for this
 234 cell preparation was modified from a previously published study (Hammond et al., 2019). Briefly,
 235 halves of Cx3cr1-NuTRAP mouse brains were rinsed in ice-cold 1X HBSS with no calcium, no
 236 magnesium, and no phenol red (diluted 1:10 from #14185-052, ThermoFisher), sliced into 4
 237 sagittal sections and placed into a 7 mL Kimble Dounce homogenizer (#D9063, MilliporeSigma)
 238 containing 5 mL pre-chilled 1X HBSS. The brain tissue was homogenized using 15 strokes of
 239 the loose and tight pestles while simultaneously rotating the pestle and then passed through a
 240 pre-wet 70 μ m cell strainer (#130-095-823, Miltenyi Biotec) placed onto a prechilled 15 mL
 241 conical tube. The glass homogenizer was washed twice with 5 mL of 1X HBSS and the washed
 242 volume was transferred to the 15 mL conical tube containing the brain homogenate. Next, cells
 243 were pelleted at 300 x g for 5 minutes at 4°C. Following centrifugation, the supernatant was
 244 aspirated and the cells were resuspended in 10 mL 40% ice-cold Percoll (#17-0891-02,
 245 MilliporeSigma) in 1X HBSS (4 mL Percoll, 1 mL 10X HBSS, 5 mL RNase-free water). The cell
 246 mixture was spun at 500 x g for 30 minutes at 4°C using full acceleration and braking. The
 247 myelin and Percoll were carefully aspirated, leaving the microglia-containing cell pellet
 248 undisturbed. The cell pellet was then washed in 10 mL of ice-cold HBSS and spun again at 300
 249 x g for 5 minutes at 4°C. After aspirating the supernatant completely, the cells were
 250 resuspended in 1 mL 0.5% BSA (#130-091-376, Miltenyi Biotec) in D-PBS and filtered through a
 251 35 μ m filter (#352235, Fisher Scientific). An aliquot of 200 μ L cells was retained as “Dounce-
 252 Input” for flow cytometry. The remaining cells were pelleted at 300 x g for 10 minutes at 4°C to
 253 process for Translating Ribosome Affinity Purification (TRAP) and RNA-Seq library preparation.

254 **Cell Counting from brain tissue**

255 Filtered cells were diluted 1:10 in 0.5% BSA (#130-091-376, Miltenyi Biotec) in D-PBS prior to
 256 cell counting on a MACSQuant Analyzer 10 (#130-096-343, Miltenyi Biotec). 50 μ L of diluted
 257 cells were analyzed to determine absolute cell count. Cells were gated on FSC-A/SSC-A to
 258 determine cell count and FSC-A/FSC-H to determine singlet count. Absolute cell counts were
 259 used to determine antibody staining ratios.

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

261 Cells from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were pelleted at 300 x g for
 262 10 minutes at 4°C and resuspended in 90 μ L of 0.5% BSA in D-PBS with 10 μ L of CD11b
 263 (Microglia) MicroBeads (#130-093-636, Miltenyi Biotec) per 10^7 total cells. After gently pipetting
 264 to mix, cells were incubated for 15 minutes at 2-8°C protected from light. Cells were washed
 265 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
 266 was resuspended in 500 μ L of 0.5% BSA in D-PBS. After priming the autoMACS Pro Separator
 267 (#130-092-545, Miltenyi Biotec), sample and collection tubes were placed in a cold MACS Chill
 268 5 Rack (#130-092-951, Miltenyi Biotec) with both positive and negative fractions being collected.
 269 The double-positive selection (Posseld) program (ie., positive fraction cells are then run over a
 270 second magnetic column for higher purity) was used to elute highly pure CD11b⁺ cells in 500 μ L
 271 of autoMACS Running Buffer (#130-091-221, Miltenyi Biotec). Following separation, the positive
 272 fraction was reserved for further applications and analysis.

273 **Antibody Labeling for FACS from brain tissue**

274 Cell suspensions from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were pelleted at
 275 300 x g for 10 minutes at 4°C and resuspended in 96 μ L of 0.5% BSA in D-PBS, 2 μ L of CD11b-
 276 APC antibody (M1/70.15.11.5, #130-113-793, Miltenyi Biotec), and 2 μ L of CD45-VioBlue
 277 antibody (REA737, #130-110-802, Miltenyi Biotec). After mixing well, cells were incubated for 10
 278 minutes in the refrigerator (2-8°C) protected from light. Cells were washed with 1 mL of 0.5%
 279 BSA in D-PBS and pelleted at 300 x g for 10 minutes. Cell suspensions from half brains were
 280 processed in parallel for cartridge-based FACS (MACSQuant Tyto) and cytometer-based FACS
 281 (FACSAria).

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

283 Stained cell pellets from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were
 284 resuspended in 10 mL of 0.5% BSA in D-PBS. A MACSQuant Tyto Cartridge (#130-106-088,
 285 Miltenyi Biotec) was primed using 1 mL of 0.5% BSA in D-PBS. The cell suspension was then

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

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

308 Following staining, cells from a single hemisphere of a Cx3cr1-NuTRAP mouse brain were
 309 pelleted and then resuspended in 2 mL of 0.5% BSA in D-PBS for cytometer-based sorting
 310 (FACSAria Illu cell sorter, BD Biosciences). An aliquot of 200 μ L stained cells was saved as
 311 FACS-Input for analysis. A violet (405 nm) laser was used to gate CD45-VioBlue positive cells
 312 using a 450/40 nm filter. A blue (488 nm) laser with 530/30 nm and yellow-green (561 nm) laser
 313 with 610/20 nm filter combinations was used to gate on eGFP⁺ cells without auto-fluorescence
 314 interference. A red (640 nm) laser was used to detect CD11b-APC fluorescence with a 660/20
 315 nm filter. The gating strategy was set to CD11b⁺CD45⁺eGFP⁺ for isolation of microglia (**Figure**
 316 **1-6**). Cells were sorted into 500 μ L of 0.5% BSA in D-PBS. After sorting was completed, an
 317 aliquot (10%) of the positive fraction was analyzed on the MACSQuant Analyzer 10 Flow
 318 Cytometer.

319 **Addition of Inhibitors**

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

335 **Flow Cytometry Analysis from brain tissue**

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

346 To test the effect of cell preparation method on the relative abundance of cell types, aliquots of
 347 cells were stained with: 1) Microglial (CD11b-APC (M1/70, #130-113-793, Miltenyi Biotec) /
 348 CD45-VioBlue (REA737, #130-110-802, Miltenyi Biotec)), 2) Neuronal (CD24-VioBlue (REA743,
 349 #130-110-831, Miltenyi Biotec)), 3) Astrocytic (ACSA2-APC (REA969, #130-116-245, Miltenyi
 350 Biotec)), or 4) Oligodendrocytic (O4-APC (REA576, #130-119-982, Miltenyi Biotec))

351 fluorophore-conjugated antibodies, according to manufacturer's instructions. Cells were washed
 352 and resuspended in 500 μ L and run on the MACSQuant Analyzer 10 Flow Cytometer. Relative
 353 cell proportions with and without transcription/translation inhibitors were assessed (**Figure 6-3**)
 354 using MACSQuantify v2.13.0 software.

