FACS isolation of endothelial cells and pericytes from mouse brain microregions

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The vasculature is emerging as a key contributor to brain function during neurodevelopment and in mature physiological and pathological states. The brain vasculature itself also exhibits regional heterogeneity, highlighting the need to develop approaches for purifying cells from different microregions. Previous approaches for isolation of endothelial cells and pericytes have predominantly required transgenic mice and large amounts of tissue, and have resulted in impure populations. In addition, the prospective purification of brain pericytes has been complicated by the fact that widely used pericyte markers are also expressed by other cell types in the brain. Here, we describe the detailed procedures for simultaneous isolation of pure populations of endothelial cells and pericytes directly from adult mouse brain microregions using fluorescence-activated cell sorting (FACS) with antibodies against CD31 (endothelial cells) and CD13 (pericytes). This protocol is scalable and takes ~5 h, including microdissection of the region of interest, enzymatic tissue dissociation, immunostaining, and FACS. This protocol allows the isolation of brain vascular cells from any mouse strain under diverse conditions; these cells can be used for multiple downstream applications, including *in vitro* and *in vivo* experiments, and transcriptomic, proteomic, metabolomic, epigenomic, and single-cell analysis.

INTRODUCTION

The brain vasculature has a key role in development and aging, as well as in mature physiological and pathological states. Blood vessels are composed of two main cell types: endothelial cells, which form the lumen of blood vessels, and mural cells. In the brain, endothelial cells are connected by tight junctions and enwrapped by pericytes in the microvasculature. Pericytes are critical for the development of the blood–brain barrier[1,2] and regulate blood flow in response to neuronal activity and neurotransmitter release in adulthood[3,4]. Conversely, pericyte dysfunction or loss may contribute to neurodegenerative diseases[5–7]. Endothelial cells and pericytes also constitute key components of adult neural stem cell niches and provide both contact-mediated and diffusible signals that influence neural stem cell proliferation and differentiation[3,8–21]. As such, methods to directly probe the function and molecular identity of vascular cells are increasingly needed.

Protocols for the isolation of brain vascular cells often require the whole brain, or large regions, because of the difficulty of obtaining adequate yields of vascular cells. However, it is becoming apparent that vascular cells exhibit regional heterogeneity, both developmentally and functionally[21–23]. For example, vascular beds have different architectures in adult neural stem cell niches, as compared with the rest of the brain[24,25], with endothelial cells and pericytes from the adult ventricular–subventricular zone (V-SVZ) neural stem cell niche and cortex exhibiting functionally distinct secretomes[21]. These highlight the importance of developing methods that allow the isolation of pure primary vascular cells from different brain microregions.

Previous approaches to isolating brain vascular cells include microvessel isolation and culture[11,26,27], immunopanning[28–30], and selective *in vitro* culture conditions to enrich different populations[31–33]. However, these approaches require large amounts of starting material, are time-consuming, use harsh dissociation and digestion conditions, and yield impure vascular populations that require further subculturing. FACS allows the isolation of defined populations of cells on the basis of their expression of surface markers or fluorescent reporters. Brain endothelial cells and pericytes have been isolated using transgenic fluorescent reporter mice[28,34–37]. Importantly, the selection of appropriate markers is complicated because of the expression of commonly used markers, such as Tie2 and Nestin, by both endothelial cells and pericytes[34,35]. Moreover, in the brain, it has been especially challenging to obtain pure populations of pericytes because widely used markers, such as NG2, PDGFR-β, and Nestin, are also expressed by oligodendrocyte progenitors, adult neural stem cells and progenitors, and astrocytes and/or ependymal cells[28,34–40]. Double-transgenic reporter lines allow pure populations of pericytes to be isolated[36] but may not capture all pericyte subtypes in the brain. Finally, if transgenic fluorescent reporter mice are used to isolate vascular cells from different mouse models, time-consuming breeding is required to generate the necessary lines.

Therefore, we devised a FACS approach to prospectively isolate primary endothelial cells and pericytes from microdissected brain regions independent of transgenic reporter mice, rapidly, without subculturing, and with high purity[21]. This strategy provides a simple platform for the simultaneous obtainment of primary endothelial cells and pericytes under a variety of conditions from discrete brain areas.

Development of the protocol

We developed this protocol to compare functional differences between endothelial cells and pericytes isolated from the V-SVZ adult neural stem cell niche and those isolated from the cortex, a non-neurogenic region. However, it can be applied to any brain region of interest. Briefly, microdissected tissue is minced, digested with collagenase/dispase, labeled with antibodies specific for endothelial cells (CD31 (PECAM-1 (ref. 41))) and pericytes (CD13 (alanyl-aminopeptidase[42])), and simultaneously collected using FACS (**Fig. 1**).

We confirmed that CD13 is a surface marker for brain pericytes[21], circumventing issues with commonly used pericyte

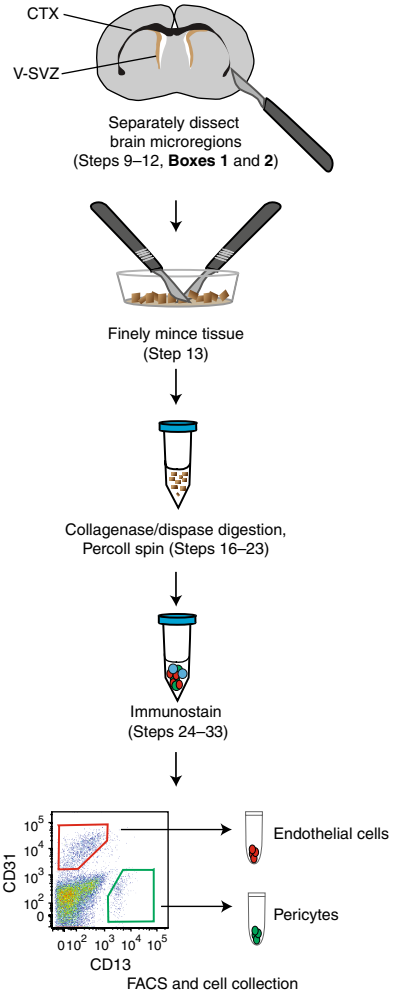

Figure 1 | Overview of protocol for purification of brain endothelial cells and pericytes by FACS. Brain regions of interest, such as the cortex and V-SVZ, are microdissected and finely minced with a scalpel. The tissue is then digested with collagenase/dispase, and myelin and debris are eliminated by centrifugation through Percoll. Finally, single-cell suspensions are incubated with fluorescently conjugated antibodies, and pure populations of endothelial cells and pericytes are collected using FACS. CTX, cortex; V-SVZ, ventricular–subventricular zone stem cell niche (brown) adjacent to the lateral ventricles. Adapted with permission from Crouch *et al.*[21], Regional and stage-specific effects of prospectively purified vascular cells on the adult V-SVZ neural stem cell lineage, *J. Neurosci.*, vol. 35, Copyright 2015; permission conveyed through Copyright Clearance Center.

