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TITLE:

Isolation and Profiling of Human Primary Mesenteric Arterial Endothelial Cells at the Transcriptome Level

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SUMMARY:

The protocol describes the isolation, culture, and profiling of endothelial cells from human mesenteric artery. Additionally, a method is provided to prepare human artery for spatial transcriptomics. Proteomics, transcriptomics, and functional assays can be performed on isolated cells. This protocol can be repurposed for any medium- or large-size artery.

ABSTRACT:

Endothelial cells (ECs) are crucial for vascular and whole-body function through their dynamic response to environmental cues. Elucidating the transcriptome and epigenome of ECs is paramount to understanding their role in development, health, and disease, but is limited in the availability of isolated primary cells. Recent technologies have enabled the high-throughput profiling of EC transcriptome and epigenome, leading to the identification of previously unknown EC cell subpopulations and developmental trajectories. While EC cultures are a useful tool in the exploration of EC function and dysfunction, the culture conditions and multiple passages can introduce external variables that alter the properties of native EC, including morphology, epigenetic state, and gene expression program. To overcome this limitation, the present paper demonstrates a method of isolating human primary ECs from donor mesenteric arteries aiming

to capture their native state. ECs in the intimal layer are dissociated mechanically and biochemically with the use of particular enzymes. The resultant cells can be directly used for bulk RNA or single-cell RNA-sequencing. Alternatively, the isolated cells can be plated for culture, with minimal contamination of cells, confirmed through fluorescence-activated cell scanning (FACS) and immunofluorescence. In addition, a workflow is described for the preparation of human arterial tissue for spatial transcriptomics, specifically for a commercially available platform, although this method is suitable for other spatial transcriptome profiling techniques. This methodology can be applied to different vessels collected from a variety of donors in health or disease stages to gain insights into EC transcriptional and epigenetic regulation, a pivotal aspect of endothelial cell biology.

INTRODUCTION:

Lining the lumen of blood vessels, endothelial cells (ECs) are crucial regulators of vascular tone and tissue perfusion. ECs are remarkable in their ability to react to the extracellular environment and adapt to changes in the dynamics and composition of blood flow. These dynamic responses are mediated through a network of intracellular signaling events, including transcriptional and post-transcriptional modulations with spatio-temporal resolution. The dysregulation of these responses is implicated in many pathologies, including but not limited to cardiovascular disease, diabetes, and cancer[1,2].

A large proportion of studies make use of cell lines or animal models to interrogate EC transcriptome. The former is a useful tool, given the relative ease of use and inexpensiveness. However, serial culturing can introduce phenotypic alterations to ECs, such as fibroblastic features and a lack of polarization, disconnecting them from their *in vivo* state[3]. The primary cells, e.g., human umbilical vein EC (HUVEC) have been a popular choice since the 1980s but are derived from a developmental vascular bed that does not exist in adults, thus unlikely to represent mature ECs. Animals, especially mouse models, better represent the physiological or pathophysiological environment of ECs and allow interrogation of transcriptomes as a result of genetic perturbation. Murine ECs can be isolated from various tissues, including the aorta, lungs, and adipose tissues using enzyme-based procedures[4–6]. However, the isolated cells cannot be used for multiple passages unless transformed[6] and are often limited in numbers, which requires pooling from multiple animals[5,8,9].

The advent of new technologies exploring the vessel architecture at a transcriptomic level, particularly with single-cell resolution, has enabled a new era of endothelial biology by revealing novel functions and properties of ECs[5, 10–14]. A rich resource built by Tabula Muris and investigators collected single-cell transcriptomic profiles of 100,000 cells including ECs across 20 different murine organs[15], which revealed both common EC marker genes and unique transcriptomic signatures with inter-and intra-tissue differences[5,13]. Nevertheless, there are clear differences between mouse and human in genome, epigenome, and transcriptome, especially in the non-coding regions[16–18]. These aforementioned drawbacks emphasize the importance of analysis of ECs using human samples in order to gain a faithful profile of ECs in their native state in health and disease.

Most EC isolation methods rely on physical dissociation through homogenization, finely cutting, and mincing the tissue before incubation with proteolytic enzymes for differing times. The enzymes and conditions also vary considerably between tissue types, from trypsin to collagenase, used alone or in combination[19–21]. Further antibody-based enrichment or purification are often included to increase the purity of ECs. Typically, antibodies against EC membrane markers, e.g., CD144 and CD31 are conjugated to magnetic beads and added to the cell suspension[22,23]. Such a strategy can be generally adapted for EC isolation from multiple human and mouse tissues, including the techniques introduced in this protocol.

In their native state, ECs interact with multiple cell types and may exist in vascular niches where cell proximity is crucial for function. While single-cell and single-nuclear RNA-sequencing (scRNA and snRNA-seq) studies have been paramount to the recent breakthroughs in describing EC heterogeneity, the dissociation process disrupts tissue context and cell-cell contact, which are also important to understand EC biology. Developed in 2012 and named Method of the Year in 2020[24], spatial transcriptome profiling has been utilized to profile global gene expression while retaining the spatial features in various tissues including brain[25], tumor[26], and adipose tissue[27]. The technologies can be targeted, using specialized probes specific for particular RNA sequences attached to affinity reagents or fluorescent tags, thus detecting select genes at subcellular resolution[28–31]. They can also be untargeted[32,33], which typically use spatially barcoded oligonucleotides to capture RNA, which together get converted to cDNA for subsequent sequencing library preparation and hence has the advantage of deducing whole tissue gene expression in an unbiased manner. However, spatial resolution is not currently achieved at a single cellular level with commercially available technologies. This can be overcome to some extent with data integration with scRNA-seq data, ultimately allowing the mapping of single-cell transcriptome in a complex tissue context while retaining its original spatial information[34].

Herein, a workflow is described to profile EC transcriptome using human superior mesenteric artery, a peripheral artery that has been used to study vasodilation, vascular remodeling, oxidative stress, and inflammation[35–37]. Two techniques are described: 1) to isolate and enrich ECs from the intima of blood vessels combining mechanical dissociation and enzymatic digestion suitable for single-cell transcriptome sequencing or subsequent *in vitro* culture; 2) to prepare arterial sections for spatial transcriptome profiling (**Figure 1**). These two techniques can be performed independently or complementarily to profile ECs and their surrounding cells. Furthermore, this workflow can be adapted for use on any major medium or large artery.