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

362 **Translating Ribosome Affinity Purification (TRAP) and RNA extraction**

363 For TRAP from whole tissue, a hemisected half-brain was minced into small pieces and
 364 homogenized in 1.5 mL ice-cold complete homogenization buffer (50 mM Tris, pH 7.4; 12 mM
 365 MgCl₂; 100 mM KCl; 1% NP-40; 1 mg/mL sodium heparin; 1 mM DTT; 100 μ g/mL
 366 cycloheximide (#C4859-1ML, MilliporeSigma); 200 units/mL RNaseOUT Recombinant
 367 Ribonuclease Inhibitor (#10777019; Thermo Fisher Scientific); 0.5 mM Spermidine (#S2626,
 368 MilliporeSigma), 1X complete EDTA-free Protease Inhibitor Cocktail (#11836170001;
 369 MilliporeSigma) with a glass Dounce tissue grinder set (#D8938; MilliporeSigma), as previously
 370 (Chucair-Elliott et al., 2020). For TRAP from cells, a single hemisphere of a Cx3cr1-NuTRAP
 371 mouse brain was used to generate a cell suspension prior to sorting. After pelleting cells at
 372 1,000 x g for 10 min at 4°C, cells were resuspended in 400 μ L of ice-cold complete
 373 homogenization buffer, transferred to a glass Dounce tissue grinder set, and homogenized 15
 374 times with pestle A. Volume was brought up to 1.5 mL with complete homogenization buffer.
 375 Homogenates (from tissue or cells) were transferred to 2 mL round-bottom tubes and
 376 centrifuged at 12,000 x g for 10 minutes at 4°C. After centrifugation, 100 μ L of the supernatant
 377 was saved as TRAP "Input". The remaining supernatant was transferred to a 2 mL round-bottom
 378 tube and incubated with 5 μ g/ μ L of anti-GFP antibody (ab290; Abcam) at 4°C with end-over-end
 379 rotation for one hour. Dynabeads Protein G for Immunoprecipitation (#10003D; Thermo Fisher
 380 Scientific) were washed three times in 1 mL ice-cold low-salt wash buffer (50 mM Tris, pH 7.5;
 381 12 mM MgCl₂; 100 mM KCl; 1% NP-40; 100 μ g/mL cycloheximide; 1 mM DTT). After removal of
 382 the last wash, the homogenate/antibody mixture was transferred to the 2 mL round-bottom tube
 383 containing the washed Protein-G Dynabeads and incubated at 4°C with end-over-end rotation

384 overnight. Magnetic beads were collected using a DynaMag-2 magnet and the unbound-
 385 ribosomes and associated RNA discarded. Beads and GFP-bound polysomes were then
 386 washed three times with 0.5 mL of high-salt wash buffer (50 mM Tris, pH 7.5; 12 mM MgCl₂;
 387 300 mM KCl; 1% NP-40; 100 µg/mL cycloheximide; 2 mM DTT). Following the last wash, 350
 388 µL of Buffer RLT (Qiagen, Germantown, MD) supplemented with 3.5 µL 2-β mercaptoethanol
 389 (#444203, MilliporeSigma) was added directly to the beads and incubated with mixing on a
 390 ThermoMixer (Eppendorf) for 10 minutes at room temperature. The beads were magnetically
 391 separated and the supernatant containing the target bead-bound polysomes and associated
 392 RNA was transferred to a new tube. 350 µL of 100% ethanol was added to the tube ("TRAP"
 393 fraction: enriched in transcriptome associated to eGFP-tagged ribosomes) and then loaded onto
 394 a RNeasy MinElute column (Qiagen). RNA was isolated using RNeasy Mini Kit (#74104,
 395 Qiagen), according to manufacturer's instructions. RNA was quantified with a Nanodrop One^c
 396 spectrophotometer (#ND-ONEC-W, Thermo Fisher Scientific) and its quality assessed by
 397 HSRNA ScreenTape (#5067-5579, Agilent Technologies) with a 4150 TapeStation analyzer
 398 (#G2992AA, Agilent Technologies).

399 **Library construction and RNA sequencing (RNA-seq)**

400 Directional RNA-Seq libraries were made according to the manufacture's protocol from 2-100 ng
 401 RNA. TRAP input and positive fraction RNA from all samples were used to create individual
 402 RNA-Seq libraries (no pooling of samples was performed). Briefly, poly-adenylated RNA was
 403 captured using NEBNext Poly(A) mRNA Magnetic Isolation Module (#NEBE7490L; New
 404 England Biolabs, Ipswich, MA). Following mRNA capture, mRNA was eluted from the oligo-dT
 405 beads and fragmented by incubating with the First Strand Synthesis Reaction Buffer and
 406 Random Primer Mix (2X) from the NEBNext Ultra II Directional Library Prep Kit for Illumina
 407 (#NEBE7760L; New England Biolabs) for 15 minutes at 94°C. First and second strand cDNA
 408 synthesis were performed sequentially, as instructed by the manufacturer's guidelines. After
 409 purification of double-stranded cDNA with 1.8X SPRISelect Beads (#B23318, Beckman
 410 Coulter), purified cDNA was eluted in 50 µL 0.1X TE Buffer and subjected to end prep. The
 411 NEBNext Adaptor was diluted 1:100 in Adaptor Dilution Buffer (provided) before ligating the
 412 adaptor to the cDNA. After purifying the ligation reaction with 0.9X SPRISelect Beads (#B23318,
 413 Beckman Coulter), cDNA was eluted in 15 µL of 0.1X TE (provided). Next, cDNA libraries were
 414 amplified with 16 cycles of PCR using the NEBNext Ultra II Q5 Master Mix (provided) and
 415 unique index primer mixes from NEBNext Multiplex Oligos for Illumina Library (#E6609L, New
 416 England Biolabs). Libraries were purified with 0.9X SPRISelect Beads (#B23318, Beckman

417 Coulter) and then sized with HSD1000 ScreenTapes (#5067-5584; Agilent Technologies).
418 Libraries had an average peak size of 316 bp. Libraries were quantified by Qubit 1X dsDNA HS
419 Assay kit (#Q33230, Thermo Fisher Scientific). The libraries for each sample were pooled at 4
420 nM concentration and sequenced using an Illumina NovaSeq 6000 system (SP PE50bp, S4
421 PE150). The entirety of the sequencing data is available for download in FASTQ format from
422 NCBI Gene Expression Omnibus (to be included on acceptance).