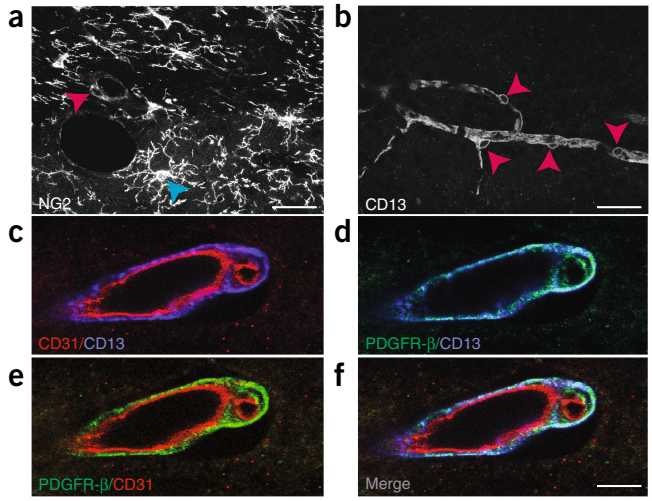

Figure 2 | CD13 is a specific pericyte marker in the adult mouse brain. Commonly used pericyte markers cannot be used to isolate pericytes from the brain because they are also expressed by other cell types. (**a**–**f**) Confocal images of immunostaining for different pericyte markers in the brain. (**a**) NG2 is expressed by both pericytes (magenta arrowhead) and oligodendrocyte progenitors throughout the brain (blue arrowhead). (**b**) CD13 labeling is found exclusively in perivascular cells (arrowheads). (**c**–**f**) Both CD13 and PDGFR-β label pericytes (**d**,**f**) but not CD31$^+$ endothelial cells (**c**,**e**,**f**). Scale bars: 25 µm (**a**,**b**); 10 µm (**f**). **c**–**f** adapted with permission from Crouch *et al.*[21], Regional and stage-specific effects of prospectively purified vascular cells on the adult V-SVZ neural stem cell lineage, *J. Neurosci.*, vol. 35, Copyright 2015; permission conveyed through Copyright Clearance Center. Experiments were performed with the approval of the Columbia University IACUC and the Basel-Stadt cantonal veterinary office.

markers that are expressed by multiple cell types in the brain[28,34–40] (**Fig. 2**). Incubation with a combination of collagenase and dispase results in vascular cell detachment from the basement membrane and preserves both CD31 and CD13 epitopes. To eliminate myelin and other debris, a brief Percoll centrifugation step gives an excellent yield of vascular cells from small amounts of starting material, in contrast to very low yields after ultracentrifugation with dextran. Finally, to exclude blood cells, CD45 and CD41 are included as negative selection markers.

We have previously analyzed the purity of FACS-isolated populations using flow cytometry for independent endothelial and pericyte markers, acute immunostaining, quantitative PCR, and microarray analysis (**Fig. 3**)[21]. By both flow cytometry (**Fig. 3a**) and acute immunostaining (**Fig. 3b–i**), endothelial cells were >97% pure and pericytes were >96% pure; they expressed the expected endothelial and pericyte genes, respectively, but not astrocytic (*Gfap*), neuronal (*Tubb3*), or microglial (*Aif1*) markers (**Fig. 3j–l**). In sum, this combination of antibodies allows the purification of endothelial cells and pericytes from any mouse strain.

Application of the method

This simple and rapid FACS isolation strategy can be applied to illuminate diverse aspects of endothelial and pericyte biology in the brain (**Fig. 4**). Using this strategy, we uncovered functional differences in endothelial cells and pericytes from different brain regions, and identified a novel role for PIGF-2, secreted by both cortical and V-SVZ endothelial cells, as a potent mitogen for adult V-SVZ neural stem cells[21].

Importantly, this protocol allows both cell types to be isolated independently of transgenic reporters from brain microregions. As such, it may be used to probe regional differences in endothelial cells and pericytes, and how these cells change in different physiological states and pathological conditions. The purified cells may be used for transcriptome analysis, proteomics, metabolomics, or epigenomics; for *in vitro* experiments, including 3D vascular assays and coculture studies; to make conditioned medium; for *in vivo* applications, including transplant experiments and blood–brain barrier experiments; and to probe the intrinsic functional and molecular heterogeneity of CNS endothelial cells and pericytes, and their relationships with other brain cell types at both the population and single-cell level (**Fig. 4**).

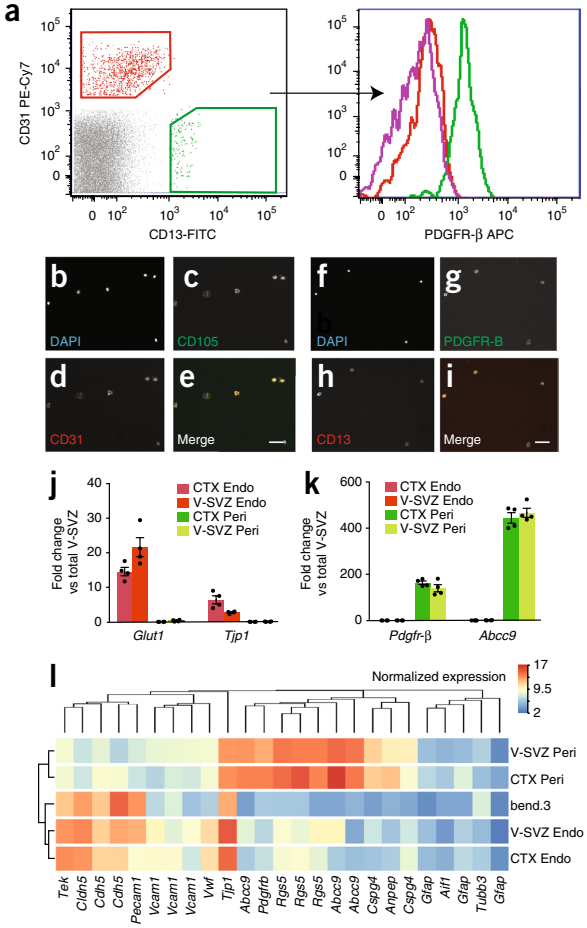

Figure 3 | Validation of FACS-purified endothelial cells and pericytes. (**a**) By flow cytometry, CD13$^+$ pericytes (green) express PDGFR-β, whereas CD31$^+$ endothelial cells (red) do not. Magenta shows CD31$^-$CD13$^-$ cells, which are also PDGFR-β negative. (**b**–**e**) Images of acutely immunostained FACS-purified endothelial cells (**b**, DAPI) showing colabeling of endothelial markers CD105 (**c**), CD31 (**d**), and merge (**e**). (**f**–**i**) Images of acutely immunostained FACS-purified pericytes (**f**, DAPI) co-labeled with PDGFR-β (**g**), CD13 (**h**), and merge (**i**). (**j**–**l**) Validation of RNA expression of endothelial and pericyte genes in acutely purified populations by quantitative PCR (**j**, **k**) or microarray (**l**). Endothelial cells and pericytes were FACS-purified, and cDNA was prepared directly from lysed cells using the NuGEN Prelude Lysis Buffer and the NuGEN Pico WTA System v2. SYBR Green Master Mix was used for quantitative PCR[21]. For microarrays, cDNA was biotinylated with the NuGEN Encore Biotin Module and hybridized to Affymetrix GeneChip Mouse 430.2 Gene Arrays. (**j**,**k**) Expression levels of endothelial genes *Glut1* and *Tjp1* (**j**) and pericyte genes *Pdgfr-β* and *Abcc9* (**k**) relative to total V-SVZ. $n = 4$ biological replicates, mean ± s.e.m. (**l**) Heat map of normalized expression values from microarray analysis of FACS-purified primary endothelial cells and pericytes from V-SVZ and cortex. Primary endothelial cells cluster with the bend.3 endothelial cell line and express characteristic endothelial genes. Note that the bend.3 cell line does not express some canonical endothelial genes. Primary pericytes cluster together and express high levels of known pericyte genes. $n = 3$ biological replicates per cell type. Microarray was normalized with Mas5 without any baseline transformation using Genespring GX11 software. Hierarchical clustering was performed with Euclidean distance metric and average linkage. Scale bars, 100 µm. **a**–**k** adapted with permission from Crouch *et al.*[21], Regional and stage-specific effects of prospectively purified vascular cells on the adult V-SVZ neural stem cell lineage, *J. Neurosci.*, vol. 35, Copyright 2015; permission conveyed through Copyright Clearance Center. Experiments were performed with the approval of the Columbia University IACUC and the Basel-Stadt cantonal veterinary office.