PROTOCOL:

Human tissue studies were conducted on deidentified specimens obtained from the Southern California Islet Cell Resource Center at City of Hope. The research consents for the use of postmortem human tissues were obtained from the donors' next of kin, and ethical approval for this study was granted by the Institutional Review Board of City of Hope (IRB No. 01046).

1. Physical dissociation (estimated time: 1–2 h)

1.1. Place a fresh artery on a 10 cm dish and wash with sterile Dulbecco's phosphate-buffered saline (D-PBS).

1.2. With sterile forceps and dissection scissors, remove the fat and outer connective tissues until the vessel is clean. Measure the length of the vessel with a ruler placed outside of the dish and take pictures for records (**Figure 1A**).

1.3. Pre-warm appropriate digestion buffer to 37 °C: for scRNA-seq use cell-dissociation enzyme; for cell culture assays use 1–6 mg/mL of Collagenase D, 3 mg/mL of bacteria derived protease, 100 mM of HEPES, 120 mM of NaCl, 50 mM of KCl, 5 mM of glucose, with 1 mM of $CaCl_2$[6,38] (pH 7.0, does not require adjustment) added 5 min prior to use.

1.4. Take the mesenteric artery (typically with a diameter of 6–8 mm) and cut lengthwise with scissors to open the vessel lumen vertically.

1.5. Using needles, attach the vessel onto black wax on all four corners, leaving the intima exposed (**Figure 1B**).

1.6. Add 1 mL of pre-warmed digestion buffer to intima. Take a sterile scalpel and scrape the lumen of the vessel gently twice.

NOTE: The purpose of this procedure is to dissociate the intimal layer, which may need practice. Enough force needs to be exerted to remove endothelium, but not so much that the deeper layers of tissue are also removed, which will reduce the EC representation.

1.7. Transfer the digestion buffer into a 5 mL tube. Add 1 mL of digestion buffer to the intima and pipette up and down carefully to collect the remaining cells and add to the 5 mL tube. Incubate cells at 37 °C, rotating at 150 rpm for 5 min.

1.8. Add 2 mL of M199 medium (or D-PBS if proceeding with scRNA-seq) to the cell suspension to quench the enzymatic reaction. Mix gently and centrifuge at 4 °C, 600 *x g* for 5 min.

1.9. Remove the supernatant and store the supernatant separately to culture as a control to observe if all cells are captured in the pellet in step 1.8. Resuspend the cell pellet in 1 mL of M199 medium (or D-PBS if proceeding with scRNA-seq).

1.10. Assess the cell viability by mixing 10 µL of trypan blue with 10 µL of cell stock. Observe the cell morphology and count the cells using a hemocytometer.

NOTE: At this point, cells can be used for protein or RNA quantification or cultured *in vitro* using EC culture media following standard protocols[39]. Alternatively, they can be prepared for sequencing as described in the next section.

1.11. For the culture, coat two wells of a 6-well plate by pipetting 500 µL of attachment reagent into wells for 30 min at room temperature. After removal of attachment reagent and washing with sterile D-PBS, dispense the full cell stock into one well, and the supernatant kept as a control into the second well.

2. Preparation for RNA-seq studies (estimated time: 3–4 h)

2.1. Centrifuge the cell stock from step 1.11 at 4 °C, 600 *x g* for 5 min. Remove the supernatant and resuspend the pellet gently with a P1000 pipette and wide-bore tip in 1 mL of 0.04% bovine serum albumin (BSA) in D-PBS. Mix well to ensure a single-cell suspension.

2.2. Pass the solution through a 40 µm strainer to remove cell debris. If debris remains, pass through a second strainer into 4 mL of 0.04% BSA in D-PBS.

2.3. Centrifuge at 4 °C, 600 *x g* for 5 min. Remove the supernatant and resuspend the pellet in 500 µL of 0.04% BSA in D-PBS using a P1000 pipette with a wide-bore pipette tip. Mix well to ensure a single-cell suspension.

2.4. Assess the cell viability by mixing 10 µL of cell stock with 10 µL of trypan blue. Using a hemocytometer, observe the morphology, determine whether there is a single-cell suspension without clusters, check for tissue debris, and calculate the number of living and dead cells.

2.5. If cells are clusters or debris remains, repeat washing with 0.04% BSA in D-PBS or pass through 40 µm strainer again.

NOTE: While this will reduce the yield, it is important that cells exist in a clean single-cell suspension for optimal sequencing.

2.6. Single-cells can be used for scRNA-seq.

3. Spatial transcriptome profiling (estimated time: 3–4 h)

3.1. Add isopentane (2-methylbutane) to a metal canister and chill in liquid nitrogen (LN_2) or dry ice (LN_2 is preferred as it will bring the temperature lower than dry ice). Pour optimal cutting temperature compound (OCT) in wells of labeled plastic cryomolds taking care to not create bubbles and remove those that appear. Leave cryomold on dry ice to chill.

3.2. Cut at least two coronal sections, 1 cm in length of the vessel. Using long (12”) forceps, submerge the tissue in isopentane until frozen. Quickly submerge the tissue in OCT at orientation with the lumen visible in the center. Take care to remove any bubbles, especially those next to the tissue.

3.3. Using long (12”) forceps, grasp the cryomolds, and hold them in the metal canister. While the base of the molds should be in the liquid, it should not be too deep to allow isopentane to

run over the tissue. Observe the freezing, the OCT will become white progressively from the outside in 1–2 min.

3.4. Store these sections in a sealed container at -80 °C for up to 6 months.

NOTE: Maintain samples on dry ice at all times and do not allow more than one freeze-thaw cycle. This tissue can be used for histological analysis, RNA/DNA fluorescence *in situ* hybridization (FISH), or spatial transcriptomics, which will be described now.