423 **RNA-Seq Data Analysis**

424 Following sequencing, reads were trimmed and aligned prior to differential expression statistics
425 and correlation analyses in Strand NGS software package (v4.0) (Strand Life Sciences). Reads
426 were aligned against the full mm10 genome build (2014.11.26). Alignment and filtering criteria
427 included: adapter trimming, fixed 2bp trim from 5' and 2bp from 3' ends, a maximum number of
428 one novel splice allowed per read, a minimum of 90% identity with the reference sequence, a
429 maximum 5% gap, and trimming of 3' end with Q<30. Alignment was performed directionally
430 with Read 1 aligned in reverse and Read 2 in forward orientation. All duplicate reads were then
431 removed. Normalization was performed with the DESeq2 algorithm. Transcripts with an average
432 read count value >5 in at least 100% of the samples in at least one group were considered
433 expressed at a level sufficient for quantitation per tissue and those transcripts below this level
434 were considered not detected/not expressed and excluded, as these low levels of reads are
435 close to background and are highly variable. For statistical analysis of differential expression, a
436 one-way ANOVA or two-way ANOVA with the factors of TRAP fraction and treatment and a
437 Benjamini-Hochberg Multiple Testing Correction followed by Student-Newman Keuls post hoc
438 test were used (FDR<0.1). For those transcripts meeting this statistical criterion, a fold change
439 >|2| cutoff was used to eliminate those genes which were statistically significant but unlikely to
440 be biologically significant and orthogonally confirmable due to their very small magnitude of
441 change. Visualizations of hierarchical clustering and principal component analyses were
442 performed in Strand NGS (Version 4.0, Bangalore, India). Cell type specific marker gene lists
443 were generated from the re-analysis published by McKenzie et al. (McKenzie et al., 2018) of
444 immunopurified and high throughput single cell data from mice. Over-representation analysis
445 (ORA) was conducted using WEB-based Gene SeT AnaLysis Toolkit (WebGestalt,
446 www.webgestalt.org) (Liao et al., 2019). Top over-represented biological processes were
447 selected from gene ontology functional database with no redundant option selected
448 (Hypergeometric test, BHMT, FDR<0.05) and background reference gene list of all expressed
449 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, BHMT, 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* activation 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* activation transcripts”. Gene set enrichment analysis (GSEA) was run using GSEA v4.1.0 (Mootha et al., 2003; Subramanian et al., 2005).

Immunocytochemistry 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 μ m-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 μ m 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

514 across the gene body. We first examined enrichment/depletion of previously published
515 microglial, astrocytic, oligodendrocytic, neuronal, and endothelial markers in the transcriptomic
516 profiles (Chucair-Elliott et al., 2020; McKenzie et al., 2018) (**Figure 2-1**). Each of the four sort
517 methods showed similar levels of enrichment of microglial marker genes (**Figure 2A**) and
518 depletion of astrocytic, oligodendrocytic, neuronal, and endothelial marker genes (**Figure 2B-E**)
519 when compared to Cell-Input. In combination with the flow cytometric data presented in **Figure**
520 **1**, this gives confidence that each of the sort methods are effective in isolating highly pure
521 populations of microglia.

522 Next, we examined the transcriptomic data in an unbiased manner. Principal component
523 analysis (PCA) of all expressed genes (>5 counts in all samples from at least one group)
524 showed clear separation of Cell-Input from all sort methods in the first component with 81% of
525 the explained variance (**Figure 2F**). Differentially expressed genes were called by One-way
526 ANOVA with Benjamini-Hochberg multiple testing correction (BHMTc) followed by Student-
527 Newman Keuls post hoc ($FDR < 0.1$, $|FC| > 2$; **Figure 2-1**). Hierarchical clustering of the 7,378
528 DEGs shows separation of Cell-Input from all sort methods with similar patterning of enrichment
529 and depletion of DEGs across all sort methods scaled to Cell-Input (**Figure 2G**). The majority of
530 pairwise DEGs (sort method v Cell-Input) were in common between all sort methods
531 ($7,084/7,378 = 96\%$) (**Figure 2H**), suggesting a high degree of similarity between each of the
532 sort methods. In addition, 5322 DEGs (72%) that were down and 1,759 DEGs (24%) were up in
533 all sort methods compared to Cell-Input. There were only 297 discordant DEGs (4%) between
534 the different sort methods as compared to Cell-Input (**Figure 2I**).

535 Over-representation analysis of gene ontology (ORA GO) of the 1,759 genes that were up
536 across all sort methods identified 177 over-represented biological processes pathways
537 (BHMTc, $FDR < 0.05$; **Figure 2-1**). The “Enrichment ratio” is the number of observed genes
538 divided by the number of expected genes from the given gene list. Examination of the top 10
539 over-represented biological processes reveals several pathways involved in microglial function,
540 including: cytokine-mediated signaling, immune response activation, and adaptive immune
541 response, among others (**Figure 2J**). Running a similar ORA GO on the 5,322 genes down
542 across all sort methods (compared to Cell-Input) revealed 252 over-represented biological
543 processes (BHMTc, $FDR < 0.05$; **Figure 2-1**). The top 10 processes include many neuron-
544 focused pathways, such as: neurotransmitter transport, neurotransmitter level regulation, and
545 membrane potential regulation (**Figure 2K**), indicating depletion of these genes in the sorted
546 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 1759 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 BNCRSTTTCANTTYY.

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-

579 NuTRAP mice is evident as CX3CR1⁺ cells are rapidly turned over following tamoxifen
580 induction. To demonstrate the loss of eGFP⁺ in circulating CX3CR1⁺ cells, we collected blood
581 from Cx3cr1-NuTRAP mice 3- and 60- days post-cessation of tamoxifen administration and
582 compared the levels of CD11b⁺CD45⁺ cells expressing eGFP to that of cre negative (WT)
583 controls. There was a marked increase in CD11b⁺CD45⁺eGFP⁺ cells 3 days post-tamoxifen
584 induction, which decreased to levels indistinguishable from WT controls after 60 days (**Figure 1-**
585 **3**). As such, brains were collected from Cx3cr1-NuTRAP mice at least 60 days following
586 tamoxifen treatment. In studies without transgenic labeling, brains can be perfused with ice-cold
587 physiological saline to eliminate contaminating cells from circulation.