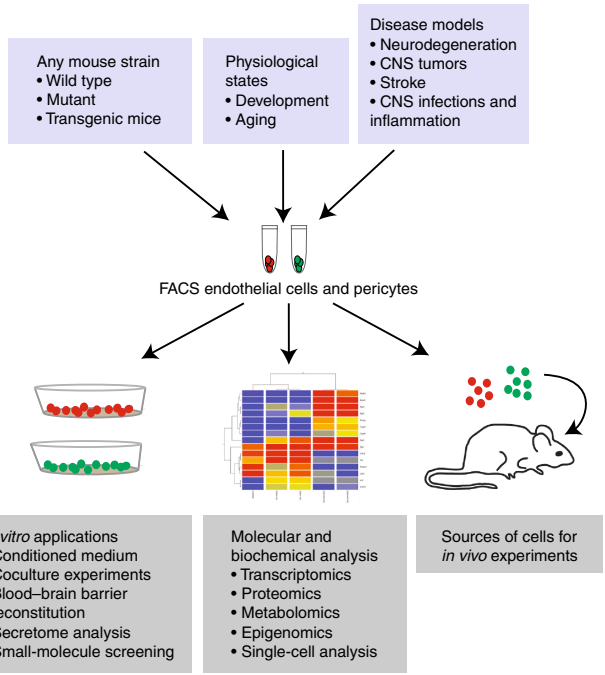

Figure 4 | Potential applications of FACS-purified primary endothelial cells and pericytes. A major advantage of this purification protocol is that it can be used to isolate vascular cells from any mouse strain. Pure populations of endothelial cells and pericytes can be used in a variety of *in vitro* and *in vivo* applications, and for molecular analysis.

Comparison with other methods

Few experiments have been performed using primary brain vascular cells because of difficulties in isolating pure populations with sufficient yield. As an alternative, immortalized cell lines and human primary umbilical vein endothelial cells have been widely used to study the interactions of endothelial cells with different cell types[8–10,12,43,44]. However, the transcriptomes and behavior of immortalized cell lines and primary cells differ greatly, as do endothelial cells from different organs and species. Moreover, little is known about pericytes in the brain. Importantly, *in vivo*, the brain vasculature undergoes changes in different states. As such, the ability to isolate primary endothelial cells and pericytes under different physiological conditions[44–50] is an advantage of our approach. FACS-purified cells can be immediately collected and processed for downstream analysis, avoiding changes that may occur upon prolonged culture *in vitro*.

Other methods, such as outgrowth of vascular cells from microvessels or *in vitro* selection[11,26,27,31–33], require days to weeks and do not yield pure cell populations. Immunopanning approaches require large numbers of cells and multiple iterations of purification. By contrast, our FACS methodology requires <5 h to obtain pure populations and allows the simultaneous purification of both endothelial cells and pericytes from even small amounts of starting material for *in vitro* or *in vivo* studies.

Importantly, unlike other pericyte markers, including PDGFR-β, NG2, and Nestin, which are also expressed by other brain cell types[28,34–40], CD13 can be used as a single marker to isolate pericytes. As such, FACS purification using CD13 antibodies circumvents the need for transgenic reporter mice. In addition, unlike other vascular isolation techniques, FACS approaches provide a quantitative readout of ratios of different cell types. This protocol

can therefore provide insight into endothelial/pericyte ratios in different brain structures, potentially revealing functional differences between vascular beds.

Limitations

This protocol should have excellent reliability in regard to the isolation and study of perivascular cells from any region of the adult mouse brain. Investigators who wish to use this protocol to obtain cells for *in vitro* applications should consider the behavior of the purified populations *in vitro* to determine how many cells and wells should be plated in order to have enough cells for experiments. For example, we observed that V-SVZ and cortical endothelial cells divided to produce a confluent monolayer but then became quiescent and could not be passaged. A limitation of all FACS experiments is that the cells experience stress during dissociation. Data obtained with FACS-purified cells should always be validated *in vivo*. Finally, translating this protocol to different organs or species will require antibody validation and optimization of enzyme dissociation and cell number. Moreover, as with any marker, *in vivo* immunostaining should be performed before FACS for different experimental paradigms to ensure the specificity of CD31 and CD13 expression.

Experimental design

A key step for FACS is the dissociation of the tissue to a single-cell suspension while preserving surface marker epitopes and optimizing yield. The vasculature is embedded in an extracellular matrix-rich environment, and blood vessel cells must be released by enzymatic dissociation. Important parameters are the particular enzymes used, duration of digestion, and amount of tissue, which must be balanced to optimize yield and epitope preservation. Collagenase and dispase, a neutral protease, allow the isolation of both endothelial cells and pericytes. By contrast, papain, a cysteine protease frequently used in FACS purification of brain cells[51,52], degrades the CD31 epitope in a time-dependent manner, and the $CD13^+$ population is greatly reduced (**Supplementary Fig. 1**).

We have used this protocol to isolate vascular cells from the adult mouse V-SVZ, cortex, and striatum. This protocol is optimized for processing two samples in parallel, in which each sample contains ~0.2 g of tissue. This corresponds to the entire cortex from one adult mouse or the V-SVZ from ten adult mice, or the striatum from five adult mice. Care should be taken if more than two samples are to be processed in parallel, as the number of tubes used for some steps can become unwieldy.

If different amounts of tissue or additional markers are to be used, some of the digestion parameters may need to be individually optimized. After digestion (Step 18), the solution should appear cloudy and viscous, without obvious intact tissue. To assess digestion efficiency, visually inspect a small volume under the microscope. Ideally, blood vessels should have the appearance of 'beads on a string'. Cells can then easily be dissociated into single cells at the trituration step. If intact blood vessel fragments are present, increase the digestion time or the concentration of enzyme. If additional markers are included, it is critical to assess how each epitope is affected by enzymatic treatment and to optimize digestion conditions to balance dissociation, epitope preservation, and cell viability. The digestion time or amount of enzyme per sample can be varied, the population ratios can be assessed by flow cytometry, and the yield can be assessed by collection of cells. In general, increasing the duration of digestion and decreasing the amount of tissue improves dissociation, but allowing digestion to proceed for too long may compromise epitope preservation and cell viability.

Controls constitute a critical part of all FACS experiments. Species-matched isotype controls should be performed for each primary antibody to ensure the absence of nonspecific binding. Single-color controls are essential to set sort parameters and compensation for each fluorophore. Another set of important controls is all fluorophores minus one (FMO) to define gates for each population (**Supplementary Fig. 2**).

MATERIALS

REAGENTS

FACS

- 70% (vol/vol) Ethanol (Fisher, cat. no. 04-355-305)
- FBS (Atlanta Biologicals, cat. no. S11550) ▲ **CRITICAL** Store at −20 °C in 50-ml or 5-ml aliquots for up to 12 months. Store thawed FBS at 4 °C for 1 week maximum.
- 10× PBS (pH 7.4; Life Technologies; cat. no. 70011-044)
- Glucose (Sigma-Aldrich, cat. no. G7021-1KG)
- 35% (wt/vol) BSA solution (Sigma-Aldrich, cat. no. A7979-50ML)
- HBSS (1×; Life Technologies, cat. no. 14175-095)
- UltraPure DNase/RNase-free dH_2O (Life Technologies, cat. no. 10977-015)
- Collagenase/dispase (Sigma-Aldrich, cat. no. 11097113001)
- DNase (Worthington, cat. no. 2139)
- Penicillin–streptomycin (100×; Life Technologies, cat. no. 15140-122) ▲ **CRITICAL** Prepare 0.5-ml aliquots and store at −20 °C for up to 12 months.
- Percoll (Sigma-Aldrich, cat. no. P1644-500ML) ▲ **CRITICAL** Store at 4 °C for up to several months.
- DAPI (4′,6-diamidino-2-phenylindole,dihydroclhoride; Life Technologies, cat. no. D1306) ! **CAUTION** DAPI is a known mutagen and should be handled with care. Collect all DAPI-containing solutions for disposal according to institutional guidelines.
- Mice. Any mouse strain of interest (male or female). We used 2-month-old CD1 mice (Charles River Laboratory, strain code 022) ! **CAUTION** All animal experiments should be performed according to relevant guidelines and regulations. All procedures in this protocol involving mice were approved by the Institutional Animal Care and Use Committee at Columbia University and by the Basel-Stadt cantonal veterinary office.

