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Journal

Nature Protocols, 19(3)

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Publication Date

2024-03-01

DOI

10.1038/s41596-023-00929-1

Peer reviewed

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HHS Public Access
Author manuscript
Nat Protoc. Author manuscript; available in PMC 2024 November 05.

Published in final edited form as:
Nat Protoc. 2024 March ; 19(3): 603–628. doi:10.1038/s41596-023-00929-1.

Profiling human brain vascular cells using single-cell transcriptomics and organoids

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Abstract

Angiogenesis and neurogenesis are functionally interconnected during brain development. However, the study of the vasculature has trailed other brain cell types because they are delicate and of low abundance. Here we describe a protocol extension to purify prenatal human brain endothelial and mural cells with fluorescence activated cell sorting (FACS) and utilize them in downstream applications, including transcriptomics, culture, and organoid transplantation. This approach is simple, efficient, and generates high yields from small amounts of tissue. When

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#These authors share equal contribution.

Author contributions statements

E.E.C. and E.J.H. conceived the project and designed the experiments. E.E.C. performed FACS experiments, 2D culture experiments, and organoid transplants. L.N.D performed FACS experiments with passaged cells and organoid transplants. E.J.V. performed FACS experiments. J.O.B., K.W-P., J.C., T.J., and A.B. performed scRNA-seq bioinformatics. L.N.D., A.B., and M.G.A. performed and designed organoid experiments. E.E.C and E.J.H. wrote the manuscript with inputs from all authors.

Competing interests: The authors have no competing interests to report.

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the experiment is completed within 24 hours post-mortem interval, these healthy cells produce high quality data in single-cell transcriptomics experiments. These vascular cells can be cultured, passaged, and expanded for many in vitro assays, including Matrigel vascular tube formation, microfluidic chambers, and metabolic measurements. Under these culture conditions, primary vascular cells maintain expression of cell-type markers for at least three weeks. Finally, we describe how to use primary vascular cells for transplantation into cortical organoids, which captures key features of neurovascular interactions in prenatal human brain development. In terms of timing, tissue processing and staining requires approximately 3 hours, followed by an additional 3 hours of FACS. The transplant procedure of primary, FACS-purified vascular cells into cortical organoids requires an additional 2 hours. The time required for different transcriptomic and epigenomic protocols can vary based on the specific application, and we offer strategies to mitigate batch effects and optimize data quality. In sum, this vasculo-centric approach offers an integrated platform to interrogate neurovascular interactions and human brain vascular development.

EDITORIAL SUMMARY:

Protocol extension describing the purification of prenatal human brain endothelial and mural cells with FACS, and their utilization in downstream applications including cell culture, organoid transplantation, and single cell transcriptomics.

PROPOSED TWEET:

Purification of prenatal human brain endothelial and mural cells with FACS and their downstream applications

PROPOSED TEASER:

Profiling human brain vascular cells

Introduction:

Initially perceived as passive conduits to deliver oxygen and nutrients, blood vessel cells are now recognized as major contributors to brain health and disease[1,2]. During brain development, genetic and environmental factors which impact brain vascular cells can evolve into long-term neurological deficits[3,4]. In the adult brain, dysfunction in brain vascular cells contributes to arterio-venous malformations (AVMs), stroke, neurodegeneration, and glioblastoma[5-8]. These diseases highlight the critical role of vascular cells for building and maintaining a healthy brain. Further investigations into the role of vasculature in many brain pathologies offer an untapped opportunity to ameliorate neurological disease.

The primary building blocks of the vasculature are endothelial and mural cells. In the brain, endothelial cells form the vascular lumen and exhibit special features, including tight junctions and highly selective transcytosis, which together establish the blood-brain barrier to insulate the brain from potentially toxic substances in the blood[9]. Situated between the endothelial cell lumen and the surrounding astrocytes, microglia, and neurons, mural

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cells serve as a critical signaling hub of the neurovascular unit. Mural cells, which include smooth muscle cells, pericytes and fibroblasts, are critical components for the development of the blood-brain barrier[10,11]. Mural cell-secreted vitronectin is one known molecular signal that regulates barrier formation[12]. Other pathways which regulate endothelial and mural cell communication in the prenatal human brain include Collagen, Laminin, Midkine, Notch, Fibronectin, and VEGF[13]. Mural cells also control neurovascular coupling[14-16] and contribute to inflammation and immune cell influx into the brain[17,18]. In addition, both endothelial and mural cells have “angiocrine” functions, secreting factors which promote neural stem cell proliferation and differentiation[2,13]. Despite the critical importance of the vasculature for brain function and dysfunction, our understanding of these cells is limited. This is due in part to the fact that vascular cells represent only 5-10% of the total cell population in the brain[13] and therefore enrichment is required to study these cells in depth. Despite the critical roles of vascular cells, there is a dire need for efficient and integrated approaches to isolate, culture, and profile these cells in a reproducible fashion.

Development of the protocol

Overview—Here, we describe an integrated strategy designed to isolate, enrich and profile vascular cells from prenatal human brain using single-cell transcriptomics and transplantation into cortical organoids. The first stage of this protocol involves purifying endothelial, mural, and perivascular immune cells with fluorescence-activated cell sorting (FACS). These vascular cells can then be cultured in isolation in 2D and 3D conditions to characterize their functional properties, including bioenergetics. In parallel, these cells could be used in single-cell transcriptomics and multi-omics technology to investigate their molecular and cellular diversity. Finally, transplantation of these primary vascular cells into cortical organoids (“vascularizing organoids") provides an effective model to study the intricate interactions between angiogenesis and neurogenesis (Figure 1).

Cell type isolation—To isolate vascular cells from prenatal human brain tissues, we adapted our previously published protocol that was designed for the adult mouse brain[19,20]. First, prenatal human brain tissue from the second trimester was minced with a scalpel and digested with collagenase/dispase, triturated with DNase, and centrifuged through 22% Percoll to remove debris (Figure 2). By mining the single-cell transcriptomics data from prenatal human brain[21], we identified CD31 (PECAM1), ANPEP (CD13), and CD45 (PTPRC) as an effective combination to isolate endothelial and mural cells from the prenatal human brain, and simultaneously exclude perivascular myeloid cells (Figure 1,2). Flow cytometry confirmed the validity of these markers to isolate distinct populations of endothelial cells ($CD45^{-}$;$CD31^{+}$;$ANPEP^{-}$), mural cells ($CD45^{-}$;$CD31^{-}$;$ANPEP^{+}$), and perivascular immune cells ($CD45^{+}$;$CD31^{-}$;$ANPEP^{-}$) (Figure 3a). As further confirmation, we found the cell surface marker CD146, an alternative mural cell marker[22], also co-labeled with ANPEP (Figure 3a). We have found this protocol is also successful with purification of fresh, adult human brain endothelial cells, but not mural cells. Likely this result is due to changes in the expression of cell surface markers. CD31+ endothelial cells could be identified and purified, but ANPEP expression was absent and CD146 expression was very low (Figure 3b), in agreement with other published data on adult human brain endothelial and mural cells[6,23]. As far as we can determine, all of the prenatal endothelial and mural

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cells are captured using CD31 and ANPEP, respectively. This conclusion is supported by the presence of all known subtypes, including fibroblasts, in the single-cell RNA sequencing (scRNA-seq)[13]. In addition, we also performed single-cell RNA sequencing of two additional populations, the perivascular immune cells ($CD45^{+}$;$CD31^{-}$;$ANPEP^{-}$) and $CD45^{-}$;$CD31^{-}$;$ANPEP^{-}$;$CD146^{+}$ cells (predominantly radial glia[21]). These populations do not express vascular markers, including *PECAM-1/CD31, CD34, TIE1,* and *RGS5* (Figure 3c). *PDGFRB* is highly expressed in mural cells, but also produced by radial glia in the human brain[24].

Cells in culture—Next, we developed in vitro cultures to characterize the morphological and functional properties of the endothelial cells and mural cells (Figure 4). Traditionally, primary endothelial cells are difficult to passage as they quickly become quiescent in culture[25]. The commercially available human umbilical vein endothelial cells (HUVEC) may be used for this purpose. However, we and others have found significant lot to lot variability in these cells[26,27]. We therefore developed our own prenatal human brain endothelial and mural cells as a cell source for experiments in culture. Interestingly, prenatal human brain endothelial and mural cells isolated with FACS can then be cultured and passaged in the EGM-2 media (Figure 4a). We have not used other media extensively, but there are other choices for vascular cells, including Smooth Muscle Cell Media (PromoCell) and Pericyte Media (ScienCell). More than 90% of mural cells in 2D cultures express PDGFR-β^{+} (Figure 4b). Similarly, >80% of endothelial cells express CD31 (Figure 4c). In addition, these vascular cells can be used in the 3D Matrigel tube formation assay, Seahorse assays, and other applications[13].

Organoid transplantation—Our recent study shows that nascent vasculatures are more enriched in the ventricular zone and subventricular zone (VZ/SVZ) in the pallium and germinal matrix, suggesting the presence of mutual interactions between angiogenesis and neurogenesis in these neurogenic niches in prenatal human brain[13,28]. Since iPSC-derived cortical organoids recapitulate early stages of human cerebral cortex development, the organoids offer a logical model to provide more mechanistic insights into these interactions. Notably, one inherent challenge in iPSC-derived cortical organoids is the lack of vasculature that provides sufficient nutrients to support neural development. While there is increasing effort to complement organoid models with vascular cells [29-31], no previous studies have added brain-specific vascular cells to the cortical organoids. To test the developmental potentials of vascular cells and their interactions with neural progenitors, we transplanted FACS-purified endothelial and mural cells into 7-9 week-old iPSC-derived cortical organoids, which mimic cortical development during the second trimester[32] (Figure 1, 5). Since the primary human brain vascular cells are also from the second trimester, this approach to vascularize cortical organoids offers an unique advantage to interrogate neurovascular interactions in prenatal human brain. Prior to transplantation, endothelial and mural cells were labeled by AAV-CMV-GFP, which allowed us to track the transplanted cells as they migrated and integrated into the organoids (Figure 5a). Following transplantation, we maintained organoids for two weeks, and then fixed and analyzed them with immunohistochemistry and RNAscope[13] (also Figure 5b, c).

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Transcriptomics—Finally, prenatal human brain endothelial and mural cells can be used in transcriptomics studies[13]. Critically, the isolation of fresh cells with FACS allows whole-cell transcriptomics, which can facilitate a wider possibility of bioinformatic data analysis, such as RNA velocity.

Applications of the method

This protocol is applicable to a wide variety of questions in neuroscience, developmental and vascular biology. With the increasing popularity of neuroimmunology, the microglia obtained through this procedure can also be employed to study vasculature-microglia interactions during brain development[33]. In addition, delivering therapeutics across the blood-brain barrier (BBB) has been a longstanding challenge for bioengineers, biologists, and chemists[34-36]. Our protocol offers direct investigations into the molecular and cellular properties of a diverse population of vascular cells that will allow us to resolve the challenges to cross the BBB.