588 **Assessment of relative levels of non-microglial macrophages within different sort** 589 **fractions from Cx3cr1-NuTRAP brains.**

590 Perivascular, choroid plexus, and meningeal macrophages also express *Cx3cr1* (Faraco et al.,
591 2017), and thus are labeled by the NuTRAP allele (including eGFP-labelled polysomes) upon
592 tamoxifen-mediated cre recombination. CD45 expression levels are often used as a FACS sort
593 criteria to separate microglia (CD45^{Mid}) from non-microglial macrophages (CD45^{High}) (Haimon et
594 al., 2018; Marschallinger et al., 2020; Pan and Wan, 2020). The relative levels of non-microglial,
595 macrophage contamination was first assessed by flow cytometry. Cells from each group shown
596 in Figure 1A were stained with CD11b and CD45 fluorescent antibodies and analyzed on a
597 MACSQuant Analyzer 10. The percent CD45^{Mid} and CD45^{High} of CD11b⁺CD45⁺ singlets were
598 assessed according to the gating strategy displayed in **Figure 1-4A**. The Cell-Input fraction had
599 the highest (16%) CD45^{High} population of CD11b⁺CD45⁺ cells, as compared to the autoMACS
600 (8%), autoMACS to Tyto (5%), Tyto (4%), and FACSARIA (6%) (**Figure 1-4B**). There was not a
601 significant difference in the proportion of CD45^{Mid} or CD45^{High} (out of CD11b⁺CD45⁺) cells by
602 sort method. The vast majority (>95%) of CD45^{Mid} cell populations co-expressed eGFP
603 regardless of sort method. In contrast, only ~74% of CD45^{High} cells from the Cell-Input and
604 autoMACS sort fraction co-expressed eGFP. The remaining 26% eGFP⁻CD45^{High} cells are likely
605 contaminating cells from blood circulation. Since the FACS-based methods used eGFP as a
606 sorting criteria, the CD45^{High} cells co-expressing eGFP in the cartridge-based Tyto (96%) and
607 cytometer-based FACSARIA (99%) were higher than the Cell-Input (**Figure 1-4C**).

608 Many recent studies have examined differences in the transcriptomic signatures of microglia
609 and non-microglial brain resident macrophages. To further refine our microglial marker list
610 constructed based off a meta-analysis of cell sorting studies (McKenzie et al., 2018), we
611 compared previously published microglial marker lists from cell sorting (Chucair-Elliott et al.,

2020), 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 *Em*) (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

645 enrichment scores were similar between the different sort methods (**Figure 3G, Figure 3-1**).
646 Based on the flow cytometric and transcriptomic data, all sort methods have high levels of
647 microglial purity and low levels of CAM and monocyte contamination.

648 **Comparison of TRAP-isolated microglial transcriptome from tissue homogenate, cell** 649 **suspension, and various microglial sort methods.**

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

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

674 In recent years, several studies have suggested that cell-isolation methods cause *ex vivo*
675 activational effects in microglia (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020). In

676 this section, our goal was to determine if different sorting techniques result in different levels of
 677 *ex vivo* activation. We used Tissue-TRAP as an “unactivated” microglial reference group, since
 678 the Tissue-TRAP method does not rely on the creation of a cell suspension or cell sorting
 679 techniques. There were 8076 DEGs between the translomes when Cell- and Sort-TRAP
 680 methods were compared to the Tissue-TRAP reference (One-way ANOVA, BHMT, SNK
 681 $FDR < 0.1$, $|FC| > 2$). Upset plot of the 8076 DEGs shows the majority of the DEGs
 682 ($7800/8076 = 97\%$) are in common between all groups (Cell-/Sort-TRAP v. Tissue-TRAP)
 683 (**Figure 5A, Figure 5-1**). Hierarchical clustering of all 8076 DEGs shows distinct clustering of
 684 Tissue-TRAP from all other groups. Cell-TRAP also clusters separately from the Sort-TRAP
 685 groups (**Figure 5B, Figure 5-1**). Comparison of DEGs from each group (Cell- and Sort-TRAP)
 686 v. Tissue-TRAP, revealed 5337 common DEGs (67%) that were depleted and 2329 common
 687 DEGs (29%) that were enriched in all groups (Cell- and Sort-TRAP) compared to Tissue-TRAP.
 688 There were only 352 discordant DEGs (4%) between the different sort methods as compared to
 689 Cell-Input (**Figure 5C**). These data, together with cell-type enrichments from **Figure 4D-H**,
 690 suggests that the act of creating a cell suspension is the largest contributor to differences seen
 691 between Tissue-TRAP and Sort-TRAP methods.

692 Next, we identified over-represented pathways among the up-regulated genes compared to
 693 Tissue-TRAP. The top 10 biological processes of the 2329 genes up-regulated in comparison to
 694 Tissue-TRAP were microglial-related pathways, including: myeloid leukocyte activation, cell
 695 activation involved in immune response, and leukocyte-mediated immunity (**Figure 5D; Figure**
 696 **5-1**). Comparing the biological processes identified in the transcriptomic analysis (**Figure 2J;**
 697 **Figure 2-1**) and the translomic analysis (**Figure 5D; Figure 5-1**) revealed 55 biological
 698 processes that were only up-regulated in the translome (**Figure 5E**). Several of the biological
 699 processes uniquely upregulated in the translome comparisons were involved in microglial
 700 activation pathways, including: response to type I interferon (IFN), response to transforming
 701 growth factor beta (TGFB), response to interleukin-6 (IL-6), and NIK/NF-kappaB signaling
 702 (NFKB) (**Figure 5F**). IL-6 is a pro-inflammatory cytokine extensively studied in brain aging and
 703 disease (Borovcanin et al., 2017; Singh-Manoux et al., 2014; Ye and Johnson, 1999). Microglia
 704 have higher IL-6 receptor (IL-6R) expression than any other cell type. As such, microglia are
 705 highly responsive to IL-6 and transition into a “primed” state when exposed to high levels of IL-6.
 706 Hierarchical clustering of the “Response to IL-6” pathway genes showed overall higher levels of
 707 expression in Cell- and Sort-TRAP groups (**Figure 5G; Figure 5-1**). The Cell-TRAP did not

708 cluster separately from the Sort-TRAP groups, providing further evidence that the *ex vivo*
709 activational signature is a function of cell preparation.

710 Next, we looked at gene expression of common cytokines (**Figure 5H; Figure 5-1**) and
711 chemokines (**Figure 5I; Figure 5-1**) across Tissue-, Cell-, and Sort- TRAP groups. We
712 observed higher levels of cytokine and chemokine transcripts across all Cell- and Sort-TRAP
713 groups when compared to Tissue-TRAP (One-way ANOVA, BHMTTC, SNK FDR<0.1, |FC|>2). In
714 an effort to cross-validate our finding with previous studies, we intersected *ex vivo* microglial
715 activational gene lists from three previous studies (Ayata et al., 2018; Haimon et al., 2018;
716 Marsh et al., 2020) and identified 21 *ex vivo* activational transcripts represented in at least two
717 of the studies (**Figure 5J; Figure 5-1**). PCA of the TRAP data from the present study on the 21
718 *ex vivo* activational genes shows clear separation of Tissue-TRAP from all other groups in the
719 first component (92.8% explained variance) (**Figure 5K**). Again, suggesting that the *ex vivo*
720 signature is a function of cell preparation. Hierarchical clustering of the 21 activational genes,
721 shows similar patterning as in the “Response to IL-6” pathway with higher levels of expression
722 across Cell- and Sort-TRAP groups compared to Tissue-TRAP (**Figure 5L; Figure 5-1**). *Zfp36*
723 was one of the *ex vivo* activational genes identified in all three previous studies (Ayata et al.,
724 2018; Haimon et al., 2018; Marsh et al., 2020). Zinc finger protein 36 (*Zfp36*) encodes for the
725 protein Tristetraprolin (TTP) which is involved in regulating immune responses through mRNA
726 destabilizing and alternative splicing (Tu et al., 2019). *Zfp36* is enriched in Cell- and Sort-TRAP
727 compared to Tissue TRAP (One-Way ANOVA, Tukey’s posthoc, ***p<0.001) (**Figure 5M;**
728 **Figure 5-1**).