3.5. Set up the cryostat temperature to -20 °C for the chamber and -10 °C for the specimen head. Equilibrate OCT embedded vessel sections, knives, brushes, and slides to -20 °C in the cryostat for approximately 30 min. While equilibrating, clean the machine and all equipment that may touch the sections, including the blade, with 70% ethanol followed by RNase decontamination solution.

3.6. Attach the sample by dispensing a small amount of OCT onto the circular cryostat block and placing the sample on top before it freezes. Place the block into the center of the specimen head and screw the block in place using a tall black handle on the left. Cutaway any excess OCT surrounding the vessel.

3.7. Setting the cutting thickness to 10 µm on the cryostat, cut approximately 60 sections and place them in a pre-cooled 1.5 mL tube. These sections will be used to assess the RNA quality. Upon removal from cryostat, immediately add 1 mL of RNA extraction reagent and vortex until the sections are completely dissolved.

3.8. Add 200 µL of chloroform and mix until the solution resembles a strawberry milkshake. Centrifuge at 11,200 *x g* for 10 min at 4 °C.

3.9. Collect the aqueous phase (clear phase on top of white and pink layers) and add 500 µL of isopropanol to it. Mix by pipetting or vortexing and place at -80 °C for at least 20 min.

3.10. Centrifuge at 11,200 *x* g for 10 min at 4 °C. Dissolve the purified RNA pellet in 5 µL of RNase-free water. Examine the RNA Integrity Number (RIN).

NOTE: Samples with RIN ≥ 7 are preferred, but RIN $\geqslant$ 6 is acceptable based on experience. The number of tissue sections and volume of solution for RNA dissolving may require optimization depending on the system used to ensure the final RNA concentration is within the RIN function range to allow for accurate RIN evaluation.

3.11. Practice cutting and placing sections properly within the fiducial frames using plain glass slides before proceeding to commercially available tissue optimization or gene expression slides. This can be achieved by drawing 6.5 mm x 6.5 mm squares on a plain glass slide, cooling to -20 °C in the cryostat, and placing sections in this capture area.

3.12. Cut 10 µm sections with anti-roll plate in place, flip and carefully flatten by gently touching the section through the surrounding OCT.

3.13. Using RNase-free cryostat brushes place the tissue section within the square, using only the surrounding OCT. Immediately place one finger (in gloves) on the backside of the capture area to melt the section to the slide.

3.14. Once the section has adhered, place the side onto the cryobar to allow the section to freeze. Care must be taken to avoid tissue folding and tissue overlying the demarcated edges of the fiducial frame. If necessary, cut the tissue into halves or quarters along the lumen using a blade to ensure the vessel section fits within the 6.5 mm x 6.5 mm frame.

3.15. Cut 10 µm sections of tissue as per 3.12–3.14 onto tissue optimization or gene expression slides. Transfer the slide to a slide mailer placed on dry ice. Store slides at -80 °C for up to 4 weeks before proceeding with published spatial protocols[33,34].

4. Sequencing data analysis (estimated time: up to 1 week depending on familiarity with software)

NOTE: For spatial transcriptomic analysis only, skip to step 4.8. scRNA-seq data is processed using the standardized pipeline aligned to human hg38 reference transcriptome. The R package Seurat (v3.2.2) is used to analyze scRNA-seq data following published guidelines[40].

4.1. Filter using well-established quality control metrics: rare cells with very high numbers of genes (potentially multiplets) and cells with high mitochondrial percentages (low-quality or dying cells often present mitochondrial contamination) are removed.

4.2. Normalize data using “sctransform”, a method to improve sample integration compared to log-normalization. These normalized data are used for dimensionality reduction and clustering, while log-normalized expression levels are used for analysis based on gene expression levels, such as cell-type classification[41].

4.3. Select marker genes per cell type, for example, *platelet endothelial cell adhesion molecule-1* (*PECAM1*) and *von Willebrand factor* (*VWF*) for ECs, *lumican* (*LUM*) and *procollagen C-Endopeptidase Enhancer* (*PCOLCE*) for fibroblasts, *B-cell translocation gene 1* (*BTG1*) and CD52 for macrophages, C1QA and C1QB for monocytes, and *myosin heavy chain 11* (*MYH11*) and *transgelin* (*TAGLN*) for vascular smooth muscle cells[36].

4.4. Compute the average expression level across single cells between each pair of markers in order to have a single marker with an average expression level associated with each cell type[42].

4.5. Apply a Gaussian Mixture Model (GMM) with two components to the expression data of each marker across single cells in order to separate cells into two sets, namely, highly expressing

the marker and lowly expressing the marker. In this way, for each marker, each single-cell is assigned to one of the two components[42].

4.6. Use statistical enrichment for the set of marker genes, a Fisher's exact test, to assign a cell type to each cluster. By doing so, a set of p-values are obtained per cluster, each corresponding to a cell type. The cell type with the lowest p-value is assigned to that cluster.

4.7. Process the spatial transcriptomic data using the Space Ranger pipelines resulting in spatial de-barcoding and generation of quality control (QC) metrics, including total aligned reads to hg38 human transcriptome, the median number of Unique Molecular Identifier (UMI) or genes per spot[35].

NOTE: The R package Seurat (v3.2.2) is used to analyze the Space Ranger-processed data following published guidelines[40].

4.8. Normalize data using “sctransform”, a method which is demonstrated to improve the downstream analysis compared to log-normalization given the heterogeneity of the tissue and the very high variance in counts across the spots.

REPRESENTATIVE RESULTS:

The analysis of ECs from mesenteric artery using a combination of mechanical and enzymatical dissociation or cryopreservation for use in various downstream assays is depicted here (**Figure 1**). ECs can be profiled in mesenteric arteries using the following steps: A) mechanical dissociation from the intima coupled with collagenase digestion to culture cells; B) generation of single-cell suspension for scRNA-seq; or C) cross-sections of the artery can be embedded in OCT to be cryosectioned to profile spatial transcriptome (**Figure 1** and **Figure 2**).