Each of three core components in this protocol – FACS, in vitro cultures, and transcriptomics – can be used in multiple applications and iterations. First, the unique protocol for tissue dissociation and FACS-based validations can be used to analyze the cellular composition of vascular and perivascular immune cells in samples of interest from different developmental ages and different pathologies. Our protocol uses only 5 fluorophores, which can be expanded to up to 16 with conventional flow cytometry or dozens more with high-parameter flow. With renewed interest in understanding different subtypes of vascular and perivascular cells, inclusion of more FACS channels in this protocol will offer flexibility to select different subpopulations. Second, this protocol provides a comprehensive approach using in vitro cultures, including the vascular cells in isolation or their interactions with neural cells in cortical organoids[13]. For instance, these cells can be grown and passaged in 2D culture for proliferation assays or in Matrigel to examine their potential for vascular tube formation[13]. In these assays, anti-angiogenic or angiogenic factors can be added or inhibited to interrogate their role on vascular proliferation and tube formation. The vascularization of organoids is increasingly popular[29-31,37]; our detailed protocol will allow widespread application of primary cells for this purpose, which we demonstrated have important implications[13]. Our protocol will open new directions for future research to improve the environmental milieu for cortical organoids and to further elucidate mechanisms that govern the interaction between angiogenesis and neurogenesis in prenatal human brain. Finally, our protocol can isolate diverse populations of primary brain vascular cells, which provide critical resources for single-cell RNA sequencing to characterize the molecular mechanisms for angiogenesis in prenatal human brain[13]. In addition to scRNA-seq, these cells could alternatively be utilized with multi-omics, in bulk or single-cell, for proteomics and epigenomics. This protocol can be easily adapted to profile vascular cells in brain tissues from different brain regions, different ages, and different pathologies.

Comparison with other methods

For vascular cell dissociation, the key advantages to this protocol are its simplicity and efficacy to isolate a diverse population of vascular cells from human brain. In our experience

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and others', vascular cell dissociation requires careful selection of enzymes as mechanical dissociation is especially detrimental to endothelial cells[38]. With relatively few steps and inexpensive reagents, robust yields of healthy vascular and perivascular cells can be obtained in 6 hours. This protocol evolved from our previous strategy to purify vascular cells from the adult mouse brain, focused on the cortex and ventricular/subventricular zone[19,20]. Surprisingly, we found that similar endothelial and mural cell surface markers could be used in the prenatal human brain. For embryonic mouse and adult human brain, different mural cell markers are required since ANPEP is not highly expressed[38] (also Figure 3a, b).

To contrast the different approaches, we compared our dissociation strategy and vascular transcriptomics data with those obtained from 3 other recent studies that also characterize human brain vascular cells [5,6,23] (Table 1). Key differences include the use of fresh tissue to isolate whole cells versus the use of frozen tissues for nuclei isolation. In the initial dissociation of the tissue, some protocols use a refined approach with scalpels and microdissection, whereas strategies with frozen tissue require homogenization. With respect to fresh tissues, we found that enzymatic digestions are much preferable to mechanical dissociation for vascular cells[13,19,20] (Figure 6a). However, choosing the correct enzyme that digests substrates of interest without also cleaving important marker proteins requires optimization. We selected collagenase/dispase for these reasons, since digestion of the basement membrane is critical[13,19,20]. We did not find any dramatic improvements with the newer liberase enzymes (Figure 6b), which are also more expensive. Papain works well for isolation of neural cells, but fails to digest the vascular basement membrane[39]. We also found very few vascular cells present after papain digestion (Figure 6c). Some protocols also use centrifugation and gradients to purify vascular cells. We found that one 10 minute Percoll step was sufficient to remove debris, and the vascular cells could be identified with FACS. Further centrifugation steps were not required in our hands.

FACS offers improved results compared to the vascular cells isolated with magnetic beads. Beads are less efficient at capturing rare cells and therefore are more suitable for enrichment of populations rather than purification (Figure 6d). Finally, ANPEP is an excellent marker for mural cells in the prenatal human brain, and does not have a magnetically conjugated antibody at this time. Unconjugated antibodies require sequential purification steps, similar to primary and secondary immunohistochemistry, which decrease yield further. We found that 50% of cells isolated with CD31 magnetic beads were $CD31^{+}$ with flow cytometry but after 3 sequential magnetic bead isolation steps, no $ANPEP^{+}$ mural cells could be identified (Figure 6d). In addition, the vascular cells purified with our strategy are sufficiently healthy and robust to permit long-term culture and passaging. This feature enables diverse and exciting potential directions with primary human brain vascular cells.

Since this protocol uses fresh tissue, post-mortem interval (PMI) is critical to obtain high quality cells and data. Based on our experience, we observe that completing the protocol within 24 hours PMI is important for cell viability (Figure 6e). Interestingly, while endothelial cells from samples with greater than 24 hours PMI are more sensitive and exhibit dramatic decreases in yield and transcriptomic data, mural cells may be able to survive in cultures and produce consistent transcriptomic data after this period (Figure 6f).

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Another method to co-culture acutely purified cells in a native microenvironment is slice culture. The main advantages to the organoid approach include the ability to study neurovascular interactions over longer periods of time and the robustness of the 3D structure[40,41]. In addition, since the organoid protocol involves a stereotyped differentiation process, the resulting neural cells are relatively homogenous at a specific stage and provide better experimental control[32,42]. In theory, like organoids in general, vascular cell transplants can be maintained indefinitely. However, potential changes in the primary cells may occur due to the in vitro environment.

Overview of the Procedure

We provide a detailed Procedure covering the dissociation of prenatal brain tissue and the isolation of cells (Steps 1-35) and the recovery of mural and endothelial cells through FACS (Steps 46-55). This includes details on the preparation of control samples using cells (Steps 36-40) and beads (41-45). Then we describe how to culture the FACS-purified cells (Steps 56-76), and characterize them with FACS (77-95). Alternatively, FACS-purified cells can be transplanted on top of organoids to study neurovascular interactions (Steps 96-114). Finally, we offer thorough instructions on bioinformatic pipelines to analyze single-cell RNA sequencing data (Steps 115-132).

Experimental design

To optimize these experiments, there are key considerations in regards to starting material (brain tissue), flow cytometry, transcriptomics, and organoid transplantation.

Starting material—This protocol as written is focused on fresh, non-frozen, prenatal human brain tissue. Any brain region may be selected; we have performed hundreds of experiments on both cortical and subcortical regions, including the ganglionic eminences, due to our interest in angiogenic and neurogenic interactions[13]. One key consideration is post-mortem interval. We have obtained high-quality sequencing data from samples, which were procured within 24 hours PMI, and were dissociated, digested, purified with FACS, and completed the first stage of the 10x protocol (Gem Generation and Barcoding). In contrast, samples with more than 24 hour PMI showed loss of endothelial cell surface markers via FACS (Figure 6e) and sequencing (Figure 6f). Adult human brain endothelial cells can be isolated with this protocol, but not mural cells (Figure 3b). We have not attempted to perform this protocol on frozen tissue, but other protocols are available for FACS-based isolation of endothelial cell nuclei from fresh frozen tissue[43].

Flow cytometry—Controls are a necessary part of any FACS experiment. Isotype controls should be included for each fluorophore to ensure that the antibody is specific. In addition, each experiment requires single color controls to compensate the flow cytometry software prior to each experiment. Fluorescence minus one (FMO) controls, in which every antibody except one is added, offer the most robust method to ensure the population of interest is collected.

Transcriptomics—We obtained high-yield transcriptomic data starting with 1,000-10,000 cells per reaction for the 10x Next GEM 3' Reagent Kits Version 3.1. More recent High

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Throughput Kits can process up to 20,000 starting cells. Each user should empirically determine the "event to cell ratio" from their FACS machine by calculating the actual number of cells obtained after FACS and comparing to the event number derived from the machine. This calcuation will ensure the transcriptomic reactions are starting with sufficient and not overwhelming amounts of RNA. For sequencing depth, we used 50,000 reads per cell as recommended by 10x. The user could employ sequencing saturation, based upon PCR duplicates in the 10x Cell Ranger software, as a readout for whether more sequencing is desired. When analyzing the transcriptomic data, there are many ways to assign cell type identities to clusters found through unsupervised clustering. In this protocol, we employ two distinct methods: ScType and manual labeling. ScType is a faster, more convenient, and more reproducible labeling approach. This approach is particularly useful when cell types are well-characterized and have clear transcriptional markers. Conversely, manual labeling allows for greater flexibility and control over the labeling process. This approach is more suitable when dealing with potentially novel or rare cell types that ScType might not detect.

Organoid transplants—For these experiments, we used a (dorsal) forebrain-directed organoid protocol to restrict the organoids to cortex like cells[13]. Unguided protocols, without manipulation of developmental signaling molecules, lead to a mix of cells from different brain regions and are challenging to use because they are not as reproducible/consistent[44]. Our transplantations were performed at 7-9 weeks in culture because this stage corresponds to human brain development during the second trimester[32]. We expect that the framework of this protocol should function well for any time of brain organoid, but scientists who are interested in other brain regions or stages of development may need to optimize their experiments. We used this experimental paradigm to determine the effects of vascular cells on neural cells, and vice versa, after 2 weeks of co-culture, and have cultured transplanted endothelial and mural cells up to 4 weeks[13]. For controls, it is important to include the transplantation of additional populations, either other FACS-purified cells or a cell line. These controls will ensure that your results cannot be attributed to the effects of any addition cells in the organoids. The commercially available 3T3 fibroblasts may be used for this purpose.

Expertise needed to implement the protocol

Isolation and purification of the cells with flow cytometry requires expertise in running the machines and setting up the software to correctly isolate the populations of interest. Flow cytometry core facilities can often assist with these standard procedures. As written, this protocol needs only 4 fluorescent markers plus DAPI or a different live/dead stain. While additional markers can be added, this relatively simple panel serves as an achievable foundation. The generation of cortical organoids is beyond the scope of this protocol[32,45,46], but the transplantation of primary brain vascular cells and their maintenance should be feasible for anyone with experience in cell culture. Finally, the isolated cells can be used in infinite omics applications depending on the current technology. We offer strategies for the initial stages of transcriptomics data analysis based on our experiments with common pitfalls, such as batch correction.

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Limitations

This protocol utilizes fresh tissues which provides the advantage of being able to perform whole-cell transcriptomics and culture of the cells after isolation. However, the tissues must be processed quickly, within 24 hours. We have not tested it extensively, but Miltenyi cell storage solution has been developed to allow an optimized storage of fresh organ and tissue samples up to 48 hours (Miltenyi Biotech). This solution may be a reasonable alternative if samples cannot be processed quickly. We have not tried our methodology on fresh frozen samples or postnatal tissue, which may require a different approach. Although we can analyze adult human brain endothelial cells with flow cytometry using this protocol[13] (also Figure 3b), we have not attempted to culture or perform omics experiments on adult brain vascular cells. Our protocol requires access to flow cytometry, although this technique is increasingly accessible[23,38,47]. In addition, flow cytometry requires cell surface markers to purify and culture cells. Intracellular markers may be feasible for analysis[48], but the current staining procedures kill the cells. Finally, we performed our experiments using 10x reagents, but there are emerging companies which also offer transcriptomic reagents at potentially lower cost. The principles described in this protocol should be applicable for a wide variety of omics experiments and vendor sources.

Materials

Critical: Downstream applications of cell dissociation/digestion/Flow Cytometry require sterility. As such we recommend all equipment and reagents be sterilized in accordance to institutional guidelines. Specific recommendations for solutions are provided.

Biological Material

- Human brain tissue from pre or postnatal ages. We have tested this protocol in first, second, and third trimester tissue as well as adult human brain samples[13]. **Caution!** Local institutional guidelines should always be followed when using human source materials since they can contain blood-borne pathogens. These protocols likely will including trainings and proper engineering and personal barrier protections. Caution! Tissues should be acquired with strict adherence to local guidelines for human subjects protection, including Institutional Review Board (IRB) and/or Human Gamete, Embryo and Stem Cell Research (GESCR) Committee approval. Critical! An equal number of male and female tissues should be studied to ensure applicability. Critical! This protocol is optimized for enzymatic digestions of approximately 1 cm^3 human brain tissue. If the enzyme cannot effectively digest the tissue due to overwhelming amount of material, the dissociation will occur with mechanical digestion. Vascular cells in our experience are very sensitive to mechanical digestion and yield will be severely compromised. More starting material requires scaling up the reagents, number of tubes used for digestion, and all subsequent steps. (see the Troubleshooting advice for step 54).