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

732 **Comparison of cellularity and microglial yield between different cell preparation** 733 **methods.**

734 Since *ex vivo* activation appears to be an artifact of cell preparation, three different methods
735 were compared for changes in cellularity within the cell preparation. The experimental design is
736 displayed in **Figure 6A**. Tissue-Input, generated by immediately Dounce homogenizing tissue in
737 TRAP lysis buffer, was used as a comparator to the three methods of cell preparation. The first
738 cell preparation method was a mechanical dissociation carried out entirely at 4°C followed by a

739 40% Percoll gradient to removed undissociated tissue and myelin debris (Dounce-Input). The
740 second cell preparation method used a papain-based enzyme mixture which requires a brief (30
741 minute) incubation at 37°C followed by myelin debris removal (Cell-Input (-) Inhibitors) which
742 was the method of cell preparation used from **Figure 1** to **Figure 5**. The third cell preparation
743 method used the same papain-based enzyme mixture, this time supplemented with
744 transcriptional and translation inhibitors (Marsh et al., 2020; Wu et al., 2017) with incubation at
745 37°C followed by myelin debris removal (Cell-Input (+) Inhibitors). Cells from each of the three
746 methods were pelleted and lysed with TRAP lysis buffer prior to RNA extraction and
747 assessment of the transcriptome. RNA yield and quality by sort method, in addition to basic
748 sequencing metrics are given in **Figure 6-1**. The average RNA yield was higher in the Tissue-
749 Input (5.8 µg/half brain) than the Cell-Input (+/-) Inhibitors (360 ng/half brain) and Dounce-Input
750 (35 ng/half brain) (One-way ANOVA, Tukey's *post hoc*, $p < 0.05$). The average RNA Integrity
751 Number (RIN) was lowest in the Dounce-Input (3.4) when compared to the Tissue-Input (7.8)
752 and Cell-Input (+/-) Inhibitors (7.3). In addition, use of an entire Cx3cr1-NuTRAP brain was
753 needed for RNA isolation from the Dounce-Input group, whereas half a brain was used for all
754 other methods. Gene body coverage plots by experimental group are shown in **Figure 6-2**. The
755 Tissue-Input and Dounce-Input groups showed more 3' bias in their gene body coverage than
756 the Cell-Input (+/-) Inhibitor groups.

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

768 Stranded RNA-Seq libraries were constructed from each of the groups displayed in **Figure 6A**.
769 Heatmaps of microglial, astrocyte, oligodendrocytic, neuronal, and endothelial cell marker lists
770 for each of the cell preparation methods were normalized to Tissue-Input. All three cell
771 preparation methods appear to be enriched for microglial transcripts as compared to Tissue-

772 Input, with the strongest enrichment in the Cell-Input (+/-) Inhibitors. The Cell-Input (+/-)
 773 Inhibitors also appear to have greater enrichment of astrocytic and endothelial markers and
 774 depletion of neuronal transcripts when compared to Tissue- and Dounce-Input (**Figure 6C**,
 775 **Figure 6-1**). CIBERSORTx, or digital cytometry, estimates cell-type abundance from bulk
 776 transcriptomics. Using publicly available cell-type specific data from mouse brain (Zhang et al.,
 777 2014) (GSE52564) as a digital cytometry reference matrix, we estimated the cellularity from
 778 each of the four groups (Zhang et al., 2014). Based on this analysis, there was no difference in
 779 the cellularity between the Tissue-Input and Dounce-Input or between the Cell-Input (-)
 780 Inhibitors and the Cell-Input (+) Inhibitors. However, there were more astrocytic,
 781 oligodendrocytic, microglial, and endothelial transcripts in the Cell-Input (+/-) Inhibitors than the
 782 Tissue- and Dounce-Input. Further, there were less neuronal transcripts in the Cell-Input (+/-)
 783 Inhibitors than the Tissue- and Dounce-Input (Two-Way ANOVA, Tukey's *post hoc*) (**Figure 6D**,
 784 **Figure 6-1**). These CIBERSORTx results are consistent with the flow cytometric data displayed
 785 in **Figure 6B** and suggest that the enzymatic and mechanical dissociation carried out at partially
 786 elevated temperatures depletes neurons from the cell preparation.

787 Lastly, we compared the cell yield per half brain between the various cell preparation
 788 techniques. The average total cell count was slightly higher in the Dounce-Input ($\sim 3.7 \times 10^6$
 789 cells/half brain) when compared to Cell-Input (+/-) Inhibitors ($\sim 1.7 \times 10^6$ cells/half brain) (**Figure**
 790 **6-1**). However, due to the increased proportion of microglia in the Cell-Input (+/-) Inhibitors
 791 compared to the Dounce-Input, there were ~ 20 times more eGFP⁺ microglia in the Cell-Input
 792 (+/-) Inhibitors compared to the Dounce-Input (**Figure 6E**, **Figure 6-1**). Thus, the enzymatic and
 793 mechanical dissociation carried out at partially elevated temperatures will minimize the need for
 794 pooling samples when using small tissue sections for microglial sorting applications.

795 **Effect of different cell preparation techniques on *ex vivo* activation profiles following** 796 **cell preparation.**

797 Recent studies have suggested that inclusion of transcriptional and/or translational inhibitors
 798 during cell preparation can prevent *ex vivo* activation confounds in microglia (Marsh et al.,
 799 2020) and other neuronal cell types (Wu et al., 2017). Additionally, *ex vivo* artifacts can be
 800 prevented by mechanical dissociation carried out entirely at low temperatures (Marsh et al.,
 801 2020). To compare the relative levels of *ex vivo* activation between various cell preparation
 802 methods, we TRAP-isolated RNA from Tissue, Dounce, and Cell (+/-) Inhibitor cell preparations
 803 as shown in **Figure 7A**. Stranded RNA-Seq libraries were constructed from TRAP-isolated
 804 translating RNA from each of the four groups. RNA yield and quality by sort method, in addition

805 to basic sequencing metrics, are given in **Figure 7-1**. Gene body coverage plots by
 806 experimental group are shown in **Figure 7-2**. The Tissue-TRAP and Dounce-TRAP groups
 807 showed more 3' bias in their gene body coverage than the Cell-TRAP (+/-) Inhibitor groups.