[Place **Figure 1** here]

[Place **Figure 2** here]

Isolated ECs cultured using the described protocol display a distinct cobblestone-like morphology with minimal contaminating cells (**Figure 3A**). Expression of EC marker vascular endothelial (VE)-Cadherin was confirmed using immunofluorescence visualizing cell-cell junctions (**Figure 3B**). Isolated ECs were subjected to flow cytometry analysis for CD31. As negative controls, unstained HUVECs (without antibody) produced a 1.1% signal and HUVECs incubated with IgG yielded a 0.8% signal. As a positive control, HUVECs stained with CD31 antibody displayed 99% purity and were used to gate the channels for human mesenteric arterial endothelial cells (HMAECs). Approximately 80% of cells were CD31 positive indicating HMAECs made up the majority of the freshly isolated cell population (**Figure 3C**). Unsuccessful isolations, through either scraping too hard/deep, seeding the cells at too low a density, or maintaining the culture beyond passage 3 results in cells with disturbed morphology, potentially expressing mesenchymal markers (αSMA) or elongating resembling a fibroblastic state (**Figure 3D**).

[Place **Figure 3** here]

ECs isolated using cell-dissociation enzyme underwent scRNA-seq. **Figure 4A** shows a representative uniform manifold approximation and projection (UMAP) isolated from mesenteric artery using the present protocol for scRNA-seq on isolated HMAECs, with 34% cells clustered as ECs using PECAM1 and VWF (**Figure 4B,C** respectively) as markers.

[Place **Figure 4** here]

For spatial transcriptomic workflow, extracted RNA from tissue sections was electrophorized on a gel to visualize RNA quality. A weak signal or absence of 18S and 28S ribosomal RNA bands, and the presence of degradation products observed in **Figure 5A**, lane A1, is an example of poor-quality RNA and should not be processed further. Samples with a concentration out of the range could lead to lower RIN (**Figure 5A**, lane B1). However, diluting the RNA by two times increased RIN sufficient for proceeding (**Figure 5A**, lane C1). Before determining gene expression, a tissue optimization was performed to determine the optimal permeabilization time to release RNA to be captured by the oligonucleotides on the gene expression slide (**Figure 5B**). Based on the fluorescence imaging of complementary DNA (cDNA) footprint, 18 min permeabilization produced the lowest background but strongest signal, and thus was selected to be the optimal duration. Hematoxylin and eosin (H&E) staining visualized the morphology of the vessel allowing regions of interest to be identified (**Figure 5C**). Library quality was assessed (**Figure 5D**) and determined to be suitable for sequencing. UMI counts per spot, number of reads per spot, and total genes are quality metrics produced by the Space Ranger pipeline, the variability of each displayed in **Figure 5E**. Finally, gene expression (using *Actin alpha-2* (*ACTA2)* as an example, **Figure 5F**) was visualized with spatial anchoring on the H&E stained vessel.

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Arterial tissue processing and spatial profiling. (**A**) A cleaned-up mesenteric artery. (**B**) Vessel cut open to expose intima. (**C**) Hematoxylin and Eosin (H&E) staining of mesenteric artery cross-section in the fiducial frame. Image captured using a 5x lens under widefield fluorescence inverted microscope. (**D**) Representative Space Ranger output file of total gene expression.

Figure 2: Overview of EC isolation and profiling techniques. Flow diagram showing different methods of processing mesenteric artery. Processing techniques result in cell suspensions suitable for single-cell (sc) RNA-seq, endothelial cell (EC) culture, or whole tissue is embedded in optimal cutting temperature (OCT) compound for spatial transcriptome profiling.

Figure 3: Validation of isolated cultured mesenteric arterial ECs. (**A**) Brightfield image of isolated ECs, 10x lens, scale bar = 50 µm. (**B**) Immunofluorescence of VE-Cadherin expression with 4',6-diamidino-2-phenylindole (DAPI) as a nuclear marker. Image captured using a 10x lens under widefield fluorescence inverted microscope. Scale bar = 50 µm. (**C**) FACS plots of unstained HUVEC (top left), HUVEC stained with IgG control (top right), HUVEC stained with CD31 (bottom

left), and the isolated human mesenteric arterial endothelial cells (HMAEC) stained with CD31 (bottom right) (**D**) Immunofluorescence images of HMAECs with DAPI (nuclear marker), alpha-smooth muscle actin (αSMA) and CD31. 40x lens, scale bar = 100 µm.

Figure 4: scRNA-seq of mesenteric arterial ECs. (**A**) Uniform Manifold Approximation and Projection (UMAP) plot of scRNA-seq data from mesenteric artery. A 2-dimensional projection of the manifold in a high dimensional space, where each point represents a single cell and the proximity to other points indicate how similar the transcriptome is with each other. Cells are color-coded by their cell-type. (**B**) PECAM1 expression projected onto UMAP. (**C**) VWF expression projected onto UMAP. Color bars represent the expression of levels of genes where the RNA levels are depicted by log-normalized unique molecular identifier counts.

Figure 5: Spatial transcriptomic workflow and representative results (**A**) Quality assessment of RNA extracted from two samples of mesenteric artery, A1 from one sample, B1 from the second sample, C1 diluted 2x from B1. Red arrows indicate ribosomal 28S and 18S bands. (**B**) Spatial tissue optimization (TO) workflow. (**C**) Gene expression (GEX) slides showing the tissue sections (left panel); H&E images of the tissue sections loaded on the GEX slide before permeabilization for 18 min, reverse transcription and library construction (right panel), scale bar = 1cm. (**D**) Quality assessment of the libraries prepared. (**E**) Output metrics from Space Ranger show range between average reads, UMIs, and genes per spot for different libraries. Error bars denote min and max. (**F**) *ACTA2* expression across vessel section. All microscopy images were taken using a widefield inverted microscope, from a 5x lens, tilescan module.