Reagents

- 100X Antibiotic-Antimycotic (Gibco cat. no. 15240096)

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- 1X PBS pH 7.4 (Gibco cat. no. 10010031)
- 10X PBS pH 7.4 (Gibco cat. no. 70011044)
- 1X HBSS (Life Technologies cat. no. 14175-095)
- 35% (wt/vol) BSA solution (Sigma-Aldrich cat. no. A7979-50ML)
- 70% (vol/vol) Ethanol (Fisher Scientific cat. no. 04-355-305)
- Bovine Collagen Solution (Stem Cell Technologies cat. no. 4902)
- Collagen/Dispase (Sigma-Aldrich cat. no. 11097113001)
- UltraComp eBeads (Invitrogen cat. no. 01-2222-42)
- DAPI (Invitrogen cat. no. D1306)
- DNAse I (Worthington cat. no. LS002139)
- EBM-2 (Lonza cat. no. 00190860)
- EGM-2 BulletKit (Lonza cat. no. cc-3162)
- Glucose (Sigma-Aldrich cat. no. G7021-1KG)
- Percoll (Sigma-Aldrich cat. no. P1644-500ML)
- Sterile ddH_2O
- UltraPure DNase/RNase-free dH_2O (Life Technologies cat. no. 10977-015)
- Matrigel (GFR) (Corning cat. no. 356230)
- 0.05% Trypsin (Fisher Scientific cat. no. 25300054)
- AAV-CMV-GFP (Vector Biolabs cat. no. 7117)
- PFA 4% (Thermo Scientific cat. no. JL9943-K2)
- D-Sucrose (Fisher Scientific cat. no. BP220-212)
- Tissue-Tek O.C.T. Compound (Sakura cat. no. 4583)
- Blue O.C.T. (Epredia Neg-50) (Fisher Scientific cat. no. 22-050-451)
- Anti-adherence Rinsing Solution (Stemcell Technologies cat. no 07010)

Antibodies

- Anti-CD45 PE-Cy7 (BD Pharmigen------ cat. no. 557748, RRID:AB_396854)
  - https://scicrunch.org/resolver/RRID:AB_396854
- Anti-CD146 PE (BD Pharmigen------ cat. no. 550315, RRID:AB_393604)
  - https://scicrunch.org/resolver/RRID:AB_393604
- Anti-ANPEP APC (BD Pharmigen------ cat. no. 557454, RRID:AB_398624)
  - https://scicrunch.org/resolver/RRID:AB_398624

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- Anti-CD31 FITC (BD Pharmigen------ cat. no. 555445, RRID:AB_395838)
  - https://scicrunch.org/resolver/RRID:AB_395838
- Anti-CD140b (PDGFR-β) PE (BD Pharmigen------ cat. no. 558821, RRID:AB_397132)
  - https://scicrunch.org/resolver/RRID:AB_397132
- Anti-ANPEP PE-Cy7 (Biolegend------ cat. no. 301711, RRID:AB_10900061)
  - https://scicrunch.org/resolver/RRID:AB_10900061

Equipment

- Dissecting microscope – such as the Nikon SMZ-U
- Sterile Micropipettes – such as Rainin Pipet-lite XLS
- Sterile 0.65 μL Microcentrifuge Tubes such as Eppendorf cat. no. CLS3206
- Sterile 1.5 mL Microcentrifuge Tubes – such as Eppendorf cat. no. L201351L
- Sterile 15 mL Conical Tubes – such as Falcon cat. no. 720005
- Sterile 50 mL Conical Tubes – such as Avantor cat. no. 525-1075
- Sterile 5 mL FACS Tubes – such as Falcon cat no. 352235
- Sterile 70 μm Filters – such as Falcon cat. no. 352350
- Sterile 6-Well Plate (flat bottom) – such as Falcon cat. no. 353046
- Sterile 24-Well Plate (flat bottom) – such as Corning cat. no. 3524
- 37°C Incubator – such as NuAire 8700
- Sterile 60 mm Cell Culture Dish – such as Falcon cat. no. 353002
- Microsurgery Knives - Sharpoint
- Sterile Serological Pipettes – Thermo Fisher Nunc
- Pipette Aid – Power Pipette Plus
- Sterile Transfer Pipettes – such as Fisher cat. no. 13-711-20
- Rotating Oven – such as Robbins Scientific model 400
- Refrigerated Centrifuge – such as Thermo Sorvall legend XTR centrifuge
- Refrigerated Microcentrifuge – such as Eppendorf centrifuge 5415D
- Sterile Micropipette Tips – such as VWR Universal Pipet tips
- Wide-Opening Micropipette Tips (1000 μL)
- Microscope – Leica DM IL LED
- Ice Container
- 0.2μm filter – such as Millipore cat. no. 52GPT02RE

- Cell Culture Vacuum Aspiration System
- Vacuum
- Biological Safety Cabinet – Labconco
- FACS Sorter – such as FACS Aria II
- Millicell Hanging Inserts such as cat. no. PICUM03050
- Disposable Plastic Base Molds – such as Fisher cat. no. 2236355Z

Software and Algorithms

- Cell Ranger (RRID:SCR_017344)
- R Project for Statistical Computing (RRID:SCR_001905)
- RStudio (RRID:SCR_000432)
- Seurat (RRID:SCR_016341)
- ScType (https://github.com/IanevskiAleksandr/sc-type)
- Harmony (https://github.com/immunogenomics/harmony)

Reagent Setup

30% (wt/vol) Glucose Stock Solution—Dissolve 15 g of Glucose in 20 mL of UltraPure DNase/RNase-free dH_2O. Once glucose is dissolved, add water up to 50 mL. Filter through a 0.2 μm filter and store at 2-8°C for up to 6 months.

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. Store at 2-8°C for up to 1 month. **Critical!** Prepare under sterile conditions.

DNAse Stock Solution (10 mg/mL)—Dissolve 100 mg of DNAse I in 10 mL of sterile ddH_2O. Make 302 μL aliquots and store the DNAse stock solution at −20°C for at least 2 months. **Critical!** Avoid freeze–thaw cycles that could diminish the enzyme activity. **Caution!** DNAse I is a known irritant. Handle with care.

Critical! DNAse is also essential to successful digestion[37]. See the Troubleshooting advice for steps 9 and 54.

Collagenase/Dispase Solution (100 mg/mL)—Prepare a 100 mg/mL stock solution in UltraPure DNase/RNase-free dH_2O. Make 502 μL aliquots and store at −20°C for at least 2 months. **Critical!** Use aliquoted collagenase/dispase within 1 month of resuspension; otherwise, enzyme activity may be compromised. **Caution!** Collagenase/dispase is a known irritant. Handle with care.

Critical! Vascular cells are very sensitive to mechanical dissociation[37]. See the Troubleshooting advice for steps 9 and 54.

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DAPI Stock Solution (5 mg/mL)—Dissolve one vial (10 mg) of DAPI in 2 mL of ddH_2O to make a 5 mg/mL DAPI stock solution. Make 250 μL aliquots and store at –20°C for up to 6 months. In-use aliquots can be stored at 2-8°C. **Caution!** DAPI is a known mutagen. Handle with care.

22% (vol/vol) Percoll Solution—Mix 110 mL of percoll, and 50 mL of 10X PBS with 340 mL of UltraPure DNase/RNase-free dH_2O to obtain 500 mL of 22% (vol/vol) percoll solution. Filter through a 0.2 μm filter and store at 2-8°C for up to 1 month.

Collagen Coating Solution (0.03 mg/mL)—Dilute 3 mg/mL bovine collagen solution in 1X PBS appropriately to obtain desired volume. Prepare fresh before use.

Endothelial Growth Medium (EGM-2 Medium)—Add all growth factors provided in the EGM-2 BulletKit (10.00 mL of FBS, 0.20 mL of Hydrocortisone, 2.00 mL of hFGF-B, 0.50 mL of VEGF, 0.50 mL of R3-IGF-1, 0.50 mL of Ascorbic Acid, 0.50 mL of hEGF, 0.50 mL GA-1000, and 0.50 mL of Heparin) per the manufacturer's instructions and 5 mL of Antibiotic-Antimycotic (100X stock) to the EBM-2 basal medium. The medium can be stored at 2-8°C for up to 1 month. **Critical!** Do not use media after one month. See the Troubleshooting advice for step 94.

30% (wt/vol) Sucrose Solution—Dissolve 3 g of sucrose in 7 mL of 1X PBS. Once sucrose is dissolved, bring the volume up to 10 mL with 1X PBS and mix well. Store at 2-8°C for up to 1 month.

30% (vol/vol) Sucrose/OCT Solution—For a 60% (wt/vol) sucrose solution in PBS, dissolve 6 g of sucrose in 5 mL of 1X PBS. Once sucrose is dissolved, bring the volume up to 10 mL with 1X PBS and mix well. Combine the 60% solution with blue OCT at a 1:1 ratio to make 10 mL of 30% sucrose OCT:PBS. Store at room temperature (20-22°C) for up to 1 month.

Procedure

Timing approximately 2.5 hours.

Dissociation and digestion

Timing approximately 1 hour, add an additional 20 minutes per sample.

1. Turn on rotating oven and allow it to heat up to 37°C while you prepare your sample.
2. Obtain a 60 mm cell culture dish containing ~7 mL of HBSS/BSA/Glucose.
3. Add 150 μL of pre-aliquoted DNAse (10 mg/mL) to each dish.
4. Obtain ~250 mg of human brain tissue per sample. Document case details.

   Caution! Local institutional guidelines should always be followed when using human source materials since they can contain blood-borne pathogens. These

protocols likely will including trainings and proper engineering and personal barrier protections.

5. Place in dish containing HBSS/BSA/Glucose + DNAse. **Critical step!** It is crucial DNase is added to the solution at this step otherwise digestion will be inadequate. See also the Troubleshooting advice for steps 9 and 54.

6. Cut tissue into small (~2 mm x 2 mm chunks) in HBSS/BSA/Glucose + DNAse solution in a sterile environment (Figure 2).

   Critical step: Ideally, dissection should occur in a cell culture biosafety cabinet. If this is not possible, the tissue can be dissected outside of a hood with attention paid to best aspectic technique.

7. Distribute tissue into three 15 mL conical tubes.

   - Aliquot the tissue-buffer solution in dish into the tubes first.
   - Use extra HBSS/BSA/Glucose buffer to wash the dish and make up to 5 mL per tube.

8. Add 167 μL of collagenase/dispase (100 mg/mL) to each tube. Please note, 167 μL of collagenase/dispase per 5 mL is equivalent to 33.4 μL of solution per 1 mL

9. Digest for 30 minutes in rotating oven at 37°C. *TROUBLESHOOTING.* **Critical step!** Optimal enzymatic digestion is the most important factor to maximize yield. Therefore, this step may need optimizing to achieve sufficient but not too much digestion. See also the Troubleshooting advice for step 54.

10. Spin down for 5 minutes at 300g, 4°C.

Percoll density gradient centrifugation

Timing approximately 35 minutes

11. After samples have finished spinning, discard the supernatant by pouring out the liquid into a waste container. **Critical step!** Do not aspirate the supernatant during the cell dissociation and digestion, as the cell pellet may be aspirated and lost.