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

830 In order to study *in vivo* pathophysiological microglial responses, it is necessary to reduce the
 831 *ex vivo* confounds of cell preparation. For example, with aging and disease microglia increase
 832 production of inflammatory cytokines, chemokines, and reactive oxygen species (Von Bernhardi
 833 et al., 2015). Response to oxidative stress and reactive oxygen species were also identified as
 834 pathways significantly over-represented in the 85 prevented *ex vivo* activational transcripts.
 835 Induction of the response to oxidative stress pathway (**Figure 7E, Figure 7-1**) and generation of
 836 common chemokines (**Figure 7F, Figure 7-1**) were prevented in the Dounce-TRAP and Cell-
 837 TRAP (+) Inhibitors as compared to the Cell-TRAP (-) Inhibitors group.

838 Another active area of research in glial biology is that of cellular senescence, characterized by
 839 cell-cycle arrest and secretion of pro-inflammatory cytokines and chemokines (Hou et al., 2021;
 840 Hu et al., 2021). Cell preparation method altered expression of cellular senescence-related
 841 genes identified from a previously published study (Aird et al., 2016). Inclusion of transcription
 842 and translation inhibitors during cell preparation reduced the induction of these senescence-
 843 associated genes (**Figure 7G, Figure 7-1**).

844 Heatmap of the 21 *ex vivo* activational transcripts identified in at least two of the three examined
 845 previous studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020) from **Figure 5J**
 846 shows prevention of *ex vivo* activation with the addition of transcriptional and translational
 847 inhibitors or mechanical dissociation carried out entirely at low temperatures (**Figure 7H, Figure**
 848 **7-1**). PCA of the 21 *ex vivo* activational transcripts identified shows strong separation of Cell-
 849 TRAP (-) Inhibitors from all other groups in the first component (86.1% explained variance) and
 850 clustering of Dounce-TRAP and Cell-TRAP (+) Inhibitors together (**Figure 7I**).

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

854 Discussion

855 Microglia have emerged as key players in brain disease, including age-related
 856 neuroinflammation and neurodegeneration (Colonna and Butovsky, 2017; Lana et al., 2021). As
 857 a minority cell population in the brain, in depth microglial molecular and biochemical analyses
 858 benefit from enrichment strategies to provide cell-specific data (Okaty et al., 2011). While *in vitro*
 859 cell culture models are useful for mechanistic studies, they fail to recapitulate the complexity of
 860 the nervous system milieu. As such, models and methods to 'debulk' microglia from brain tissue
 861 have become a focus in the field (Chucair-Elliott et al., 2020; McKinsey et al., 2020). The most
 862 common methods for isolating microglia include enzymatic and/or mechanical dissociation of
 863 brain tissue followed by immunolabeling with magnetic beads for MACS (Bordt et al., 2020) or
 864 fluorescent-conjugated antibodies for FACS-based enrichment (Bohlen et al., 2019). As these
 865 methods continue to evolve, quantitative comparisons of cell preparation and sort strategies are
 866 needed to aid decision making of what approaches to use in specific studies. As well, there are
 867 legitimate concerns that these isolation approaches introduce artifacts, especially in glial cell
 868 populations which by nature are sensitive to changes in their microenvironment (Wu et al.,
 869 2017). Determining the degree of *ex vivo* activational artifacts, and how they may vary between
 870 isolation approaches, has been challenging because the field lacked a resting cell type-specific

871 reference (absent of *ex vivo* activational confounds related to enzymatic/mechanical
872 dissociation) as a comparator.

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

884 Cartridge-based FACS is a new iteration of FACS and produced nearly equivalent cell purities
885 as traditional cytometry-based FACS but with greater cell yield. Given the higher cell yield,
886 cartridge-based FACS may be beneficial for applications requiring large amounts of starting
887 material, including ChIP-seq, HiC-based techniques, proteomics, and primary microglial
888 cultures. Another advantage of this cartridge-based approach is the self-contained nature of the
889 system that does not produce aerosols thus not requiring biosafety containment (Roberts et al.,
890 2021). Cytometry-based FACS produced the purest cell population and has the highest
891 capabilities for dimensions of labeling. However, this approach is also the slowest and had the
892 lowest cellular yield. A strict comparison of FACS methods relies on details of the gating
893 strategies used and these can likely be tuned to emphasize highest purity or cell yield.
894 Cytometer-based FACS easily incorporates various staining controls, which allows for detailed
895 refinement of gating without additional cost. For cartridge-based FACS, separate cartridges are
896 needed for staining controls, increasing the cost. Additionally, the cartridge-FACS does not have
897 traditional forward scatter detection, but instead utilizes back scatter, making it difficult to gate
898 cell populations from debris. Validation of cartridge-FACS sorted cells should be performed on a
899 traditional cytometer as a control.

900 Combining MACS with cartridge-based FACS, to initially de-bulk microglia and then further
901 purify, did not result in higher purity of microglia and the presence of the magnetic beads shifted
902 signals in the FACS. Taken together, these data demonstrate that all of these approaches are

903 valid for microglial isolation from brain and return highly pure cell populations that are suitable
904 for molecular and biochemical analyses.

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

917 Using TRAP isolation of the microglial transcriptome as a baseline measure of microglia, induction
918 of *ex vivo* activation pathways occurred during enzymatic and mechanical cell preparation and
919 was sustained during microglial isolation, independent of sort method. Compared to previous
920 studies of these activation artifacts (Ayata et al., 2018; Haimon et al., 2018; Marsh et al.,
921 2020) we found a common set of activation markers centered on immediate early genes. This
922 unified set of markers can be used in future studies as markers of artifactual microglial cell
923 activation. More broadly the use of ribosomal tagging methodologies to provide a comparator for
924 optimizing cell preparation could be applied to any relevant organ.

925 The use of transcriptional and translational inhibitors (Marsh et al., 2020; Wu et al., 2017) during
926 the cell preparation was investigated in the context of acutely isolating cells for immediate use in
927 downstream molecular and biochemical analyses. Addition of transcriptional and translational
928 inhibitors blocked much of the *ex vivo* activation artifacts without otherwise changing the cell
929 phenotype. Whether this is a valid approach for cells that will subsequently be cultured was not
930 assessed. Nonetheless, as studies also delve into microglial heterogeneity at the single cell
931 level (T. Masuda et al., 2020; Stratoulis et al., 2019) inclusion of inhibitors in the preparation
932 and enrichment of microglia can reduce artifacts in these single cell studies as well (Marsh et
933 al., 2020). Application of transcriptional and translational inhibitors in the context of specific CNS
934 pathologies should be validated on a case-by-case basis, potentially using the same
935 approaches presented here. As an alternative to enzymatic and mechanical dissociation,

936 mechanical dissociation carried out entirely at 4°C also avoids *ex vivo* activation of microglia.
937 However, the cell/RNA yields and RNA integrity associated with mechanical dissociation was
938 much lower than that obtained with enzymatic and mechanical dissociation.