Antibodies

- Anti-CD13-FITC (BD Biosciences, cat. no. 558744; RRID: AB_397101) ▲ **CRITICAL** Store at 4 °C for up to 1 month; protect from light.
- Anti-CD31-APC (BD Biosciences, cat. no. 551262; RRID: AB_398497) ▲ **CRITICAL** Store at 4 °C for up to several months; protect from light.
- Anti-CD41-PE (BD Biosciences, cat. no. 558040; RRID: AB_397004) ▲ **CRITICAL** Store at 4 °C for up to several months; protect from light.
- Anti-CD45-PE (BD Biosciences, cat. no. 553081; RRID: AB_394611) ▲ **CRITICAL** Store at 4 °C for up to several months; protect from light.

Secondary-only control antibodies

- FITC rat IgG1, κ isotype control (BD Biosciences, cat. no. 552916)
- APC rat IgG2a κ isotype control RUO (BD Biosciences, cat. no. 553932)
- PE rat IgG2b, κ isotype control RUO (BD Biosciences, cat. no. 553989) ▲ **CRITICAL** Store at 4 °C; protect from light.

Culture of purified endothelial cells and pericytes

- Collagen (bovine collagen solution; Stem Cell Technologies, cat. no. 4902) ▲ **CRITICAL** Store at 4 °C for up to several months.
- EGM-2 medium (EGM-2 BulletKit; Lonza, cat. no. cc-3162) ▲ **CRITICAL** Store at 4 °C for up to several months without growth factors.
- Corning Co-Star flat-bottom cell culture plates (24 well; Fisher Scientific, cat. no. 3524)

• Vybrant dye (Invitrogen, cat. no. V35003)
• RNase AWAY (Ambion, cat. no. 10328011)

EQUIPMENT

Dissection

• 10-cm Petri dish (Fisher Scientific, cat. no. FB0875713)
• 35- × 10-mm Cell culture dish (Corning, cat. no. 430165)
• Surgical straight standard scissors (serrated, sharp/blunt; FST, cat. no. 14007-14)
• Fine scissors (tough cut, straight, sharp/sharp; FST, cat. no. 14058-09)
• Delicate bone scraper (FST, cat. no. 10075-16)
• Standard Dumont fine forceps (angled at 45°; FST, cat. no. 11251-35)
• Stereomicroscope, ideally with a transilluminated base (Leica, model no. TL5000)
• Fiber-optic light guide unit (Leica, model no. KL300)
• Two-well concavity slide (Electron Microscopy Sciences, cat. no. 71878-08)
• Razor blade (Personna, cat. no. 94-115-71)
• Microsurgery knife (22.5°; Wilson Ophthalmic, cat. no. 622-0WR2250-06)
• Parafilm (Bemis, cat. no. PM992)
• Sterile transfer pipette (Fisher Scientific, cat. no. 13-711-20)

Cell suspension preparation and sorting

• Refrigerated centrifuge with brake (Eppendorf, model no. 5804 R) with holders for 15-ml Falcon tubes and 5-ml FACS collection tubes (see below)
• Blue Max Jr. 15-ml polypropylene conical tubes (Falcon, cat. no. 352097)
• Blue Max 50-ml polypropylene conical tubes (Falcon, cat. no. 352098)
• Cell strainer (40-µm, nylon; Falcon, cat. no. 352340)
• FACS collection tubes, 5-ml polypropylene round-bottom tubes (Falcon, cat. no. 352063)
• P1000 pipette (Rainin)
• P200 pipette (Rainin)
• P20 pipette (Rainin)
• Hybridization oven with rotisserie (Bellco, model no. 7930-00100)
• FACS sorter (e.g., Becton Dickinson, model no. FACS Aria II)

REAGENT SETUP

▲ **CRITICAL** All solutions, including stock solutions, should be prepared under sterile conditions in a laminar flow hood to avoid contamination.

▲ **CRITICAL** The total volumes of 1× PBS, 2% (vol/vol) FBS in PBS, HBSS/BSA/glucose buffer, and tissue collection solution should be calculated before the start of the protocol, as they will depend on the number of samples. The volumes outlined in the protocol are optimized for ~0.2 g of tissue (cortex from one adult mouse or the V-SVZ from ten adult mice). Multiple regions can be dissected from each brain. If more than one brain region/sample is to be processed in parallel, multiply the volumes by the total number of brain regions per sample to be processed.

30% (wt/vol) Glucose stock solution Dissolve 15 g of glucose in 50 ml of UltraPure DNase/RNase-free dH_2O. To help dissolve the powder, add glucose to 20 ml of water, let the glucose dissolve, and then add water up to 50 ml. Filter and store at 4 °C for up to 6 months.

DNase stock solution (10 mg/ml) Dissolve 50 mg of DNase in 5 ml of sterile ddH_2O. The DNase stock solution can be stored at –20 °C for at least 2 months. ▲ **CRITICAL** Avoid freeze–thaw cycles that could diminish the enzyme activity; make 150-µl aliquots, and thaw only the necessary amount for each experiment.

Collagenase/dispase for digestion Prepare a 100 mg/ml stock solution in UltraPure DNase/RNase-free dH_2O, make 0.5-ml aliquots, and store at −20 °C. ▲ **CRITICAL** Use aliquoted collagenase/dispase within 1 month of resuspension; otherwise, enzyme activity may be compromised.

DAPI Dissolve one vial (10 mg) in 2 ml of deionized water to make a 5 mg/ml DAPI stock solution. Make aliquots and store at −20 °C for up to 6 months. ! **CAUTION** DAPI is a known mutagen and should be handled with care. Collect all DAPI-containing solutions for disposal according to institutional guidelines.

22% (vol/vol) Percoll solution Mix 11 ml of Percoll and 5 ml of 10× PBS with 34 ml of UltraPure DNase/RNase-free dH_2O to obtain 50 ml of 22% (vol/vol) Percoll solution. Filter through a 0.2-µm filter and store at 4 °C for up to 1 month.

Endothelial medium (EGM-2 medium) Add growth factors provided in the EGM-2 BulletKit per the manufacturer's instructions and penicillin–streptomycin (100× stock) to the EGM-2 medium. The medium can be stored at 4 °C and used for up to 2 weeks.

Trituration solution Add 50 µl of DNase per 1 ml of 2% (vol/vol) FBS in PBS. Total volume required = 1 ml per digestion tube. ▲ **CRITICAL** Prepare the trituration solution fresh at Step 19 of the PROCEDURE.

Percoll tubes Prepare two Percoll tubes per digestion tube. Add 4 ml of 22% (vol/vol) Percoll to each 15-ml Falcon tube. ▲ **CRITICAL** Prepare the Percoll tubes fresh at Step 19 of the PROCEDURE.

FACS collection tubes Add 3 ml of endothelial medium per FACS collection tube, and place on ice. ▲ **CRITICAL** If samples are to be collected for RNA analysis, they can either be collected in FACS tubes and centrifuged, or collected directly in 96-well plates containing appropriate solutions. ▲ **CRITICAL** Prepare the FACS collection tubes fresh at Step 19 of the PROCEDURE.

1× PBS Approximately 10 ml of 1× PBS is needed for dissection. This can either be prepared from a 10× stock, autoclaved, and used as needed, or purchased as 1× PBS. ▲ **CRITICAL** Prepare fresh at Step 2 of PROCEDURE under sterile conditions.