12. Add 1 mL of HBSS/BSA/Glucose and 50 μL of pre-aliquoted DNAse (10 mg/mL) into each sample tube.

13. Gently mix/resuspend 50 times using a micropipette, taking care not to introduce bubbles, which may detrimentally affect the cells[49].

14. Obtain two additional 15 mL conical tubes per each sample tube. For example, for 3 sample digestion tubes, obtain 6 tubes for Percoll filtration. **Critical step!** Yield will be compromised if each digestion isn't distributed into two Percoll tubes.

15. Dispense 4 mL of 22% Percoll into each new conical tube.

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16. Carefully dispense cell suspension from each sample tube into the new conical tubes containing 22% Percoll in equal amounts. Use ~750 μL per tube.

    Critical step! Cell suspension should be dispensed **on top of the Percoll**.

17. Spin down for 10 minutes at 500g, 4°C with no deceleration.

18. Discard supernatant.

19. Reunite contents of all percoll tubes into one tube by resuspending the cell pellet in the HBSS/BSA/Glucose remaining after pouring out the supernatant.

20. Spin down the sample tube for 5 minutes at 300g, 4°C.

21. Do not discard the remaining tubes. The small amount of cells left over after combining the majority of cells into one tube for the master mix can be used for unstained and DAPI controls (Steps 36-45).

Master mix preparation

Timing approximately 1-5 minutes

22. Add 200 μL of HBSS/BSA/Glucose into a fresh tube.

23. Add 2 μL of each antibody and 1 μL of DAPI into the tube containing 200 μL of HBSS/BSA/Glucose per sample.

Ab/Stain	Dilution	Volume
DAPI	1:200	1μL
CD45 PE-Cy7	1:100	2μL
CD146 PE	1:100	2μL
ANPEP APC	1:100	2μL
CD31 FITC	1:100	2μL

Immunostaining

Timing approximately 30 minutes.

24. After samples have finished spinning, discard supernatant.

25. Resuspend in ~200 μL of HBSS/BSA/Glucose.

26. Add the master mix into the tube of resuspended cells.

27. Incubate for 15minutes **on ice.** During this sample incubation start preparing controls by following steps 36-45.

28. Wash with 5 mL of HBSS/BSA/Glucose and mix.

29. Spin down for 5 minutes @ 300g, 4°C.

30. Discard supernatant.

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- Note the amount of supernatant remaining in the tube. Need 500 μL of supernatant to resuspend.
- Add additional HBSS/BSA/Glucose if below the recommended 500 μL needed to resuspend.

31. Resuspend cells.

32. Obtain 1 FACS tube and a 70 μm filter.

33. With the filter over the FACS tube, dispense the resuspended cells through the filter and into the FACS tube.

34. Seal the tube and place on ice.

Critical step! Cells should be analyzed as soon as possible. Longer periods of dissociation may compromise cell viability.

35. Take samples to FACS.

Control (Cell) tube preparation

Timing approximately 5 minutes

Critical! 6 total controls are needed (see step 43). We recommend only using cells for the unstained and DAPI controls because they cannot be performed with beads. This strategy does not waste cells on fluorescence controls and optimizes yield. However, if the user prefers not to use beads, cells can be used for all controls. In that case, a small part of the sample should be removed for control staining.

36. Obtain the three leftover 15 mL tubes after combining sample contents into one tube (step 19).

37. Sequentially, rinse each tube with ~500 μL of HBSS/BSA/Glucose and combine into one tube.

38. Obtain 6 fresh FACS tubes and fresh 70 μm filters.

39. With the filters on top of the FACS tubes, dispense 250 μL of combined buffer rinse into two of the FACS tubes.

40. Proceed to step 41.

Control (Beads) tube preparation and immunostaining

Timing approximately 5 minutes

41. Dispense 250 μL of HBSS/BSA/Glucose into each of the 4 remaining FACS tubes. **Critical!** If using cells rather than beads as controls, add at least 10,000 cells per tube with the HBSS.

42. Add 2.5 μL of a single antibody to each single color control tube into the FACS tubes containing 250 μL of HBSS/BSA/Glucose.

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43. For the live/dead stain, add 1.25 μL of DAPI into the FACS tubes containing 250 μL of combined buffer/cell rinse. 1 tube will be unstained.

FACS Tube	Ab/Stain	Dilution	Buffer Vol.	Ab/Stain Vol.
1	DAPI	1:200	250 μL	1.25 μL
2	CD45 PE-Cy7	1:100	250 μL	2.5 μL
3	CD146 PE	1:100	250 μL	2.5 μL
4	ANPEP APC	1:100	250 μL	2.5 μL
5	CD31 FITC	1:100	250 μL	2.5 μL
6	Unstained	N/A	250 μL	N/A

44. After adding antibodies as explained in step 43, shake UltraComp eBead vial and add one drop of beads to each single color control with the “Ready to Use” bottle. Please note, beads are designed to bind antibodies and eliminate the need to use cells for many controls. Therefore, do not add beads to dapi and unstained controls. If cells are being used for all controls, then do not add beads to any of the controls.

45. Seal the tubes and place on ice.

FACS

Timing ~2 hours.

CRITICAL Every flow cytometry system will have slightly different procedures. Here are high-yield principles to execute a successful flow cytometry experiment.

46. Set the laser levels for each of your fluorophores with unstained and single color controls.

47. Once the laser levels are set, then run the single color controls again to record each fluorophore. After every single color control has been run, the machine can perform compensation, the method of correcting for fluorescence spill over.

48. Once the laser levels are set and compensation performed, the user can draw gates to select sequential populations (Figure 3a). For this strategy, we recommend first eliminating debris with SSC and FSC gates. These are size and complexity parameters.

49. Next, eliminate doublets also with SSC or FSC.

50. Eliminate dead cells. We use DAPI but a number of live/dead stains exist in different fluorophores.

51. After eliminating debris, doublets, and dead cells, then the user should set up FACS plots to select their population of interests. In our studies, endothelial cells were isolated as $CD45^{-}CD31^{+}ANPEP^{-}$ and mural cells were isolated as $CD45^{-}CD31^{-}ANPEP^{+}$.

52. (Optinal) Run additional controls to determine the true population. These may include "FMO" controls and unstained controls.

53. After the population has been defined, use a 100 μm nozzle to perform cell sorting. **Critical step!** A smaller filter may damage the cells.

54. Translate the "event number" readout from the cell sorter into a true cell number, a parameter termed "efficiency"[20]. This calculation may be necessary for downstream applications.

    TROUBLESHOOTING.

55. After sorting several downstream procedures can be performed:

    - Follow steps 56-95 for 2D culture of purified mural and endothelial cells. 3D culture or metabolic measurements can also be performed as described previously[13].
    - Follow steps 96-114 for the transplantation of cells into brain organoids.
    - Follow steps 115-132 for single cell transcriptomic analysis.

Plating cells

Timing approximately 1 hour.

Collagen coating—Timing approximately 35 minutes

56. Obtain a 24-well plate.

57. Calculate the amount of collagen coating solution needed. We recommend 500 μL of collagen coating solution (0.03 mg/mL) per well.

58. Add 500 μL of collagen coating solution (0.03 mg/mL) into each well.

59. Incubate plate w/ collagen for 30 minutes at 37°C.

60. After incubation, remove collagen coating solution from all wells.

61. Wash wells once with 1X PBS and then they are ready to use. (If not using immediately, keep PBS in the wells and store in 37°C incubator until the sorted cells are ready to be plated).

Plating—Timing approximately 10 minutes

62. Spin down sorted mural and/or endothelial cell tubes (from step 54) by centrifuging for 5 minutes at 300g, 4°C.

63. After spinning, remove supernatant from microcentrifuge tubes containing cells.

64. Add 500 μL of prewarmed (37°C) growth media into micro tubes containing mural/endothelial cell pellet.

65. Resuspend.

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66. Add desired amount of cell solution into collagen-coated wells. We recommend filling each culture well with cell solution to approximately half volume.
67. Add additional 500 μL of growth media into wells containing cells for 1 mL total per well.
68. Add 500 μL of 1X PBS to all other empty wells to keep them from drying.
69. Place in 37°C incubator.

    Pause point Cells should be maintained in the incubator until they are used for experiments, with media changes approximately 3x a week (see Steps 70-76 below).

Cell culture

Changing media—Timing approximately 10 minutes

70. Determine the amount of EGM-2 media required for passaging based on plates and/or wells required. Aliquot endothelial cell growth media in a 50 mL conical tube.
71. Obtain plates with cells from 37°C incubator.
72. Place new tip on vacuum. Aspirate the old growth media from the plates.
73. Using a serological pipette, add 1X PBS to each plate. For a 24-well plate, we recommend 0.5 mL per well. Swirl each plate, then vacuum off the majority of PBS. *TROUBLESHOOTING.*
74. Using another serological pipette, add fresh growth media to each plate. For a 24-well plate, we recommend 0.5 mL per well. Swirl each plate once.
75. Place plates back into the 37°C incubator.
76. Repeat steps 70-75 to change media approximately every other day. Monitor confluency and when confuency has reached 75-80%, proceed to passaging (step 77).

Passaging and FACS—Timing approximately 30 minutes plus FACS

CRITICAL Ideal cell confluency for high yield FACS and cell health is 70% plate coverage.

77. Aliquot endothelial cell growth media in a 50 mL conical tube.
78. Obtain plates with cells from 37°C incubator.
79. Place new tip on vacuum. Vacuum out the old growth media from the plates. *TROUBLESHOOTING.*
80. Using a serological pipette, add 1X PBS to each plate. For a 24-well plate, we recommend 0.5 mL per well. Swirl each plate, then vacuum out the PBS. *TROUBLESHOOTING.*

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81. Using another serological pipette, add 0.5 mL of trypsin to each well. Swirl each plate once.

82. Place plates back into the 37°C incubator.

83. In approximately five minutes, remove the plates from the incubator.

84. View the plates under the microscope. Ensure that cells are no longer adhered to the bottom of the plate. Tap the bottom of the plates if necessary.

    TROUBLESHOOTING. **Critical!** Ensure that cells are beginning to round up before attempting to dislodge them from the plate. If cells are over-digested, their viability will be compromised. If cells are not sufficiently digested, then they will not come up from the plate.

85. Set up the appropriate number of 15 mL conical tubes (1 tube per well).

86. Add 1 mL of the HBSS/BSA/Glucose buffer to each 15 mL conical tube (or 6 mL to each 50 mL tube).

87. Tap bottom of plates once again to ensure cells aren't stuck on the bottom.

88. Gently tilt each plate and remove trypsin and cells using a serological pipette. Add to the appropriate conical tube containing the HBSS/BSA/Glucose buffer.

89. Wash the plate one more time with 1 mL HBSS/BSA/Glucose buffer to catch remaining cells, then discard empty plates.

90. Spin down tubes in centrifuge for 5 minutes at 300g, 4°C

91. To validate cell-surface marker expression of the vascular cells for downstream assays, perform FACS as in steps 46-54. Otherwise proceed directly to step 93.

    - If evaluating mural cells use this master mix and plate for all live cells after FACS:

Ab/Stain	Dilution	Volume
DAPI	1:200	1 μL
CD140b PE	1:100	2 μL
ANPEP PeCy7	1:100	2 μL
CD31 FITC	1:100	2 μL

    - If evaluating endothelial cells use this master mix and plate for all live cells after FACS:

Ab/Stain	Dilution	Volume
DAPI	1:200	1 μL
CD45 PE-Cy7	1:100	2 μL

Ab/Stain	Dilution	Volume
CD146 PE	1:100	2 μL
ANPEP APC	1:100	2 μL
CD31 FITC	1:100	2 μL

For passaged mural cells, we have also performed staining for PDGFR-β (2 μL at a 1:100 dilution; Figure 4b).