939 Collectively, our data demonstrate that a variety of microglial isolation methods can be used
940 with equivalent results and tuned to the needs of the specific study. In addition, activational
941 artifacts occur during cell preparation and can be prevented by inclusion of specific inhibitors
942 early in the cell preparation protocol or by avoiding high temperatures. The different cell sorting
943 methods did not show additional activational effects or differences between methods, indicating
944 that concerns over artifacts should not drive isolation method selection. Addition of
945 transcriptional and translational inhibitors during cell preparation reduces *ex vivo* artifacts and is
946 an easily implementable approach to avoid potential confounds.

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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 CD45^{mid} and CD45^{high} 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**), FACS Aria sort

1120 (Figure 1-6), and flow cytometry (Figure 1-7) are given as supplements. **C-D**) All sort positive
1121 fractions were enriched for: (C) eGFP⁺ singlets and (D) CD11b⁺CD45⁺ singlets (as compared to
1122 input) (Two-Way ANOVA, Main effect of TRAP Fraction, *** $p < 0.001$)(Figure 1-1). When
1123 comparing positive fractions, the autoMACS positive fraction had lower % eGFP⁺ singlets as
1124 compared to all other sort methods. FACSARIA had higher percentage of eGFP⁺ singlets than all
1125 other sort methods. FACSARIA had higher percentage of CD11b⁺CD45⁺ singlets as compared to
1126 all other sort methods positive fractions (Two-way ANOVA, Tukey's post-hoc, * $p < 0.05$). **E**)
1127 Microglial yield was significantly higher in the MACSQuant Tyto positive fraction as compared to
1128 the autoMACS to MACSQuant Tyto and FACSARIA positive fractions (One-Way ANOVA,
1129 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

1137 and depletion of: **(B)** astrocytic, **(C)** oligodendrocytic, **(D)** neuronal, and **(E)** endothelial
1138 transcripts when compared to Cell-Input. **F)** Principal component analysis of all expressed
1139 genes shows clear separation of Cell-Input from all other sort methods in the first component
1140 with 81% of explained variance. **G)** Hierarchical clustering of differentially expressed genes
1141 (DEGs) (One-Way ANOVA, BHMTTC, SNK FDR<0.1, |FC|>2) shows separation of Cell-Input and
1142 sort methods. Each of the sort methods show very similar patterning of expression across
1143 DEGs. **H)** Comparison of SNK post-hocs from each of the sort methods v. Cell-Input, showed
1144 the majority of enrichments/depletions (ie. DEGs) (7084/7378 = 96%) were in common between
1145 all sort methods. **I)** There were 5322 DEGs (72%) that were depleted and 1759 DEGs (24%)
1146 that were enriched in all sort methods compared to Cell-Input. There were only 297 discordant
1147 DEGs (4%) between the different sort methods as compared to Cell-Input. **J)** Top 10 biological
1148 processes over-represented in the 1759 genes upregulated in all sort methods compared to
1149 Cell-Input (Gene Ontology Over-Representation Analysis, BHMTTC FDR <0.05). **K)** Top 10
1150 biological processes over-represented in the 5322 genes downregulated in all sort methods
1151 compared to Cell-Input (Gene Ontology Over-Representation Analysis, BHMTTC FDR <0.05). **L)**
1152 Top 5 transcription factor targets over-represented in the 1759 genes upregulated in all sort
1153 methods compared to Cell-Input (Transcription factor target network over-representation
1154 analysis, BHMTTC FDR<0.05). **M)** Top 5 transcription factor targets over-represented in the 5322
1155 genes downregulated in all sort methods compared to Cell-Input (Transcription factor target
1156 network over-representation analysis, BHMTTC 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.

1167 The union of all macrophage markers (174 genes, green) was used for further analysis (**Figure**
 1168 **3-1**). **C)** RNA-Seq gene expression of brain-resident macrophage marker genes from: 1)
 1169 microglia (*Hexb*, *Tmem119*, *P2ry12*, *Siglech*) and 2) non-microglia macrophages (*Lyve1*, *Ly6c1*,
 1170 *CD163*, *Mrc1*) from each sorting method (AutoMACS, AutoMACS to Tyto, Tyto, and FACSARIA)
 1171 were compared to Cell-Input. **D)** RNA-Seq gene expression of peripheral macrophage marker
 1172 genes from: 1) infiltrating macrophages (*Emp1*, *Scarf1*, *Emb*, *Clec4e*) and 2) peripheral
 1173 macrophages/monocytes (*Emilin1*, *F5*, *Fn1*, *Gda*) from each sorting method (AutoMACS,
 1174 AutoMACS to Tyto, Tyto, and FACSARIA) were compared to Cell-Input (One-Way ANOVA,
 1175 Tukey's post hoc, * $p < 0.05$). All bars represent the mean normalized gene expression $\pm$ SEM. **E)**
 1176 Gene set enrichment analysis (GSEA) of the 113 microglial marker genes identified in Figure 3A
 1177 comparing all sort positive fractions (AutoMACS, AutoMACS to Tyto, Tyto, and FACSARIA) to
 1178 Cell-Input. **F)** Gene set enrichment analysis (GSEA) of the 174 macrophage marker genes
 1179 identified in Figure 3B comparing all sort positive fractions (AutoMACS, AutoMACS to Tyto,
 1180 Tyto, and FACSARIA) to Cell-Input. **G)** GSEA enrichment scores compared to Cell-Input for each
 1181 of the sorting methods for the microglial marker genes identified in Figure 3A and macrophage
 1182 marker genes identified in Figure 3B.