DISCUSSION:

The presented workflow details a set of techniques to profile ECs from a single piece of human artery with single-cell and spatial resolution. There are several critical steps and limiting factors in the protocol. One key to transcriptome profiling is the freshness of the tissue and RNA integrity. It is important to maintain tissues on ice as much as possible prior to processing to minimize RNA degradation. Typically, the post-mortem tissues are processed between 8–14 h after the time of death. However, beginning the isolation or the cryopreservation as soon as possible post extraction from the donors is recommended. Specifically for spatial transcriptome mapping, the tissue should be kept on dry ice and more than one freeze-thaw cycle should not be allowed, and more than one vessel cross-section should be embedded in OCT, to allow repeats if necessary. Efforts should also be made to promote an RNase-free environment during tissue processing and cell isolation. Secondly, the size of the vessels can be a limiting factor. For spatial transcriptomics, the protocol can be performed on 1 mm of the vessel in total, regardless of the diameter. For the dissociation protocol for cell culture and scRNA-seq, the lower limit would be if the vessel is too narrow (i.e., below 50 mm in diameter) to enable insertion of dissection scissors. Based on these principles, the dissociation protocol can be adapted for any medium or larger size artery or vein as long as the size of vessels allows the opening up, and the spatial transcriptome procedure can be applied to any vessels that can fit either entirely or partially into the fiducial frame.

To process tissue for single-cell transcriptome analysis, several groups have described the use of

liberase and elastase to obtain all cell populations within the vessel wall[9, 4344]. However, the resulting datasets contain on average only 3%–7% of the total cell population annotated as ECs[9,45]. One way to improve the limited EC representation in the scRNA-seq data is by enriching the EC fraction by leveraging the EC surface markers *via* FACS[44,46]. However, this usually requires large amounts of starting tissue and expensive equipment that may not be routinely available for all laboratories. This protocol takes advantage of the unique anatomical position of ECs, *viz.* primarily in the intimal layer, which allows for enrichment of ECs without the need for harsh tissue digestion and enhances cell viability for scRNA-seq or subsequent culture. The percentage of ECs in the final cell mixture for scRNA-seq can be increased by adding a step using VE-Cadherin/CD144 antibody-conjugated magnetic beads. However, this step may not exclude all other cells that interact with ECs due to the native cell-cell interaction. Nonetheless, although traditionally the other cell types (e.g., macrophages, smooth muscle cells, fibroblasts) would be regarded as "contamination" in EC isolation, they can provide useful information for cell-cell interactions in the tissue context. In the scRNA-seq data analysis, ECs can be efficiently annotated using the EC markers and the cell-cell interactions can be inferred bioinformatically using several popular bioinformatics methods (e.g., CellChat[47], CellphoneDB[48], CytoTalk[49] among others). Furthermore, the inclusion of these data can enable more effective integration with and deconvolution of the spatial transcriptome profiling data, which are not at single-cell resolution (see below)[34].

For spatial transcriptome, there is currently no gold standard for preparing any tissue for this technique and limited research in preparing vasculature, with most of the published research using animal tissue[50,51]. The technique requires commercially available slides with specialized fudicial frames with a capture area containing approximately 5,000 barcoded spots with a diameter of 55 µm. The spots are placed 100 µm apart from the center of the spot to adjacent spots leaving approximately 45 µm gaps between spots that will not be profiled, limiting the resolution. Furthermore, a single spot will contain multiple cells, as the majority of the cells are below 55 µm in area. Thus, as aforementioned, this is not a single-cell sequencing technique. However, by integrating the data with scRNA-seq, the resolution can be enhanced, and the heterogeneity within a spot can be revealed[34]. Although the present protocol focuses on one spatial transcriptomic profiling assay, this technique could be adapted for other emerging spatial profiling methods[52,53] as the protein and RNA quality is maintained in this procedure.

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DISCLOSURES:

S.Z. is a founder and board member of Genemo, Inc.