Replating—Timing approximately 10 minutes

92. Spin down tubes containing sorted cells for 5 minutes at 300g, 4°C.

93. Discard supernatant, add growth media to tubes containing the pellet of cells so total tube volume is 1 mL. For one well of a 24-well plate, we recommend 20,000-30,000 cells which should become confluent within 5 days.

94. Add growth media to each plate. Place into incubator.

TROUBLESHOOTING

95. Repeat steps 56-94 to maintain cells in 2D culture. Alternatively:

- Follow steps 96-114 for the transplantation of cells into brain organoids.
- Follow steps 115-132 for single cell transcriptomic analysis.
- The cells can also be used in Matrigel 3D angiogenesis cultures or metabolic studies (such as Seahorse assays)[13].

Organoids

Cell transplant—Timing approximately 2 hours.

CRITICAL Grow and maintain organoids as described previously[32,44,45]. We perform transplantations after the organoids have been differentiated and maintained for 7-9 weeks. Each user will need to determine their targeted neural population for vascular cell co-culture and grow the organoids to that stage.

96. Collect mural and/or endothelial cells sorted in step 54.

97. Immediately after sorting, infect the cell population of interest with AAV-CMV-GFP. Mix the cell solution thoroughly using a pipette. We use 1 μL (stock titer of 1×10^{13} GF/mL; 200 MOI) for 50,000 cells. Place cells at 37°C for 30 minutes.

98. To avoid viral leakage into the organoids, wash cells three times with 1X PBS, each time spinning down at 300g for 5 minutes at 4°C.

99. Select organoids for transplantation. Preferred organoids should display spherical shape and clear edges without any cell debris. Organoids vary in size, so a variety of organoid sizes will be used within a given experimental condition; ensure that you distribute different sizes across conditions.

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100. Using a wide-opening pipette tip, move number of desired organoids onto Millicell culture inserts in a 6-well flat bottom plate. Transwells help prevent the organoid from moving as the cells are invested during the transplants. We recommend 3-6 organoids per condition. **Critical step!** Use a wide-opening **p1000 μL** pipette tip to maintain the organoid structure. *TROUBLESHOOTING.*

101. Immediately before adding cells, gently remove as much media surrounding the organoids within the well, as possible, using a p1000 pipette.

102. Gently add desired amount of cells on top of the organoids and incubate at 37°C for 30 minutes. **Critical step!** Per organoid, we recommend not adding more than 50 μL to ensure that the cells stay on top of the organoid initially and don't float away.

103. After incubation, gently add media to well insert until the organoid floats off of the insert, approximately 3 mL.

104. Maintain organoids in a 37°C incubator. Transwell can be removed the day after transplantation. After transplantation, maintain the organoids submerged in media. Change media three times a week for up to 2 weeks, or the duration of the transplantation experiments (Figure 5b, Day 1, Stage 1). When transplantation experiments are complete, if samples need to be prepared for frozen sectioning proceed to step 105.

TROUBLESHOOTING

Freezing (Figure 5b)—Timing approximately 8 hours

Critical! See figure 5b for an overview of the workflow.

105. After two weeks and using wide-opening pipette tips, transfer organoids to a 24-well flat bottom plate and aspirate excess media. Up to 6 organoids can be transferred per well. PBS washing prior to fixation is not necessary (Figure 5b, Day 1, Stages 2, 3).

Critical step: Do not combine different conditions or transfer types into the same well.

106. Add 0.5-1 mL of 4% PFA to the organoid wells to cover organoids. Leave the organoids for 30 minutes at room temperature (Figure 5b, Day 1, Stage 4).

107. Remove PFA with a pipette and wash with 0.5-1 mL 1X PBS 3 times for 10 minutes (Figure 5b, Day 1, Stage 5).

108. Remove PBS with a pipette and resuspend in 0.5-1 mL 30% sucrose. Store overnight (or ~6 hours) at 4°C until organoids sink to the bottom of the well (Figure 5b, Day 1, Step 6). Please note that the incubation will depend on organoid size; larger organoids take longer to sink.

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109. Remove excess sucrose from wells and replace with 0.5-1 mL 50/50 mix of 30% sucrose and blue OCT. Store overnight (or ~6 hours) at 4°C (Figure 5b, Day 2, Stages 7, 8).

110. Remove excess 50/50 sucrose/OCT mix and resuspend in 0.5-1 mL clear OCT.

111. Fill embedding blocks with OCT and label appropriately

112. Using wide-opening tips, gently pipet out organoids and place in embedding blocks (Figure 5b, Day 3, Stages 9, 10).

TROUBLESHOOTING

113. Use a pipette tip to help them sink to the bottom of the OCT and place on dry ice (Figure 5b, Day 3, Stage 11).

114. Store in −80°C freezer until time for sectioning. We use standard procedures to section samples and for subsequent analysis using immunofluorescence[13].

Pause point Slides may be kept at −80°C for up to one year without compromising their quality.

Single-Cell RNA Sequencing

Single-Cell library preparation—Timing approximately 8 hours

115. Collect mural and/or endothelial cells sorted in step 54. Immediately after sorting, begin library preparation.

116. Perform library preparation using a 10x Genomics Chromium Next GEM Single Cell 3' Reagent Kit based on the manufacturer's instructions. For our experiments, we used v3.1 10x Genomics kits.

Sequencing—Timing varies (can be a few hours to a few days depending on the instrument).

117. Perform qPCR-based quantification, fragment analysis, and sequencing (our prepared libraries were sent to the UCSF Institute for Human Genetics Genomics Core to do this):.

- Perform qPCR-based quantification to determine the concentration of each library using the KAPA Library Quantification Kit for Illumina Platforms.
- Determine sequencing depth based on your desired coverage for each library. 10x Genomics recommends sequencing a minimum of 20,000 reads/cell for most single-cell 3' gene expression libraries.
- After using the 10x Genomics reagent kits to prepare your libraries, perform paired-end sequencing using Illumina's constructs.

118. Obtain sequenced files in FASTQ format, ready for downstream bioinformatic analysis.

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TROUBLESHOOTING

Analysis of single-cell RNA sequencing data

Aligning FASTQ files and generating feature-barcode matrices using Cell Ranger—Timing varies depending on computing power

Critical! For downstream analysis, FASTQ files must be aligned to a reference transcriptome and converted into feature-barcode matrices, also known as count matrices. 10x Genomics provides Cell Ranger, a collection of analysis pipelines, to process its Chromium single-cell data. Cellranger count is a pipeline within Cell Ranger that aligns sequencing reads in FASTQ files to a reference transcriptome and generates feature-barcode matrices.

119. Using the cellranger count pipeline, align FASTQ files to the reference transcriptome of interest and generate count matrices for each sample.

- We used the human reference transcriptome GRCh38 for our sequencing reads. Species-relevant transcriptomes are available for download from the 10x Genomics website or a comparable resource.
- A more detailed description of the cellranger count pipeline can be found here: https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/count

Preprocessing of single-cell RNA sequencing data using Seurat in R—Timing varies depending on computing power

120. Seurat is an R package that offers methods for quality control, analysis, and exploration of single-cell RNA-seq data[50]. Using the Read10x function within Seurat, import the feature-barcode matrices for each sample into R. A guided tutorial on clustering data with Seurat can be found here: https://satijalab.org/seurat/articles/pbmc3k_tutorial.html

121. Convert each feature-barcode matrix into individual Seurat objects using the CreateSeuratObject within Seurat. Note that once a seurat object is created for a given sample, any additional sample information (e.g. age of the collected sample, brain region, etc.) can be added to the metadata of that seurat object. One can add this information using: samplefilename@meta.data["Columnname"] <- "sample information".

122. Filter out low-quality cells. We filtered using the following criteria:

- Greater than 200 unique genes (features) detected in each cell. Empty droplets or dead cells will have very few genes, while doublets will have many.
- Greater than 500 unique molecular identifiers (UMIs) detected in each cell. Empty droplets or dead cells will have very few UMIs.

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- Less than 10% mitochondrial reads in each cell. A high mitochondrial percentage in a given cell typically indicates cellular stress or damage.

  Our workflow to filter out low-quality cells is adapted from guidelines provided by the Harvard Bioinformatics core (https://github.com/hbctraining/scRNA-seq_online/tree/master/lessons).

123. If necessary, combine multiple datasets using the merge function within Seurat.

124. Perform data normalization using the NormalizeData function within Seurat

125. Identify highly variable features using the FindVariableFeatures function within Seurat and specify the selection method. We used the default method (vst) and default number of variable features (2,000) to identify 2,000 highly variable genes for this dataset.

126. Scale data using the ScaleData function within Seurat

Dimensionality reduction and clustering—Timing varies depending on computing power

127. Run principal component analysis (PCA) with all high variable genes found in step 123. Selecting the number of principal components (PCs) to add to UMAP analysis can be done using Jackstraw plot analysis to remove any PCs that are insignificant[51].

128. If necessary, perform batch correction. We recommend Harmony, which uses an iterative clustering approach to account for batch effects arising from multiple samples. Harmony can be run within our Seurat workflow based on the standard integration pipeline in Harmony's documentation. A guided tutorial on removing batch effects with Harmony can be found here: https://github.com/immunogenomics/harmony. *TROUBLESHOOTING*

129. Perform unsupervised clustering using Seurat's FindNeighbors and FindClusters functions. The FindClusters function allows one to control the resolution of clustering, where a higher resolution value leads to a larger number of smaller clusters, while a lower resolution value leads to a smaller number of larger clusters. This parameter needs to be optimized for each dataset, depending on the desired specificity for cell type identification. For our dataset, The optimal resolution for our dataset was 0.5. We selected a resolution that produced well-separated and biologically meaningful clusters.

130. Visualize clusters via UMAP using Seurat package.

Labeling clusters

Timing approximately 1.5 hours

131. *Labeling using ScType*. Run the ScType package to label clusters using a reference database of cell-type signatures. This package offers several reference databases for labeling, covering various tissues and cell types; however, one can also create their own reference database. We created a reference database

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specific to our cell types of interest based on gene sets from the literature. A more detailed description of the ScType package can be found here: https://github.com/IanevskiAleksandr/sc-type

132. *Manual labeling*. Similar to ScType, this method involves identifying marker genes for each cluster and comparing them with known cell type signatures. To manually label clusters using Seurat:

- Identify marker genes using the FindAllMarkers function within Seurat.
- Using the writexl package create an excel file with your marker genes by cluster.
- Compare the marker genes from each cluster with known cell type signatures from the literature or reference databases.
- Assign labels to each cluster based on the best match with the known cell type signatures using the RenameIdents function within Seurat.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Timing

In our experience, tissue dissociation and digestion will take approximately 3 hours when starting with a piece of tissue approximately 1 cm^3. Larger pieces of tissue will likely require more time because the procedure must be scaled up proportionally (please see troubleshooting). FACS including establishing compensation with controls requires another 3 hours. The endothelial and mural cells can be placed in culture immediately after FACS. The procedures for organoid transplants will require an additional 2 hours.