2% (vol/vol) FBS in PBS Prepare 50 ml of 1× PBS by adding 5 ml of 10× PBS to 45 ml of sterile UltraPure DNase/RNase-free dH_2O. Remove 1 ml and replace with 1 ml of FBS. 15 ml of 2% (vol/vol) FBS in PBS is needed per brain region per sample. ▲ **CRITICAL** Prepare fresh at Step 2 of the PROCEDURE under sterile conditions. Unused solution can be stored at 4 °C and used for 2 d.

HBSS/BSA/glucose buffer (HBSS/1% (wt/vol) BSA/0.1% (wt/vol) glucose) Add 1.666 ml of 30% (wt/vol) glucose stock solution and 14.33 ml of 35% (wt/vol) BSA solution to 500 ml of HBSS. ▲ **CRITICAL** Prepare fresh at Step 2 of the PROCEDURE under sterile conditions and keep at 4 °C.

Tissue collection solution Prepare one 35- × 10-mm cell culture dish per brain region per sample to be dissected. To each dish, add 150 µl of 10 mg/ml DNase stock solution to 3 ml of HBSS/BSA/glucose buffer. ▲ **CRITICAL** Prepare fresh at Step 2 of the PROCEDURE under sterile conditions.

Collagen coating of tissue culture plates If endothelial cells and pericytes will be cultured after FACS, tissue culture plates should be coated with collagen 1 d before FACS. Dilute bovine collagen 1:100 (vol/vol) in 1× PBS (final concentration is ~30 µg/ml). Cover the bottom surface of wells with diluted collagen and place the plate in a tissue culture incubator at 37 °C overnight. The following day, remove residual collagen solution, and wash once with 1× PBS before use. Collagen-coated tissue culture plates should be used within 1 week after preparation.

EQUIPMENT SETUP

The Becton Dickinson FACS Aria II is operated using 13-p.s.i. pressure and with a 100-µm nozzle aperture. Gating strategies are specified in Steps 34–39.

PROCEDURE

Reagent preparation ● TIMING ~30 min

1| Clean all dissection tools with 70% (vol/vol) ethanol and wipe them dry.

2| Prepare the working solutions (1× PBS, 2% (vol/vol) FBS in PBS, HBSS/BSA/glucose buffer, and tissue collection solution) as described in the Reagent Setup.

3| Pour ~10 ml of 1× PBS into a 10-cm Petri dish and place on ice.

4| Prepare one 35- × 10-mm cell culture dish containing 3 ml of tissue collection solution for each sample and place on ice.

5| Add ~10 ml of 2% (vol/vol) FBS in PBS to a 15-ml conical tube and place on ice for use during dissection.

Harvesting of brains ● TIMING ~10 min for a group of five mice

6| Euthanize mice according to institutional guidelines, such as by CO_2 euthanasia, following American Veterinary Medical Association Guidelines.

! CAUTION Follow appropriate institutional guidelines with regard to animal husbandry and experimental procedures. Our experiments were performed with the approval of the Columbia University Institutional Animal Care and Use Committee (IACUC) and the Basel-Stadt cantonal veterinary office.

7| Spray 70% (vol/vol) ethanol onto the head/neck area and remove the brain using standard procedures, as described in Mirzadeh *et al.*[53].

▲ CRITICAL STEP Be careful when opening the skull to avoid damaging the brain.

8| Transfer the brain to the 10-cm Petri dish filled with cold 1× PBS on ice (from Step 3). Harvest up to five brains at a time, placing them in the same 10-cm Petri dish.

Dissection of brain area of interest ● TIMING 3–5 min per brain area per mouse

▲ CRITICAL Steps 9–12 describe a general brain dissection protocol. For specific dissection instructions for the cortex and V-SVZ, see **Box 1** and **Supplementary Figures 3** and **4** (cortex), and **Box 2** and **Supplementary Figures 5** and **6** (V-SVZ)[53,54].

9| Place the brain onto a piece of Parafilm and cut it into coronal slices several millimeters thick with a razor blade.

10| Using a transfer pipette, add a drop of 2% (vol/vol) FBS in PBS to each well of a two-well glass concavity slide.

11| Using fine 45° angled forceps, transfer individual coronal slices to a two-well glass concavity slide for microdissection of the area of interest under the dissection microscope.

12| After dissection of the region of interest, use fine 45° angled forceps to transfer it to a 35- × 10-mm dish filled with 3 ml of tissue collection solution on ice (from Step 4).

Box 1 | Cortex dissection ● TIMING ~3–5 min per brain

This box describes the detailed procedures for cortex dissection (see also **Supplementary Figs. 3** and **4**).

Procedure

1. After harvesting of the brain in Step 8 of the main PROCEDURE, place the brain into one well of a two-well concavity slide containing 2% (vol/vol) FBS in PBS and place it under the dissection microscope. Remove the meninges with fine 45° forceps. Insert the flat portion of the forceps into a major sulcus to obtain a firm grasp on the meninges. A good place to do this is between the two hemispheres on the top surface of the brain, or on the lower surface of the brain. Peel away the meninges.

▲ CRITICAL STEP Ensure that the brain stays moistened or removing the meninges becomes very difficult.

? TROUBLESHOOTING

2. After the meninges are removed, place the brain on a piece of Parafilm, and make four coronal cuts with a razor blade. Discard olfactory bulbs and caudal-most piece, which is mostly midbrain and cerebellum. The cortex (and V-SVZ, if desired) can then be easily dissected from each remaining coronal slice.
3. Using a transfer pipette, add a drop of 2% (vol/vol) FBS in PBS to each well of a two-well glass concavity slide . Using forceps, transfer the coronal slices to a well and place the slide under a dissection microscope.
4. Make sure to remove any residual meninges, if present. Dissect the cortex using the corpus callosum as a guide to the ventral boundary of the cortex. The V-SVZ can be harvested from the same coronal slice (**Box 2**).
5. Using forceps, gently transfer the dissected cortex to a 35- × 10-mm cell culture dish prefilled with tissue collection solution on ice. Proceed with the main PROCEDURE from Step 13 onward.

Box 2 | V-SVZ dissection ● TIMING ~3–5 min per brain

This box describes the detailed procedures for V-SVZ dissection (see also **Supplementary Figs. 5** and **6**).

Procedure

1. After harvesting of the brain in Step 8 of the main PROCEDURE, place the brain on a piece of Parafilm, and make four coronal cuts with a razor blade. Discard olfactory bulbs and the caudal-most piece, which contains mostly midbrain and cerebellum.
▲ CRITICAL STEP Alternatively, the coronal slices from **Box 1** step 2 can be used directly.
2. Using a transfer pipette, add a drop of 2% (vol/vol) FBS in PBS to each well of a two-well glass concavity slide. Using forceps, transfer the coronal slices to the two-well glass concavity slide and place the slide under a dissection microscope.
3. In each coronal slice, use the lateral ventricles as a guide to make a downward cut from the tip of the ventricle with a microsurgery knife.
4. Gently pull up on the overlying corpus callosum to free the dorsal boundary of the V-SVZ.
5. Once exposed, separate the V-SVZ from the striatal tissue by cutting away the adjacent striatal tissue. Note that the V-SVZ has a different color and a velvety consistency, as compared with the adjacent striatum. Transillumination is helpful to visualize the V-SVZ. Minimize contamination with striatal fibers.
6. Using forceps, gently transfer the dissected V-SVZ to a 35- × 10-mm cell culture dish prefilled with tissue collection solution on ice. Proceed with the main PROCEDURE from Step 13 onward.

▲ CRITICAL STEP As a general rule, the total dissection time for all brains should not exceed 2 h or cell viability will be compromised.