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Figure 1

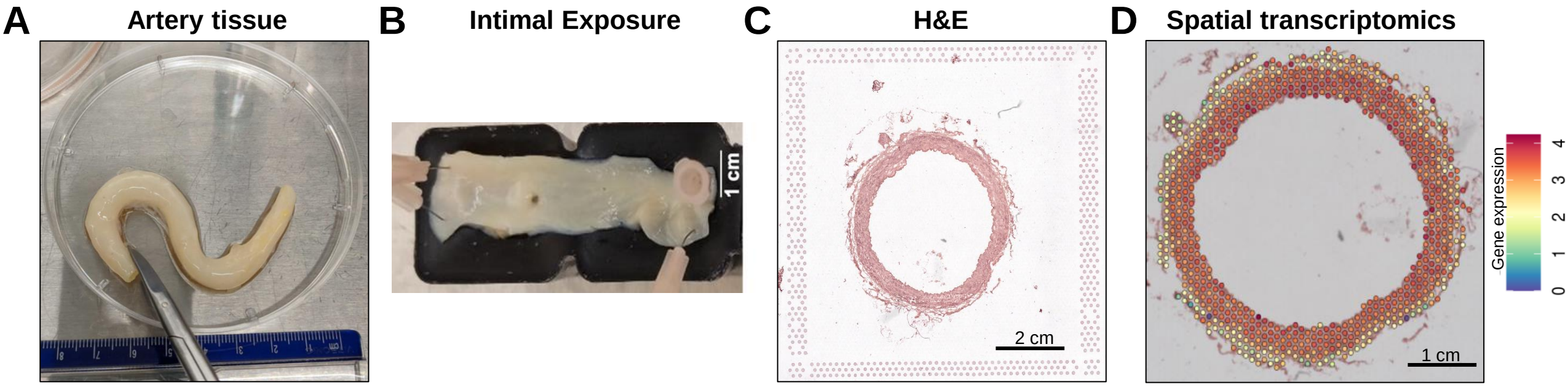


Figure 2

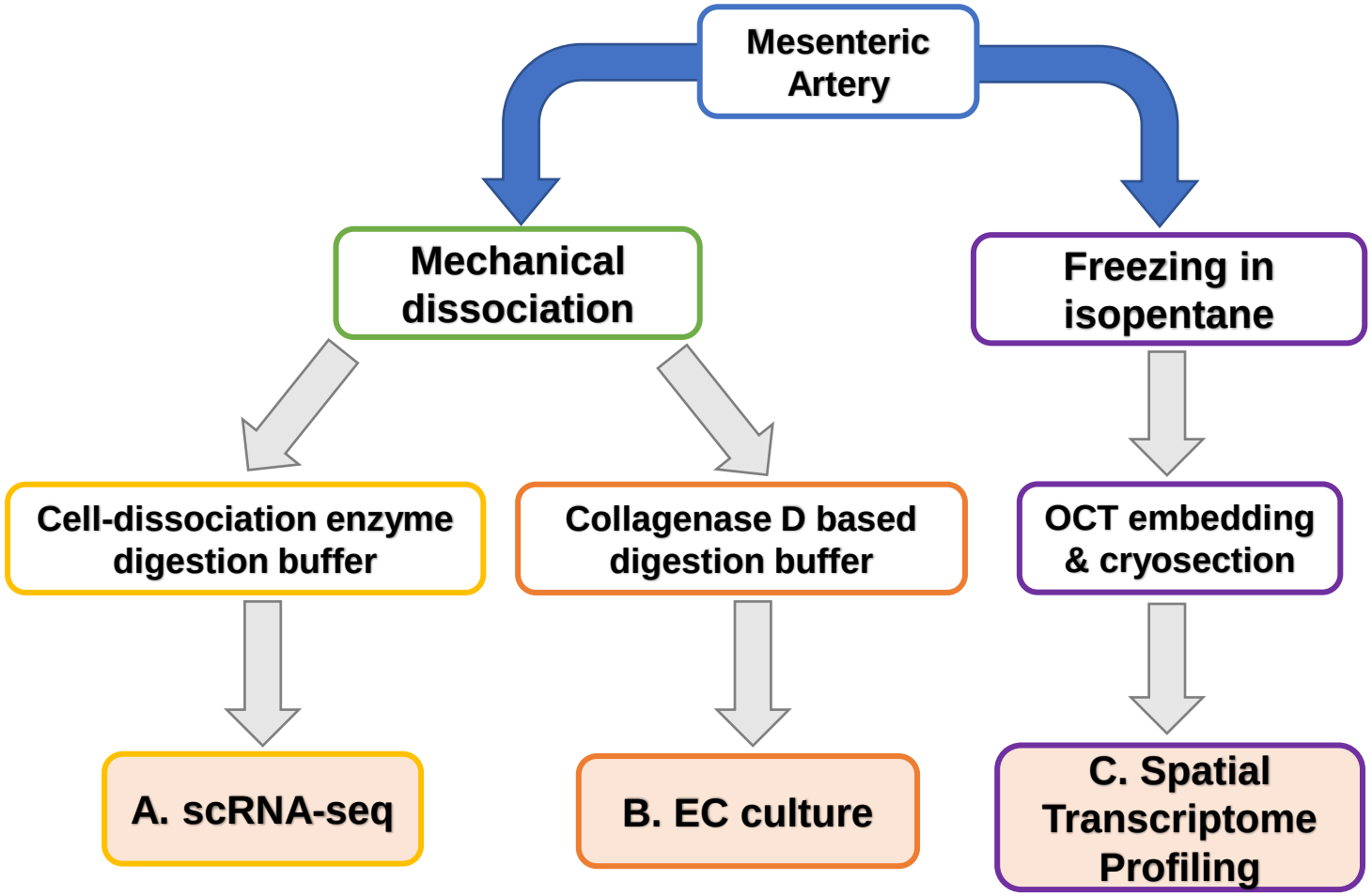


Figure 3

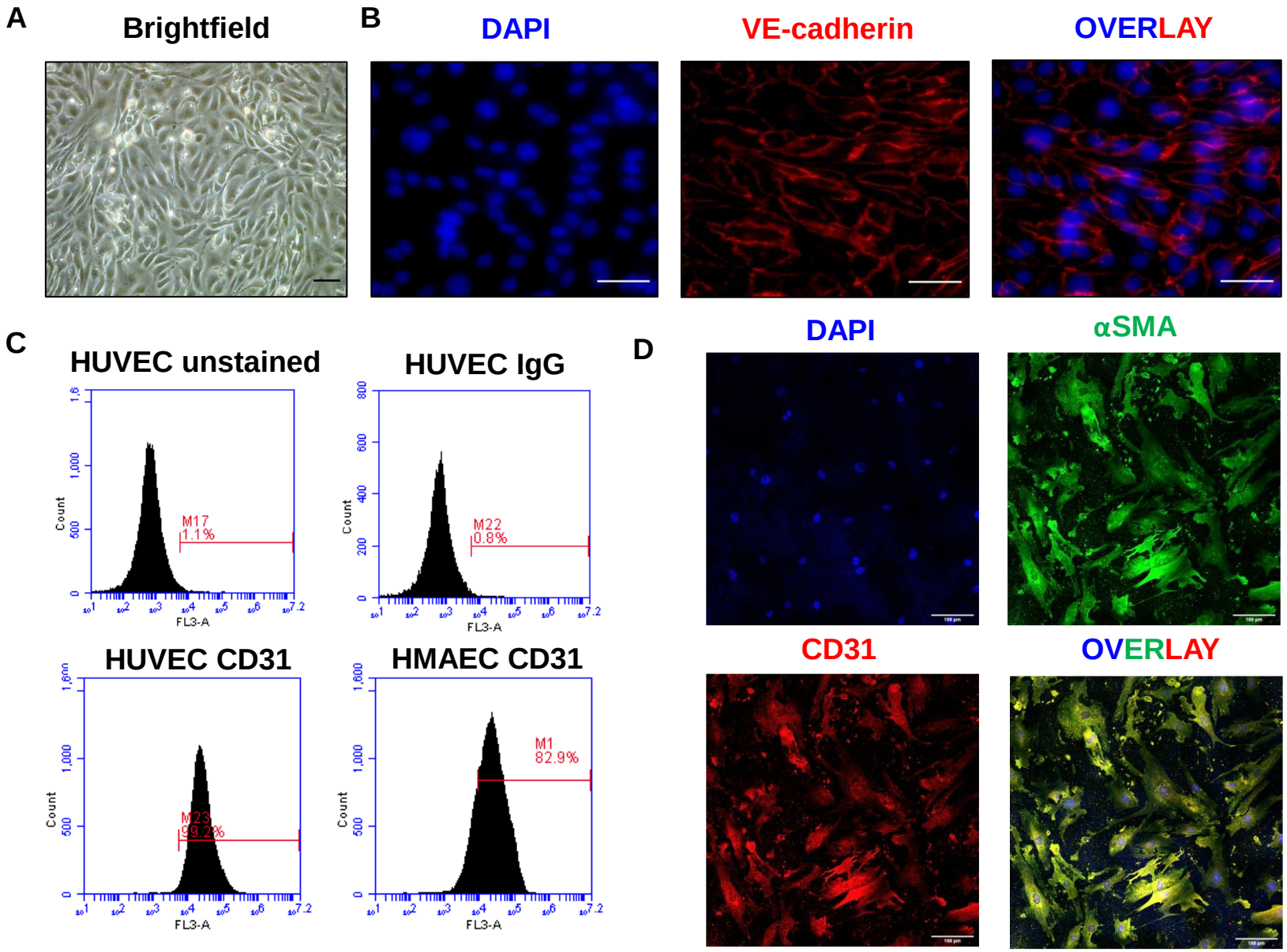


Figure 4

Click here to access/download;Figure;Figure 4.pdf

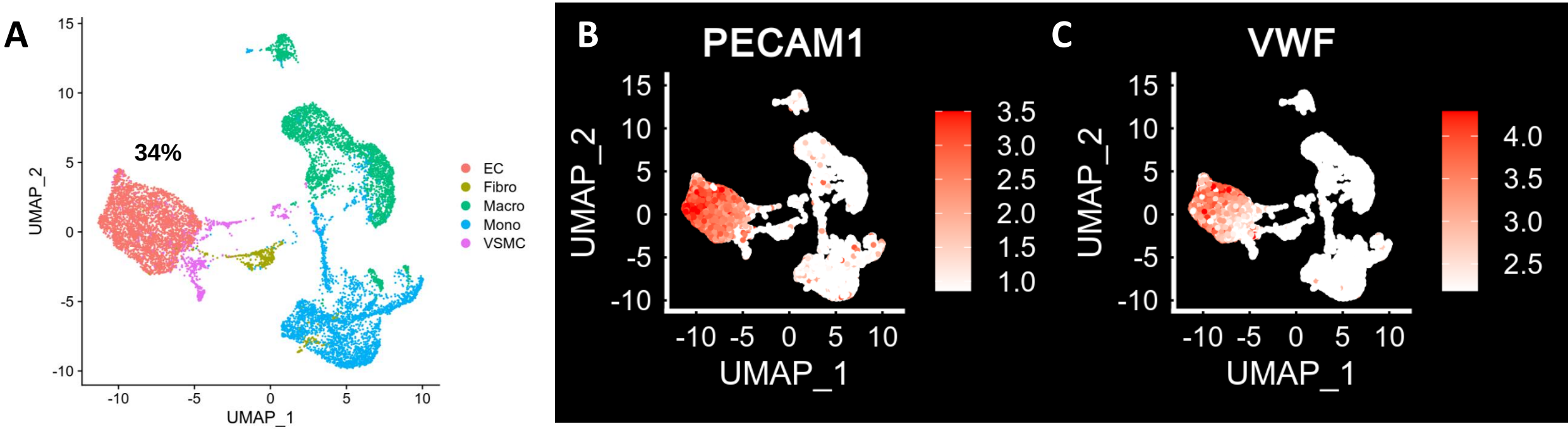


Figure 5

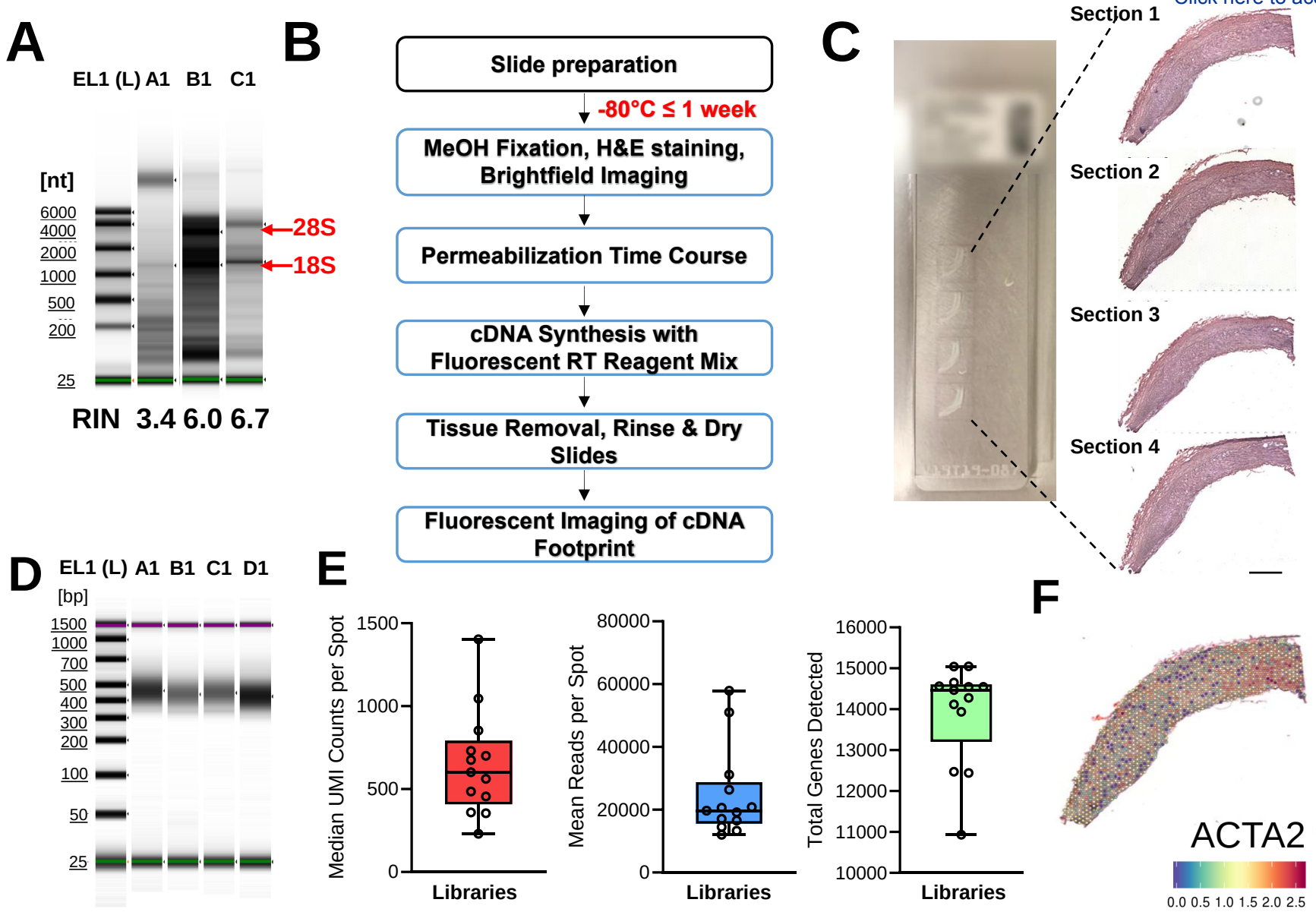


table of materials

Point-to-point response to Editors' and Reviewers' comments

We appreciate every comment from the editors and reviewers and have made our best effort to address the questions and concerns within the required time frame, we have marked all the changes in red in the revised manuscript, as detailed below.

Editorial comments to Authors:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly proofread the article and checked and corrected any spelling or grammatical errors.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the manuscript to be completely in third person.

3. Please provide at least 6 keywords or phrases.

Endothelial cell, transcriptome, artery, single cell profiling, spatial transcriptome, vasculature

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. (e.g., 10x Genomics Visium, 10x Genomics (v6.0.1) Agilent TapeStation, Zeiss Axio Observer ZII Inverted Microscope, Eppendorf, etc.)

We have removed all commercial language from the methods text and replaced with generic terms.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have revised the protocol accordingly.

6. Please insert single-line spacing between individual steps and sub-steps in the Protocol. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have adjusted the spacing for the steps in the protocol as requested and highlighted the essential steps most useful for readers to see the visualization.

7. Please add more details to your protocol steps to ensure you answer the "how" question, i.e., how is the step performed.
a. 1.2: please specify the instruments used

The instruments required are forceps and dissection scissors. We have specified this in 1.2.

b. 1.4 and 1.5: which instrument/tool is used to cut the vessel? Please consider providing a figure showing the dissected vessel with exposed intima.