Cell dissociation and digestion

Steps 1-10 Cell Dissociation and digestion: approximately 1 hour

Steps 11-21 Percoll density gradient centrifugation/filtration: approximately 35 minutes

Steps 22-23 Master mix preparation: approximately 5 minutes

Steps 24-35 Immunostaining: approximately 40 minutes

Steps 36-40 Control (cell) tube preparation: approximately 5 minutes

Steps 41-45 Control bead tube preparation and immunostaining: approximately 5 minutes

FACS

Steps 46-55 FACS: approximately 3 hours

Culture of FACS-purified vascular cells

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Steps 56-61 Collagen coat: approximately 35 minutes

Steps 62-69 Plating: approximately 10 minutes

Steps 70-76 Changing media: approximately 10 minutes

Steps 77-91 Passaging and FACS: approximately 30 minutes + FACS

Steps 92-95 Replating: approximately 10 minutes

Organoid transplants

Steps 96-104 Cell transplantation: approximately 2 hours

Steps 105-114 Freezing: approximately 8 hours

Transcriptomic analysis

Steps 115-116 Single cell library preparation: approximately 8 hours

Steps 117-118 Sequencing: Timing varies

Steps 119-126 Preprocessing of single-cell RNA sequencing data using R: Timing varies

Steps 127-130 Dimensionality reduction and clustering: Timing varies

Steps 131-132 Labeling clusters: approximately 1.5 hours

Anticipated results

When starting with approximately 1 cm^3 of tissue, approximately 30,000 endothelial cell events and 100,000 mural cell events should be obtained after FACS. Once the cells are placed in culture, they will initially double in approximately 5 days (Passage 1), and then decrease their doubling time (subsequent passages, Figure 4a). Mural cells in general are more proliferative than endothelial. In regards to the choice of CD31 and ANPEP as cell surface markers for flow cytometry, respectively, we find that they are suitable “pan” endothelial and mural cell markers based on the following criteria: 1. All of the endothelial and mural cell subtypes are captured[13]. 2. Other human brain single-cell RNA sequencing datasets do not find expression of these markers in non-vascular cells in prenatal[21] or adult human brain[6,23]. We also did not find vascular genes expressed in other cell populations (Figure 3c). Of note, we and others[6,23] find that the human brain has more mural cells than endothelial, in contrast to the adult mouse brain[19]. Endothelial cells also seem to be more delicate and sensitive to PMI (Figure 6e, f). If cells purified as endothelial cells do not express bona fide endothelial cell markers at the transcriptomic level, consider whether your protocol is too harsh or the PMI was too long.

For organoid transplants, vascular cells should integrate into the organoids quickly. GFP turns on within 24 hours and can be visualized easily within 3 days. Unfortunately, we have not found other methods of visualization to be helpful: we do not see morphological differences on brightfield microscopy, for example. Since adenoviruses do not integrate into

DNA, the GFP expression is these experiments is not stable. If the cells divide, the signal is depleted over time. In non-dividing cells, expression is maintained for the life of the cell. We did not observe CD34 or PDGFR-β in cells which did not express GFP in our experiments after two weeks[13] (also Figure 5c).

In our experiments, the organoid environment enriched for one dominant endothelial and mural cell subtype[13], but alternative culture conditions may enrich for different subtypes. Of note, it is important to perform experiments to determine that endothelial and mural cells maintain their identity upon transplantation as organoids have transcriptomic differences, including cellular stress, that may lead to phenotypic differences in vascular cells[32]. This could be determined via immunohistochemistry or RNAscope. We performed immunohistochemistry for CD34 for endothelial cells and PDGFR-β for mural cells[13] before proceeding with experiments to determine their subtypes and their effects on neural cells (Figure 5c). We and others have consistently found that vascular cells influence neural cell differentiation and proliferation in culture and in organoids[2,13,19]. Given the pervasiveness of these effects in different model systems, we would anticipate these results across a variety of neurovascular interactions. However, probing specific stages of neurovascular interactions may reveal new discoveries. In addition, vascular cells should improve cell stress in the organoids[13].

Acknowledgements

The authors wish to thank Ethan Winkler and Andrew Yang for their discussion on the strengths and challenges of different methods to purify human brain vascular cells (Table 1). Some schema icons in Figures 2 and 5 were created in Biorender.com.

Funding:

This study has been supported by the Eli and Edythe Broad Regeneration Medicine and Stem Cell Fellowship, Pediatric Scientist Development Program (5K12HD000850-34), UCSF Physician Scientist Scholars Program (PSSP), American Heart Association Career Development Grant 857876, CIRM Alpha Stem Cell Clinic Fellowship, and NIH K08 NS116161 to E.E.C., NIH K99/R00 NS111731 to A.B., NIH K99/R00 MH125329 to M.G.A., California Institute of Regenerative Medicine (CIRM) Bridges Science Master's Fellowship (SFSU EDUC2-12693) to E.J.V., P01 NS083513 and U01 MH105989 to E.J.H. The UCSF Flow Cytometry Core is supported by DRC Center Grant NIH P30 DK063720.

Data availability

Single-cell RNA-seq data have been deposited at GEO and are publicly available with code GEO: PRJNA803255. In addition, the data can be downloaded at https://cells.ucsc.edu/?ds=vascular-dev. No original code was generated using this data. The flow cytometry data can be accessed at Mendeley data (Crouch, Elizabeth; Diafos, Loukas (2023), “Vascular cell flow cytometry data (Crouch et al)”, Mendeley Data, V1, doi: 10.17632/9j6chfbvxm.1). Other data produced during and/or analyzed in this study are available from the corresponding authors upon request.

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Key data used in this protocol:

Crouch EE, Bhaduri A, Andrews MG, Cebrian-Silla A, Diafos LN, Birrueta JO, Wedderburn-Pugh K, Valenzuela EJ, Bennett NK, Eze UC, Sandoval-Espinosa C, Chen J, Mora C, Ross JM, Howard CE, Gonzalez-Granero S, Lozano JF, Vento M, Haeussler M, Paredes MF, Nakamura K, Garcia-Verdugo JM, Alvarez-Buylla A, Kriegstein AR, Huang EJ. Ensembles of endothelial and mural cells promote angiogenesis in prenatal human brain. Cell. 2022 Sep 29;185(20):3753–3769.e18. doi: 10.1016/j.cell.2022.09.004. [PubMed: 36179668]

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KEY POINTS:

- This protocol extension describes the purification of prenatal human brain endothelial and mural cells with FACS, and their utilization in downstream applications including cell culture, organoid transplantation, and single cell transcriptomics.
- This simple, efficient protocol has relatively few steps compared to other methods, and uses inexpensive reagents. Robust yields of healthy vascular and perivascular cells can be obtained in 6 hours.

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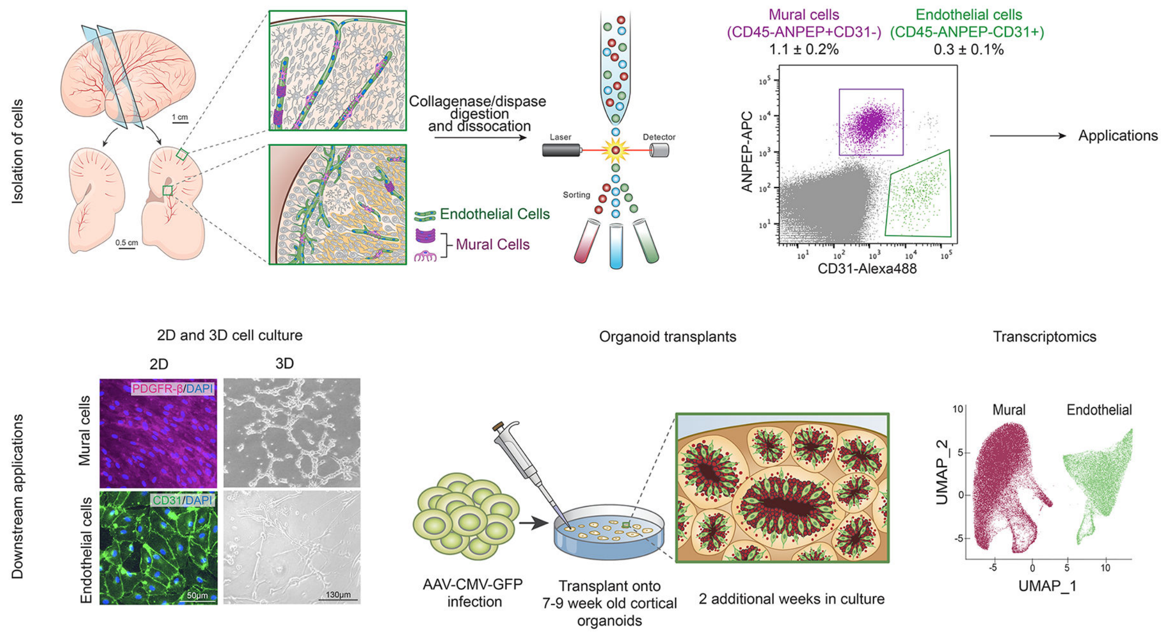

Figure 1: Capture, culture, and profiling of developing human brain vascular cells.

FACS strategy to simultaneously purify endothelial, and mural cells with ANPEP and CD31 (Steps 1-55) and downstream applications: 2D and 3D culture (Steps 56-96), organoid transplants (Steps 96-114), and transcriptomics (Steps 115-132).

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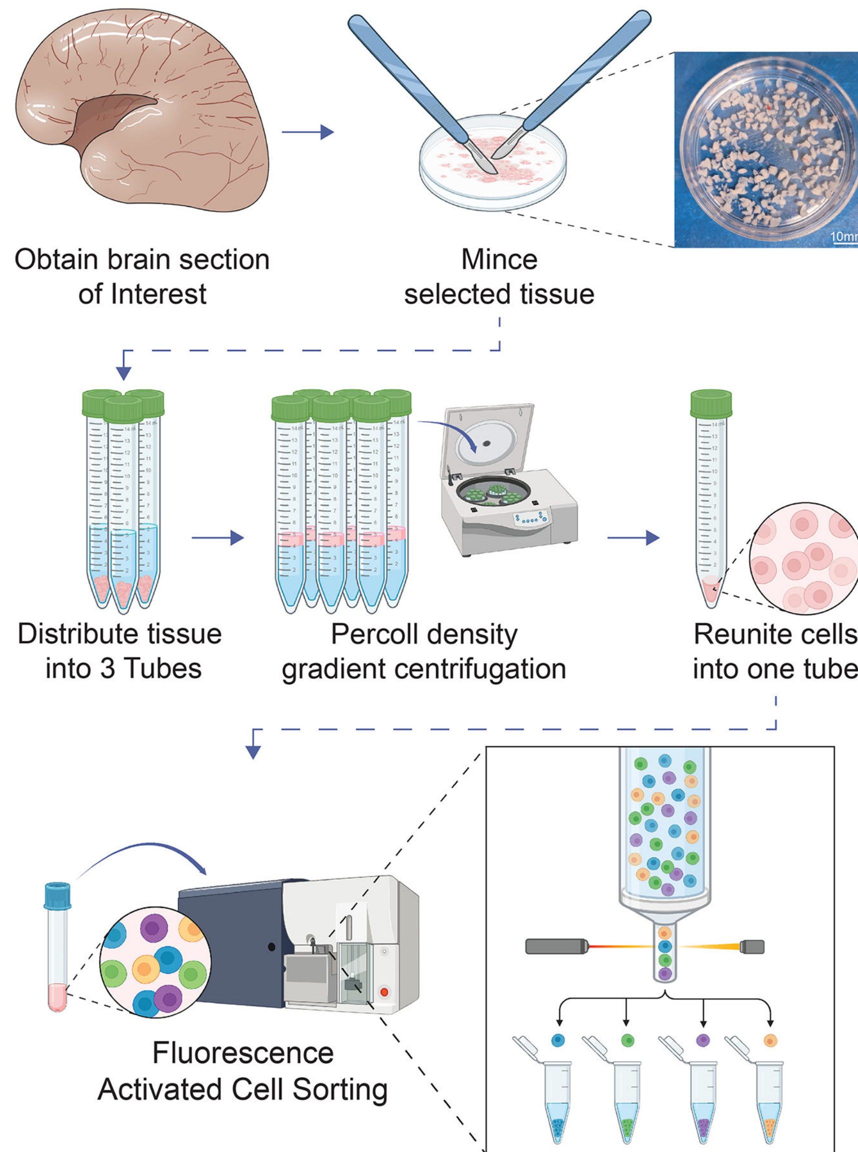

Figure 2: Detailed steps for dissociation, digestion, and isolation of prenatal human brain vascular cells.