Tissue dissociation ● TIMING ~45 min

13| Once all brains have been microdissected, use two microsurgery knives to mince the tissue into small pieces of ~1 × 1 mm in size (**Fig. 1**).
▲ CRITICAL STEP Do not mince tissue for longer than 5 min, as cell viability will be compromised.

14| Pre-wet a sterile transfer pipette with tissue collection solution and use this to transfer the minced pieces to a 15-ml Falcon tube. Carefully and slowly aspirate the minced pieces into the lowest part of the transfer pipette. Allow the pieces to settle at the tip and eject them into the Falcon tube.
▲ CRITICAL STEP Do not aspirate pieces too far up the transfer pipette or they will stick to the walls.

15| Rinse the dissection dish once or twice with 2–3 ml of 2% (vol/vol) FBS in PBS to collect the remaining pieces and add them to Falcon tube. Pellet the tissue by centrifugation at 300*g* at 4 °C for 5 min. In the meantime, proceed to Step 16.

16| While minced pieces are being centrifuged, prepare the collagenase/dispase solution. For each sample, add 0.5 ml of collagenase/dispase stock solution to 14.5 ml of 2% (vol/vol) FBS in PBS.

17| After the centrifugation step is complete, discard the supernatant by decanting and resuspend the tissue in each sample in 3 ml of collagenase/dispase solution prepared in Step 16. Split the contents of each tube into three separate 15-ml Falcon tubes (1 ml per tube) and add an additional 4 ml of collagenase/dispase solution to each tube for a total volume of 5 ml per tube (**Fig. 5**).

18| Incubate for 30 min at 37 °C in a hybridization oven with constant rotation. In the meantime, proceed to Step 19.
▲ CRITICAL STEP The timing of this step may need to be optimized for brain regions other than the cortex or V-SVZ.

19| During incubation, prepare the trituration solution, the Percoll tubes, and the FACS collection tubes needed for the rest of the protocol (Reagent Setup).

20| After the enzymatic digestion is complete, pellet the tissue by centrifugation at 300*g* at 4 °C for 5 min, and discard the supernatant by decanting.

▲ CRITICAL STEP Add a small volume of digested sample, ~10–20 µl, to a small cell culture dish or glass slide and visually inspect the sample under the microscope. Ideally, blood vessels should have the appearance of 'beads on a string'. If intact vessel fragments are present, increase the digestion time or the concentration of enzyme. If the sample has only single cells, the sample may have been overdigested.
? TROUBLESHOOTING

21| Resuspend the pellet in 1 ml of trituration solution per digestion tube, and then triturate (pipette up and down) ~100× with a P1000 pipette.
▲ CRITICAL STEP Set the P1000 pipette to less than the total volume to avoid introducing air bubbles, which will compromise cell viability.

Removal of myelin and debris with Percoll
● TIMING ~20 min
22| Split the contents of each digestion tube into two 15-ml Falcon tubes containing 4 ml of 22% (vol/vol) Percoll each, to ensure maximum cell yield (**Fig. 5**). Gently add half of the solution containing the triturated cells on top of the Percoll in each Percoll tube. Centrifuge at 560*g* at 4 °C for 10 min.
▲ CRITICAL STEP Set the centrifuge to 'no brake'; otherwise, the pellet will be contaminated with myelin and other debris.

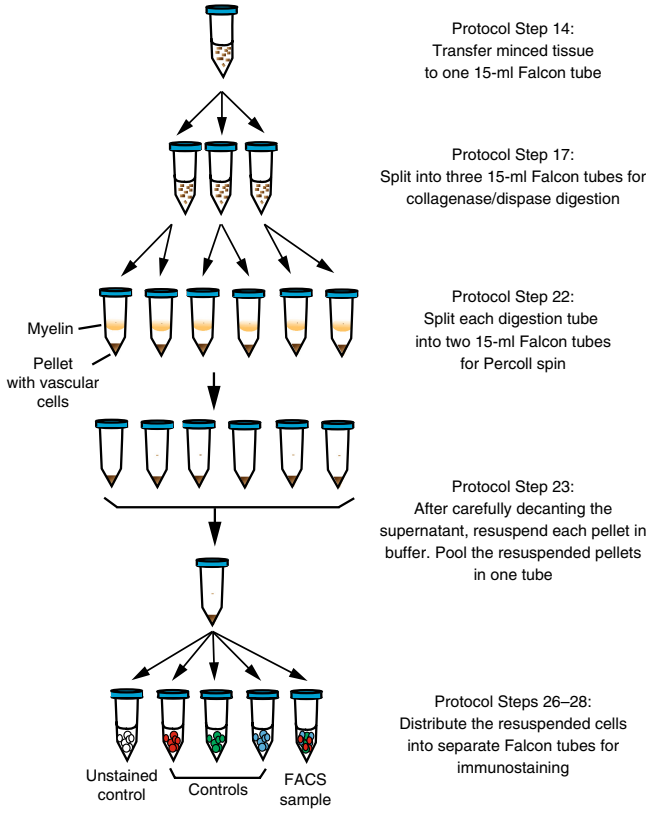

Figure 5 | Schema outlining splitting and pooling of samples at key steps in the protocol. The schema shows how each individual sample is split and pooled at different steps in the protocol to optimize cell yield during the dissociation and labeling steps.

23| Remove the supernatant by decanting, resuspend the pellet in 1 ml of HBSS/BSA/glucose buffer, and transfer the cells to a new 15-ml Falcon tube. Rinse each Percoll tube two to three times with 3 ml of HBSS/BSA/glucose buffer. For each brain region, pool the resuspended pellets and rinses in one 15-ml Falcon tube (**Fig. 5**).

Immunostaining ● TIMING ~20 min
24| Pellet the cells by centrifugation at 300*g* at 4 °C for 5 min.

25| Discard the supernatant by decanting, and resuspend the pellet in HBSS/BSA/glucose buffer. The total resuspension volume should be 400 µl for the FACS sample plus 100 µl per control. Avoid air bubbles during resuspension by setting the volume of the pipette to less than the total volume.

26| Distribute the resuspended cells into labeled 15-ml Falcon tubes for each control (100 µl) and the FACS sample (400 µl).

27| Add antibodies to the resuspended cells at the following concentrations:

	CD13-FITC	CD31-APC	CD41-PE	CD45-PE
Cat. no. (BD)	558744	551262	558040	553081
Recommended dilution (vol/vol)	1:20	1:50	1:200	1:200
Final concentration	25 µg/ml	4 µg/ml	1 µg/ml	1 µg/ml

▲ **CRITICAL STEP** The FACS sample is immunostained with all antibodies. Important controls include unstained cells, and single-color positive controls for each antibody (CD13-FITC, CD31-APC, CD41-PE, and CD45-PE). FMO controls (each combination of all antibodies but one), as well as isotype controls, should also be performed. Running all controls is essential for setting up the gates and compensation, and for each new batch of antibody (**Supplementary Fig. 2**).

28| Incubate cells for 20 min on ice without agitation. Cover to protect from light.

29| Wash the cells by adding 4 ml of HBSS/BSA/glucose buffer and then centrifuge at 300*g* at 4 °C for 5 min.

30| While cells are centrifuging, prepare DAPI working solution (HBSS/BSA/glucose buffer plus 1:500 (vol/vol) DAPI stock solution). Make the same total volume used for resuspending the cells in Step 26.
! **CAUTION** DAPI is a known mutagen and should be handled with care. Collect all DAPI-containing solutions for disposal according to institutional guidelines.

31| Discard the supernatant by decanting. Resuspend the cells in DAPI working solution.

32| Filter the cells through a 40-µm cell strainer.

33| Place samples and FACS collection tubes on ice for transport to a FACS facility. Keep cells on ice and in the dark at all times.
▲ **CRITICAL STEP** FACS should be performed as soon as possible.

FACS ● TIMING ~1 h
34| Use unstained controls and single-color controls to set the FACS parameters (**Supplementary Fig. 2**).