1184

1185 **Figure 4. Comparison of TRAP-isolated microglial transcriptome from tissue homogenate,**
 1186 **cell suspension, and various microglial cell sorting methods. A)** Schematic of the
 1187 experimental design. Cx3cr1-NuTRAP brains were hemisected and processed in halves for
 1188 whole-tissue homogenization or enzymatic and mechanical dissociation to create a single cell
 1189 suspension. Single cell suspensions were then sorted using MACS- and/or FACS-based
 1190 isolation of microglia. Tissue homogenate, mixed-cell suspension, and microglia sorted by each
 1191 of the four depicted methods were subjected to TRAP to isolate microglial-specific ribosomally-
 1192 bound RNA for creation of RNA-Seq libraries. Gene body coverage plots showed no 3' bias in
 1193 any groups (**Figure 4-2**). **B)** PCA of all expressed genes (>5 read counts in all samples from at

1194 least one group) separates Tissue-TRAP from all other groups in the first component (79%
1195 explained variance) and Cell Suspension-TRAP from all other groups in the second component
1196 (10.9% explained variance). **C**) Third component of PCA on all expressed genes separated
1197 autoMACS-TRAP from all other groups (7.2% explained variance). Each of the sort strategies
1198 had similar levels of **(D)** enrichment of microglial transcripts and depletion of: **(E)** astrocytic, **(F)**
1199 oligodendrocytic, **(G)** neuronal, and **(H)** endothelial transcripts when compared to Tissue-TRAP.
1200 All of the sort methods showed stronger depletion of: **(E)** astrocytic, **(F)** oligodendrocytic, **(G)**
1201 neuronal, and **(H)** endothelial transcripts when compared to Cell-TRAP (One-way ANOVA,
1202 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 transcriptome 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 transcriptomes 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, BHMT, 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.

1215 **D)** Top 10 biological processes over-represented in the 2329 genes upregulated in Cell-TRAP
 1216 and Sort-TRAP compared to Tissue-TRAP (Gene Ontology Over-Representation Analysis,
 1217 Hypergeometric test, BHMTTC FDR <0.05). **E)** Comparison of upregulated transcriptomic
 1218 pathways (Figure 2J; Figure 2-1) and upregulated translome pathways (**Figure 5D, Figure 5-**
 1219 **1)** reveal 55 biological processes that are upregulated in the translome only. **F)** Selection of 10
 1220 biological processes that are uniquely upregulated in the translome (from the 55 identified in
 1221 Figure 4E). **G)** Heatmap of genes involved in “Response to Interleukin-6 (GO:0070741)”
 1222 biological process. **H)** Cytokines (*Il1a*, *Il1b*, *Il6*, *Il10*, *Il16*, *Il27*) that are upregulated in the Cell-
 1223 and Sort-TRAP groups compared to Tissue-TRAP (One-Way ANOVA, BHMTTC, SNK FDR<0.1,
 1224 |FC|>2). **I)** Chemokines (*Cxcl1*, *Cxcl2*, *Cxcl10*, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl7*, *Ccl12*) that are
 1225 upregulated in the Cell- and Sort-TRAP groups compared to Tissue-TRAP (One-Way ANOVA,
 1226 BHMTTC, SNK FDR<0.1, |FC|>2). **J)** Intersection of activational genes identified in three previous
 1227 studies (Ayata et al., 2018; Haimon et al., 2018; Marsh et al., 2020) identified 21 *ex vivo*
 1228 activational transcripts represented in at least two of the studies. **I)** PCA of 21 *ex vivo*
 1229 activational genes shows clear separation of Tissue-TRAP from all other groups in the first
 1230 component (92.8% explained variance). **J)** Heatmap of 21 activational genes shows high levels
 1231 of *ex vivo* activational transcripts across Cell- and Sort-TRAP methods compared to Tissue-
 1232 TRAP. **K)** *Zfp36* is enriched in Cell-TRAP and Sort-TRAP compared to Tissue TRAP (One-Way
 1233 ANOVA, Tukey’s posthoc, ***p<0.001).

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1236 **Figure 6. Changes in cellularity based on cell preparation method.** Cx3cr1-NuTRAP brains
1237 were hemisected and single cell suspensions were generated by three different methods. Cell
1238 preparations were compared via flow cytometry and transcriptomic analyses. The three cell
1239 preparation methods were also compared to whole tissue using transcriptomic analysis. Basic
1240 sequencing metrics and all source data for Figure 6 are provided in **Figure 6-1**. Gene body
1241 coverage plots showed greater 3' bias in the Tissue- and Dounce-Input groups than the Cell-
1242 Input (+/-) Inhibitors groups (**Figure 6-2**). **A**) Schematic of experimental design presented in this
1243 figure. **B**) Flow cytometry on cell type-specific markers for microglia (CD11b⁺CD45⁺), astrocytes
1244 (ACSA2⁺), oligodendrocytes (O4⁺) and neurons (CD24⁺) for each of the cell preparation
1245 methods (One-Way ANOVA, Tukey's *post hoc*, ***p < 0.001). Representative gating strategies
1246 for flow cytometry are provided in **Figure 6-3**. **C**) Heatmap of cell type-specific markers for
1247 microglia, astrocytes, oligodendrocytes, neurons, and endothelial cells. **D**) CIBERSORTx
1248 cellularity estimates based on whole-transcriptome RNA-Seq from whole brain tissue and each
1249 of the cell preparation techniques. **E**) Comparison of the number of eGFP⁺ singlets in each half
1250 brain for each of the cell preparation methods (One-Way ANOVA, Tukey's *post hoc*, ***p <
1251 0.001). There was no overall difference in cell viability between different cell preparation
1252 methods. eGFP⁺ cells had slightly higher viability in the Cell-Input (+/-) Inhibitors than the
1253 Dounce-Input method (**Figure 6-4**). **Boxplots** represent median, Q1, Q3, minimum, and
1254 maximum of each data set. Created with BioRender.com.

Figure 7. Ex vivo activation 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* activation

1265 transcripts prevented by the addition of inhibitors and are given in the heatmap. **C)** Top 10
1266 biological processes over-representation in the *ex vivo* activational genes prevented by the
1267 addition of inhibitors (hypergeometric test, BHMTTC, FDR<0.05). **D)** Transcription factors over-
1268 represented in the *ex vivo* activational transcripts prevented by the addition of inhibitors
1269 (hypergeometric test, BHMTTC, FDR<0.05). **E)** Heatmap of select response to oxidative stress
1270 pathway genes. **F)** Heatmap of common chemokines. **G)** Violin plots of senescent cell marker
1271 expression (One-way ANOVA, Tukey's *post-hoc*, *p<0.05). **H)** Heatmap of 21 *ex vivo*
1272 activational genes identified in at least two previous studies (Ayata et al., 2018; Haimon et al.,
1273 2018; Marsh et al., 2020). **I)** PCA of 21 *ex vivo* activational genes identified in at least two
1274 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'-b-b'**) and CD45 (red signal, **c-c'-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 1-5. Cartidge-based FACS gating strategy for microglial sorting on Miltenyi Biotec MACSQuant Tyto.

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1306 **Figure 1-6. Cytometer-based FACS gating strategy for microglial sorting on FACSria**

1307 **III. A) Unstained cells from a Cx3cr1-cre/ERT2^{Neg};NuTRAP⁺ (WT) brain were used to establish**

1308 gating for CD45⁺eGFP⁺CD11b⁺ microglia. **B)** Gating strategy for Cx3cr1-NuTRAP brain cells
1309 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 6-4. 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.

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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.