Schema with detailed steps for dissociation, digestion, and isolation of prenatal human brain vascular cells. Once selected, pieces of 1 cm^3 brain tissue should be finely minced with a scalpel (Step 6), then distributed into 3 conical tubes with collagenase/dispase in solution (Step 8). At the completion of the digestion, triturate the solution (Step 13). Layer the digested cells on top of 22% Percoll solution, distributing each digestion reaction into two Percoll tubes (Steps 14-16). After the Percoll centrifugation, pool the cells (Steps 19-20). Perform immunostaining (Steps 22-35) and Fluorescence Activated Cell Sorting (Steps 46-55).

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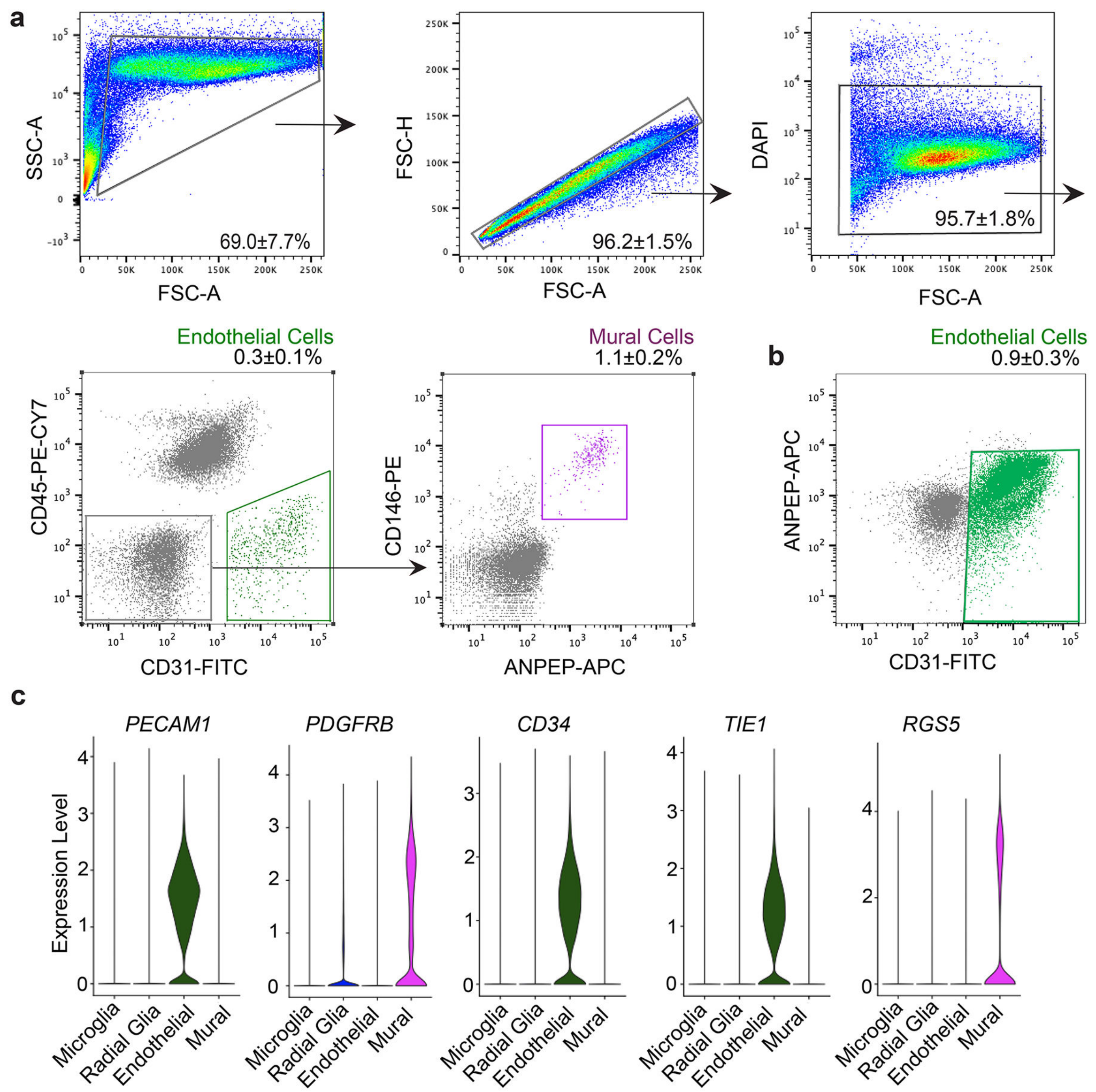

Figure 3: Prenatal and adult human brain vascular cells can be purified with FACS.

a. FACS plots showing the initial purification of prenatal human brain vascular cells. **b.** FACS plots showing CD31 expression in adult human brain endothelial cells and absence of ANPEP. **c.** Violin plots of *PECAM1/CD31, PDGFRB, CD34, TIE1,* and *RGS5* in scRNA-seq data of endothelial and mural cells, microglia and radial glia. Experiments were performed with n>5 samples and data are represented as mean ± S.E.M.

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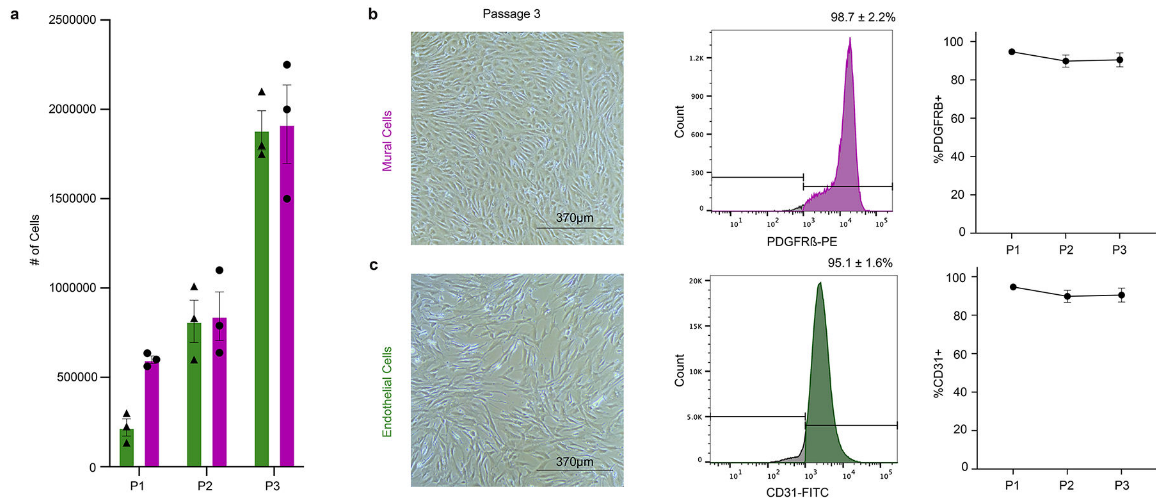

Figure 4: Prenatal human brain vascular cells can be maintained in culture.

a. Growth of passaged endothelial and mural cells after 3 passages in vitro. P1, passage 1; P2, passage 2; and P3, passage 3. **b.** Mural cells in culture. Left: Microscopic image of mural cells in culture, passage 3. Right: Flow cytometry and quantification of PDGFR-β expression in mural cells up to passage 3. **c.** Endothelial cells in culture. Left: Microscopic image of endothelial cells in culture, passage 3. Right: Flow cytometry and quantification of CD31 expression in endothelial cells up to passage 3. Data are mean ± SEM, n=3.

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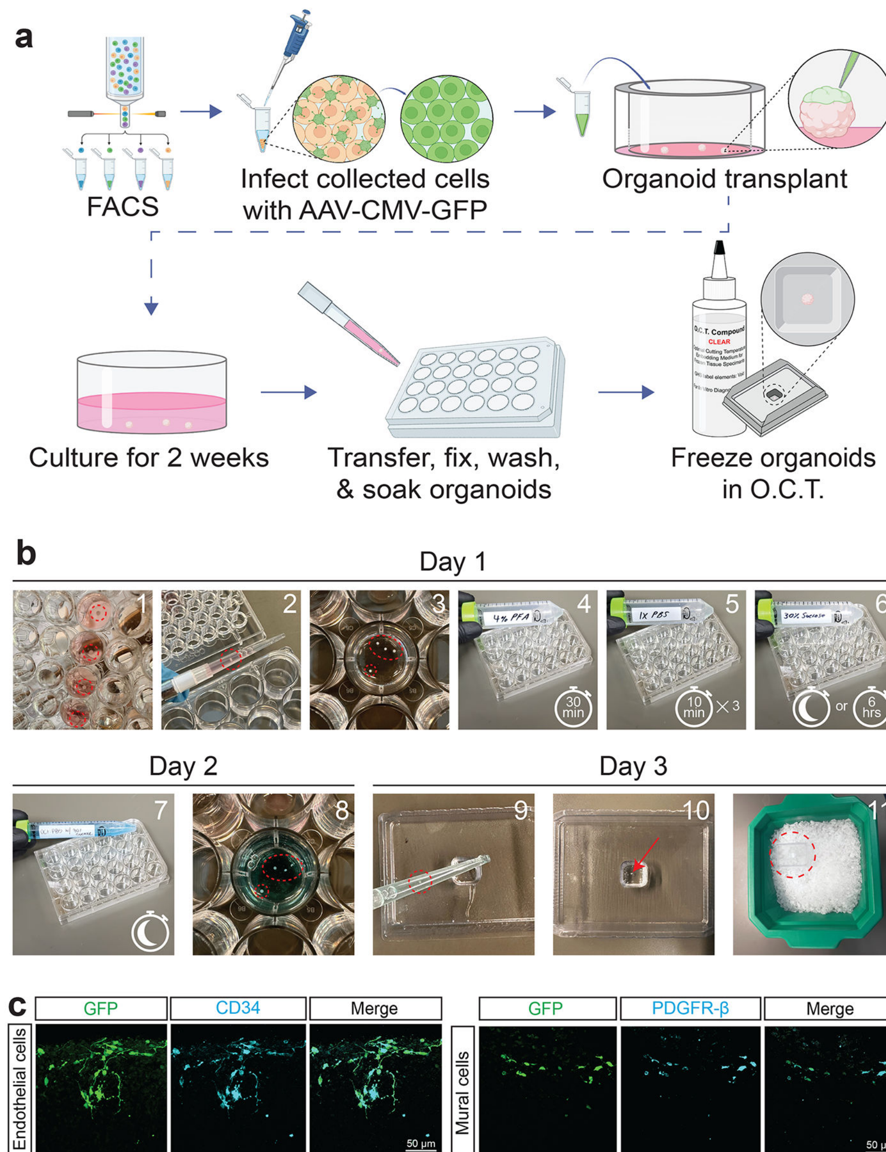

Figure 5. Organoid transplantation, embedding, and immunostaining.

a. Schema with detailed steps for prenatal human brain vascular cell viral infection, organoid transplantation, and freezing. Once desired cells are sorted and acquired via FACS (Steps 46-55), immediately infect them using AAV-CMV-GFP and transplant them onto the organoids (Steps 96-104). After culturing the organoids for two weeks, organoids should be transferred on to a 24-well plate, fixed, washed, and soaked in 30% sucrose solution. Organoids are frozen using O.C.T. within a disposable embedding mold (Steps 105-114).

b. Fixing and Embedding Organoids. 1. Images of organoids in culture (circled in red, step 104) 2. Transfer organoids using wide-opening pipette tips (Step 105). 3. Fix the organoids in a 24-well plate (Step 105). 4. Submerge for 30 minutes in PFA (Step 106). 5. Wash with 1X PBS (Step 107). 6. Immerse the organoids in 30% sucrose for 6 hours or overnight (Step 108). 7. On Day 2 remove all the sucrose and add blue OCT/sucrose solution (Step 109). 8. Immerse the organoids in blue OCT/sucrose solution overnight (Step 109). 9. Transfer

blue stained organoid to a base mold with a wide-opening pipette tips (Step 112). 10. Image demonstrating how the organoid sits in the base mold (Step 112). 11. Freeze over dry ice (Step 113). **c.** Confocal images for GFP and CD34 (endothelial cells) or PDGFR-β (mural cells) in the transplanted organoids.