35| Use the Forward-scatter-A (FSC-A) and the Side-scatter-A (SSC-A) channels to exclude debris (**Fig. 6**, top left).

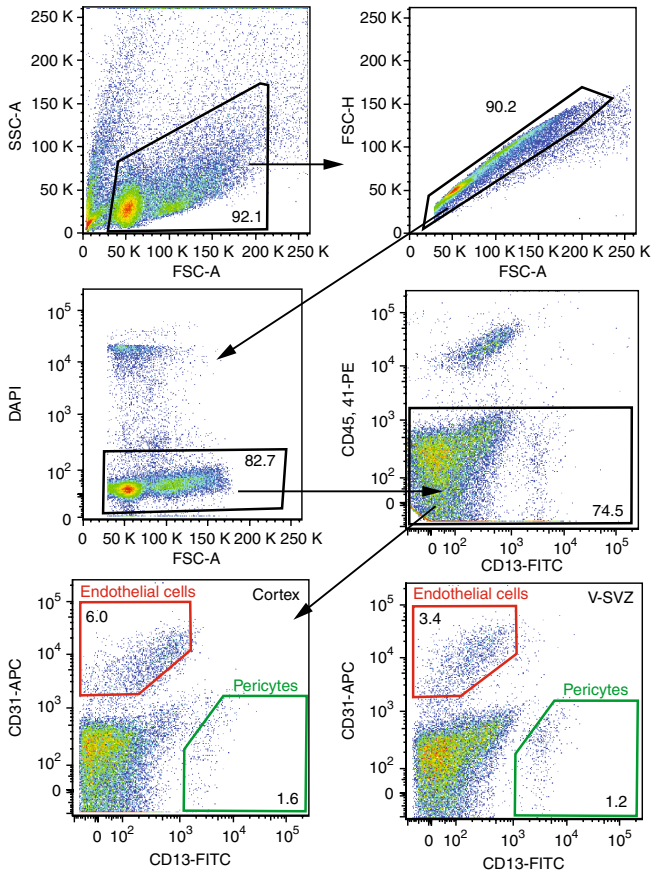

Figure 6 | Representative FACS plots showing the gating strategy for the purification of endothelial cells and pericytes from the adult mouse brain. After excluding debris (top left), doublets (top right), and dead cells (DAPI, center left), CD45⁻CD41⁻ cells are selected (center right) and CD31⁺ CD13⁻ endothelial cells and CD31–CD13⁺ pericytes are collected (cortex, bottom left; V-SVZ, bottom right). Center right and bottom images were displayed using auto biexponential display. Percentages refer to the proportion of cells in the previous parent gate. Plots show 100,000 events. FSC-A, forward-scatter area; FSC-H, forward-scatter height; SSC-A, side-scatter area. Adapted with permission from Crouch *et al.*[21], Regional and stage-specific effects of prospectively purified vascular cells on the adult V-SVZ neural stem cell lineage, *J. Neurosci.*, vol. 35, Copyright 2015; permission conveyed through Copyright Clearance Center. Experiments were performed with the approval of the Columbia University IACUC and the Basel-Stadt cantonal veterinary office.

36| Use pulse-processing of Forward-scatter-A (FSC-A) versus Forward-scatter-H (FSC-H) to exclude cell doublets and aggregates (**Fig. 6**, top right).

37| Gate the population that is DAPI-negative to exclude dead cells (**Fig. 6**, center left).
? TROUBLESHOOTING

38| Run FMO control samples and isotype controls to set appropriate gates for each antibody (**Supplementary Fig. 2**).

39| Exclude CD45⁺ and CD41⁺ cells (**Fig. 6**, center right), and collect endothelial cells (CD31⁺CD13⁻ CD41⁻CD45⁻) and pericytes (CD13⁺CD31⁻ CD41⁻CD45⁻) (**Fig. 6**, bottom panels) in 5-ml FACS collection tubes.

Box 3 | Culturing FACS-purified cells and preparing conditioned medium

This box describes how to culture FACS-purified cells and how to prepare conditioned medium.

Procedure

1. Prepare collagen-coated dishes (Reagent Setup) at least 1 d before FACS.
2. After collection of cells by FACS in Step 40 of the main PROCEDURE, centrifuge at 300*g* at 4 °C for 10 min. Resuspend FACS-purified cells in 500 µl of EGM-2 media and plate in one well of a 24-well plate that has been coated with collagen. One cortex yields enough cells for one well of a 24-well plate. For V-SVZ, which has a much lower cell yield, FACS-purified cells must be pooled from separate sorts. We pooled purified endothelial cells or pericytes from four separate FACS experiments in one well of a 24-well plate. After 1 h in culture, the cells will start to attach, and they will begin to assume their stereotypical shape after 24 h in culture. Change the medium every 2–3 d.

? TROUBLESHOOTING

3. To prepare conditioned medium, culture cells until they are >50% confluent.

▲ **CRITICAL STEP** Cells must be >50% confluent to obtain adequate concentrations of soluble factors in conditioned medium.

4. Wash the cultured cells with 1× PBS to remove the EGM-2 medium, which contains many exogenous growth factors and serum.
5. Replace with minimal medium appropriate for the particular experiment and condition the medium for 24 h.

▲ **CRITICAL STEP** We recommend using fresh conditioned medium for experiments with V-SVZ cells.

■ **PAUSE POINT** Conditioned medium can be stored at –80 °C for up to 3 months. Fresh and frozen conditioned media should be compared when first used for a new assay.

▲ **CRITICAL STEP** Do not make conditioned medium from cells that have been in culture longer than a month. For conditioned medium experiments that lack growth factors, we recommend using primary vascular cells for no longer than 2 weeks.

6. (Optional) After conditioned medium experiments, vascular cells may be fixed and immunostained for quantification or to examine the expression of different markers in culture (see **Fig. 7** for an example).

▲ **CRITICAL STEP** Cells must be collected at low pressure (13 p.s.i.) to optimize viability. Use a 100-µm nozzle aperture.

▲ **CRITICAL STEP** Collect cells in prefilled FACS tubes to cushion them. It is important to note that the volume of liquid in the collection tube will increase as cells are collected.

? TROUBLESHOOTING

40| Keep sorted cells on ice after FACS. We recommend that cells be plated in cell culture or processed for other applications as soon as possible, as further delays may compromise viability.

Post-FACS processing ● TIMING ~15 min

41| After FACS, spin down cells at 300*g* at 4 °C for 10 min. Resuspend the cell pellet in an appropriate volume for downstream applications. For culture, resuspend them in EGM-2 medium (**Box 3** and **Fig. 7**). For single-cell profiling, resuspend cells at the desired concentration in a solution appropriate for platform to be used. Alternatively, cell pellets may be used immediately for RNA isolation (as described in Codega *et al.*[52]).