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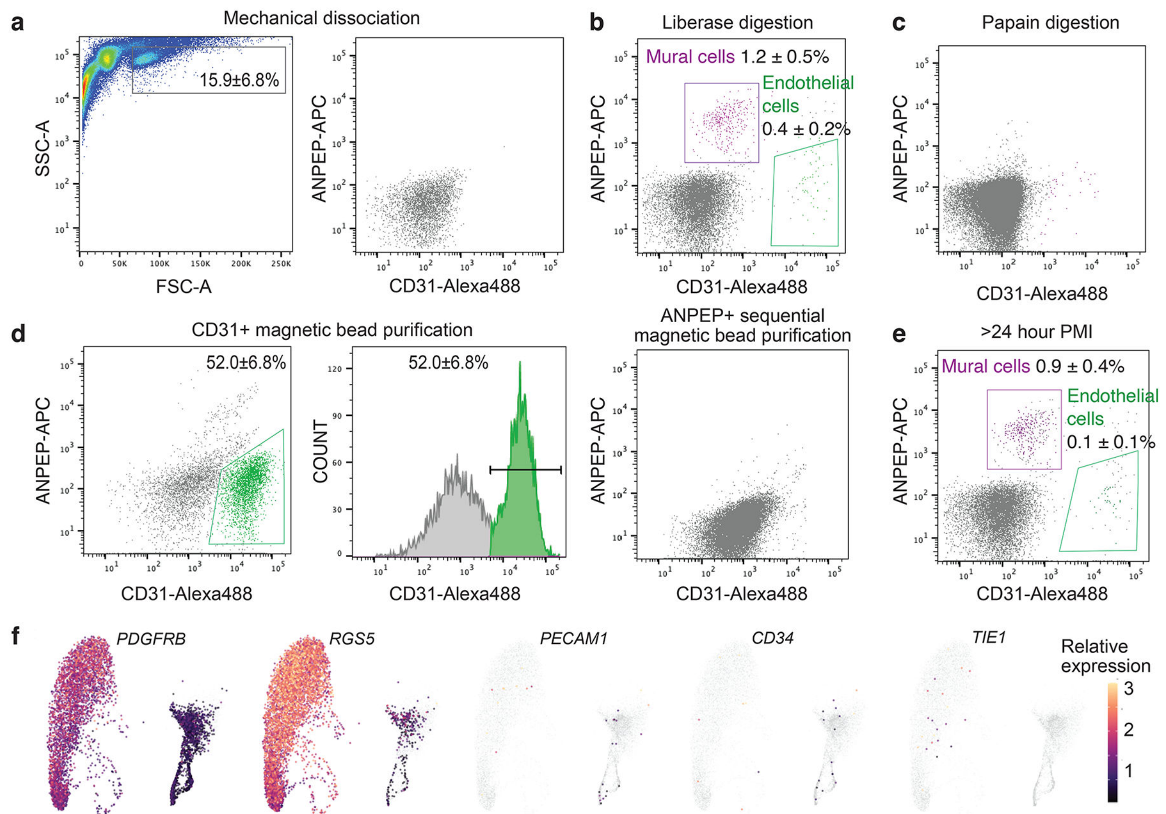

Figure 6: Alternative digestion and purification methods.

a. Example FACS plot with mechanical dissociation. Left plot shows that approximately 85% of FACS events are debris with this approach. Middle FACS plot shows the absence of vascular cells. **b.** Example FACS plot with liberase. **c.** Example FACS plot with papain. **d.** Example FACS plot with magnetic bead purification with CD31 conjugated antibody (left, middle) and sequential purification with ANPEP-APC and anti-APC magnetic antibody (right). Left and middle panels show the same data as a pseudocolor plot and histogram for clarify. Note how endothelial cells are enriched but not purified with CD31 magnetic antibody and ANPEP+ cells are completely lost. Data are mean ± SEM and n=4 replicates. **e**. Example FACS plot with >24 hour PMI. Mural cells survive the dissociation and digestion but endothelial cells do not. f. Feature plots for mural (*PDGFRB, RGS5*) and endothelial (*PECAM1, CD34, TIE1*) genes in a sample with >24 hour PMI.

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Table 1:

Comparison of this protocol with other human brain vascular isolation methods

	Crouch et al	**Winkler et al**	**Yang et al**	**Garcia et al**
Human sample type	Normal prenatal	Normal adult brain and tissues from patients with arteriovenous malformation (AVM)	Normal adult brain and brain tissues from patients with Alzheimer's disease	Normal adult brain and brain from patients with Huntington's disease
Number of cells	~147K	~181K control cells, ~101K AVM cells	~143K cells	~17K
Fresh vs frozen tissue	Fresh	Fresh	Frozen	Frozen
Nuclei vs whole cell	Whole cell	Whole cell	Nuclei	Nuclei
Isolation method	Manual mincing, Enzymatic digestion, FACS	Manual microdissection, homogenization, enzymatic digestion	Manual mincing, homogenization, dextran and sucrose density ultracentrifugation, FACS	Homogenization, density ultracentrifugation
Advantages compared to other methods	Dissociation and digestion protocol with fewer steps; optimizes cell yield from small amounts of tissue; captures whole cells for transcriptomics; high purity of cells obtained; can be used to isolate endothelial cells from adult human brain.	High yield of vascular and perivascular cells captured without FACS; captures whole cells for transcriptomics.	Can process large amounts of fresh frozen tissue, including archived and diseased samples.	Can process large amounts of fresh frozen tissue, including archived and diseased samples.
Challenges compared to other methods	Protocol can be inconvenient to scale up with larger pieces of tissue (many separate tubes will be required for digestion and Percoll); requires access to FACS facility; requires fresh, non-frozen tissue; can be used only to isolate adult human brain endothelial cells.	Technique limited to blood vessels that can be visualized by eye and vascular cells from microvessels may be under-represented; requires fresh, non-frozen tissue.	Protocol requires multiple gradient centrifugations, filtrations, washes, spins; as such, likely loses some vascular cells in these steps; captures only nuclei; includes some non-vascular cells because FACS is only performed with nuclei (dapi) staining; requires FACS machine.	Cell isolation did not target vascular cells and vascular cells were analyzed based on bioinformatic criteria alone. As such, has lower yield of vascular cells than other techniques.

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Table 2:

Troubleshooting table

Step	Problem	Possible reason	Solution
Reagent Setup	DNase and/or collagenase are not effective	DNase and collagenase may not be stored correctly.	Do not store −20 aliquots in a freezer with automated defrosting. This will result in repeated freeze/thaw cycles even without the aliquot leaving the freezer. See also troubleshooting for Steps 9 and 54.
Reagent Setup	Cells do not grow well in EGM-2 media with Antibiotic-Antimycotic.	Antibiotic-Antimycotic may have unintended effects on your cells.	We add Antibiotic-Antimycotic to our EGM-2 media because cell isolated from primary tissue may have higher risk of contamination. However, gentamicin is included with the EGM-2 media and Antibiotic-Antimycotic may affect the health of your cells. It can be excluded.
9	Digestion does not result in milky/ viscous mixture.	DNase and/or collagenase are not effective during digestion.	Consider increasing enzyme concentration or digestion time. A few microliters of digestion solution can also be obtained and examined under the microscope. Classically, digested tissue before trituration should appear as “beads on a string”. DNAse is also essential to successful digestion[38]. As cells are digested, some of them will lyse and naked DNA will be present in the solution. This DNA is sticky and can significantly damage cells with trituration if DNase is not present.
54	Poor yields from FACS	Digestion was inadequate.	Vascular cells are very sensitive to mechanical dissociation[38] (Figure 6a). If poor yields are obtained, we recommend scrutinizing the digestion for any problems first before pointing to other steps. If the enzymatic digestion is incomplete, the cells can be dissociated mechanically but the yields will decrease. The digestion may be suboptimal because too much tissue is placed in each digestion tube, because the enzyme is too old or has had repeated freeze/thaw cycles and is inactive, or because the tissue was not agitated or rotated with digestion and the enzyme was not well distributed.
		Procedure took too long.	We do not recommend one person process more than 1 cm^3 of tissue because the number of Percoll tubes become unwieldy. If the digestion and dissociation procedure takes much longer than 3 hours, the cell yield may decrease.
		Post-mortem interval (PMI) was too long.	For PMI, we have performed single-cell RNA sequencing with samples less than 24 hours PMI, but after that the cell quality declines (Figure 6e).
		Cells were collected in tubes without buffer.	Bring microcentrifuge vials containing 50 μL of HBSS/BSA/Glucose for collection. Cells can be damaged by sorting into tubes without buffer.
73, 79, and 80	Cells are lost during aspiration steps in cell culture.	Cells can be pulled off the bottom of the plate with enough force.	Do not aspirate the full volume of liquid. Leave a small amount of PBS or media in the bottom of the dish.
80	Cells do not detach from the bottom of the plate.	Serum from the EGM-2 media can inactivate trypsin.	Ensure that as much EGM-2 as possible is removed. Consider two 1X PBS washes.
94	Passaged primary human brain vascular cells do not grow well	EGM2 media may be more than or close to one month.	Do not use EGM2 media for more than one month. The hFGF-B particularly is required for Matrigel tube formation and will become inactive over time.
100, 112	Organoids are stuck in pipette tip upon transfer.	Wide-opening pipette tips were not used.	We recommend always using wide-opening pipette tips to transfer organoids. If organoids still adhere to internal pipette surface, coat the pipette tips beforehand with anti-adherent solution.
104	Vascular cells do not transplant into organoids.	Inadequate number of cells transplanted	We transplanted approximately 20,000 vascular cells per organoid.
		Organoids are in too much media volume.	Cells for transplantation should be resuspended in less than 50 μL.
		Organoids are too old.	Organoids are most optimal for use prior to week 15 of differentiation. After this time, incorporation becomes less efficient.
118	Inadequate sequencing results	Initial number of cells is too low, or read depth is too shallow.	Our prepared libraries had an average of ~10,000 cells per sample of either endothelial or mural cells. This estimated number of cells is 30-50% of the number of FACS events obtained while sorting. Our sequenced samples had an average of ~100,000 reads/cell.

Step	Problem	Possible reason	Solution
128	Cells are not integrated in UMAP.	Ineffective batch correction.	For our data[13], we used the scRNA-seq integration pipeline. However, Harmony integration is faster than scRNA-seq and is quickly becoming the more popular method for batch correcting. Both methods should be employed empirically at the biological relevance of the clustering results examined to determine the best results.

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