? TROUBLESHOOTING

■ **PAUSE POINT** Cell pellets can be stored at –80 °C for up to 6 months before RNA isolation.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

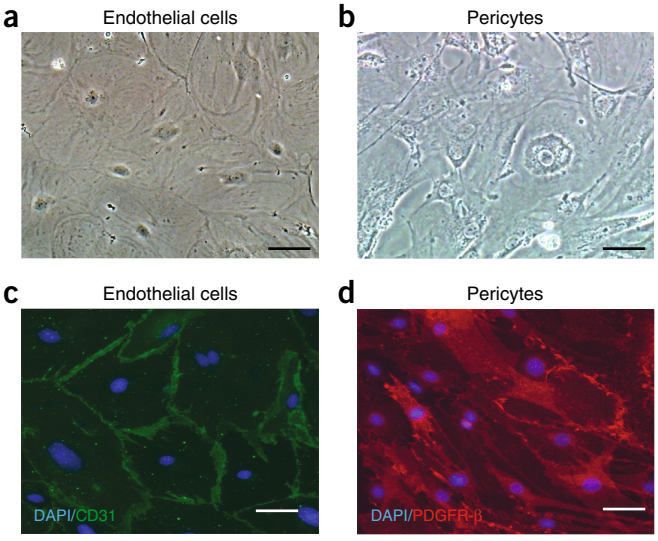

Figure 7 | Morphology and immunostaining of cultured endothelial cells and pericytes. (**a**,**b**) Phase contrast images of endothelial cells (**a**) and pericytes (**b**) after 2 weeks in culture. (**c**,**d**) Immunostaining for CD31 in cultured endothelial cells (**c**) and for PDGFR-β in cultured pericytes (**d**). Note the typical cobblestone morphology of the endothelial cells. Scale bars, 70 µm. Reproduced with permission from Crouch *et al.*[21], Regional and stage-specific effects of prospectively purified vascular cells on the adult V-SVZ neural stem cell lineage, *J. Neurosci.*, vol. 35, Copyright 2015; permission conveyed through Copyright Clearance Center. Experiments were performed with the approval of the Columbia University IACUC and the Basel-Stadt cantonal veterinary office.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
20	After collagenase/dispase digestion, large chunks of tissue remain	The tissue was not minced into small enough pieces in Step 13	Mince tissue into smaller pieces
		Collagenase/dispase digestion time was not optimized	Choose one concentration and vary the time of digestion (e.g., 15 min, 30 min, and 45 min). Then compare the yield and proportion of different cells types by FACS. With too little time, the cells will be predominantly dissociated mechanically, and cell viability will be compromised. At ideal enzyme concentrations, collagenase/dispase digests the basement membrane to liberate the cells without affecting their cell-surface receptors. With too much time, the epitopes may be cleaved, resulting in lower cell yield and altered ratios of endothelial cells and pericytes
		Falcon tubes were not properly placed in the rotisserie in the hybridization oven	Ensure that all of the pieces of tissue in each digestion tube continue to be thoroughly mixed throughout the digestion and that no pieces remain stuck to the sides of the tube
		The ratio of collagenase/dispase to tissue is incorrect	The amount of tissue in each digestion tube is important. We recommend splitting the entire cortex (both hemispheres) from one mouse into three digestion tubes. We recommend using one digestion tube for the V-SVZs (two hemispheres each) for approximately three mice (three tubes for ten mice)
		Collagenase/dispase enzyme is old	After reconstitution, frozen aliquots of collagenase/dispase should be used within 2 weeks. Thereafter, it loses activity. Once stocks are thawed from –20 °C, do not refreeze, as enzyme activity may be compromised in subsequent experiments. Make aliquots of the appropriate volume for your experiment
37	<70% of cells are viable by DAPI staining	Time of dissection was too long, or digestion and trituration were not optimal	See advice for Step 39 below
39	Endothelial or pericyte yield is less than expected	Time of dissection is too long	As a general rule, the total dissection time should not exceed 2 h, or cell viability will be compromised. It is preferable to do more rounds of FACS rather than to increase starting material beyond this point. Multiple brain areas can be obtained from a single mouse to reduce mouse numbers
		The amount of starting material is not optimal	Too much starting material will greatly increase the time of the protocol and decrease cell viability. With too little material, the conditions may be too harsh
		Trituration was not optimal	Obtain a small aliquot (~3 µl) from the triturated sample and examine under a ×10 or ×20 objective. The majority of cells should be single cells, but the occasional doublet ensures that the tissue was not overly triturated. Do not triturate >100×, and do not introduce air bubbles (i.e., avoid foaming)
		Aspiration was used to remove the supernatant	Always use decantation to remove liquids
		Fluorophore has degraded	PE and APC are bright and degrade less quickly, but FITC is dimmer and degrades quickly. Use CD13-FITC within 2 weeks of opening. New fluorophore-conjugated CD13 antibodies are now available that are brighter and more stable

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
39		Gates were not set properly	Unstained, single-color, and multicolor controls should be performed
		Too much sample was used for controls	Use fewer cells for controls
		Pressure settings incorrect on FACS machine	Use low pressure and a 100-µm aperture nozzle to collect vascular cells
		FACS events and true cell yield are not equivalent	To determine the efficiency, or actual cell/event ratio, after centrifugation of cells at Step 41, resuspend cells in 500 µl of EGM-2 medium. Plate volume containing 100 FACS-machine-determined events in one well of a 96-well plate. Then either use a live cell dye such as Vybrant dye (1:1,000) and count the number of fluorescent cells on a microscope, or fix the cells and stain with DAPI, and count the actual cell number. Divide the actual number of cells by the number of FACS events to determine sorting efficiency. We did not detect differences in the sorting efficiency between populations, but it is important to test this for each FACS machine
	Expected pericyte and endothelial cell ratios are not obtained	Digestion conditions were suboptimal	Vary digestion time and/or enzyme concentration to balance cell dissociation and epitope preservation
41	RNA yield from FACS-sorted cells is low	RNA is being degraded	Use proper technique for working with RNA. Clean all instruments and surfaces with RNase Away (or a similar product). Use RNase-free pipette tips and Eppendorf tubes
		Sorted populations have low RNA content	Increase the number of collected events per well or tube
Box 1, step 1	The meninges adhere to the brain or are difficult to peel off	The brain tissue has dried	Ensure that the brain is always covered with liquid
		Holes have been made in the meninges and they come off in small pieces	Insert the flat portion of the forceps into a major sulcus to obtain a firmer grip on the meninges. A good place to do this is between the two hemispheres on the top surface of the brain, or on the lower surface of the brain (**Supplementary Fig. 3**)
Box 3, step 2	Number of cells in culture 24 h after FACS is less than expected	Actual cell number is less than the FACS event number	Count the actual cell number after FACS to determine the FACS-sorting efficiency. Assess survival after 24 h with DAPI or Vybrant dye
		Cells at very low confluence do not survive well	Increase the amount of starting material or pool FACS experiments

● TIMING

Steps 1–5, reagent preparation: ~30 min
Steps 6–8, harvesting of brains: ~10 min for a group of five mice
Steps 9–12, microdissection of brain region of interest: ~3–5 min per brain area per mouse
Steps 13–21, dissociation to single cells: ~45 min
Steps 22 and 23, elimination of myelin with Percoll: ~20 min
Steps 24–33, immunostaining: ~20 min
Steps 34–40, FACS: ~1 h
Step 41, post-FACS processing: ~15 min

ANTICIPATED RESULTS

The protocol described here allows the simultaneous FACS purification of primary endothelial cells and pericytes from brain microregions, which can be used for diverse downstream applications. It should be easily implementable in a laboratory with standard tissue culture equipment and access to a FACS facility or sorter. An important consideration in FACS is that the final cell yield depends on the amount of starting material, as well as the dissociation and digestion conditions that must be established to preserve epitopes of interest. Moreover, FACS event number is often 10–30% higher than the actual cell number obtained. For some brain regions, samples should be pooled from multiple sorts if a larger number of cells are needed. To obtain healthy cultures, endothelial cells and pericytes should be at least 50% confluent (ideally 75%).

The approximate expected yield from one cortex or ten V-SVZs is shown below:

Yield	Cortex endothelial cells	Cortex pericytes	V-SVZ endothelial cells	V-SVZ pericytes
Number of events	100,000	20,000	30,000	10,000
Percentage (% parent ($CD41^-$ $CD45^-$))	5–10%	0.6–1.2%	1.5–3%	0.4–1%

In sum, this protocol opens up new avenues to dissect the roles of brain endothelial cells and pericytes, as well as to explore heterogeneity of endothelial cells and pericytes *in vivo*.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS E.E.C. designed the research, performed research, analyzed data, and wrote the paper. F.D. designed the research, analyzed data, and wrote the paper.

COMPETING INTERESTS The authors declare no competing interests.

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