The tool required is dissection scissors which we have included in the step. Additionally, we have included a panel showing the vessel with exposed intima in Fig. 2B.

c. 3.1: what is OCT? Please define all abbreviations before use.

OCT is optimal cutting temperature compound. We have defined OCT in the text.

d. 3.6: how do you ensure 10 µm thickness for the sections?

The cryostat has the ability to adjust the thickness of cutting the sections. We have made this clear in the text.

e. Section 4: If you want to film these steps, please add more specific details, e.g., button clicks for software actions, numerical values for settings, etc. (note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video). Alternatively, add references to published material, if available, specifying how to perform the protocol action.

We do not want to film the sequencing data analysis.

8. Figures: Please include a one-liner title referring to all the panels. Please include scale bars for all the images obtained from a microscope.

All Figure panels now have an underlined one-liner title. All microscopy images now contain a scale bar.

9. Figure 2B: please include a scale bar

We have added the scale bar to this image and stipulated the size in the figure legend.

10. Figure 3A: please provide the magnification.

We have provided the magnification in the text of the figure legend.

11. Figure 5: Text and axis labels in some of the panels are too small to read. Please ensure legibility. Please provide scale bars or magnification details for the microscope images in panel B. Please provide an x-axis in panel E.

This figure has been adjusted to ensure legibility. Text size has been increased and an x axis has been added to panel E. We have also included scale bars, of which the size is included in the text of the figure legend as well as the magnification.

12. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have revised our discussion to ensure it covers the above.

13. Please expand all the journal names in references

We have expanded all journal names in references.

Reviewer #1:

Manuscript Summary:

Malhi et al. presented a modified method to isolate endothelial cells (ECs) from human mesenteric artery for either cell culture or single cell analysis. They specifically focused on the downstream single cell transcriptomic solutions applied after the EC isolation or correct tissue preservation, which has rarely been discussed previously. Meanwhile, a series of solid data from in vitro cell culture to single-cell analysis were provided to prove the utility of their protocol. Their work lay a concrete foundation for the vasculature community to study large vessel biology and pathology in human.

We are extremely grateful for the reviewer's positive comments and recognizing the value of our work.

Minor Concerns:
1. The introduction sections could be shortened to focus on summarizing previous methods regarding EC culture and isolation from various organs, in both human and mouse.

We have taken the reviewer's suggestion and left out most of the first paragraph detailing the general role of ECs and included a section in the fourth paragraph of the introduction summarizing previous methods regarding EC culture and isolation (see Lines 81-88).

2. To enrich EC population for single cell transcriptomic profiling, the authors combined enzymatic and mechanical methods to isolate EC. However, there are clearly a more than 60% of contamination attributed by other cell types as revealed by scRNA-seq, and this is likely due to the harsh scraping. As mentioned in the discussion, the authors suggested to use CD144 antibody to purify EC before applying to downstream applications. Nevertheless, a gentle flush with pipetting after enzymatic digestion without scraping should also easily peel off the endothelial layer and solve the problem. Therefore, the mechanical process step might not be necessary if the purpose is to analyze the enriched EC population. The authors should discuss the necessity of mechanical process when it comes to EC purity.

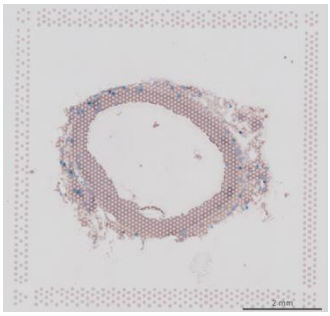
Thank you for this insightful comment. In line with the Reviewer's thought, we originally tried using a gentle flush with pipetting after enzymatic digestion (with collagenases or Trypsin)

without scraping, followed by 37°C incubation for 15 min. However, we were not able to collect enough cells for either *in vitro* culture or scRNA-seq. We reasoned that this could be due to the insufficient penetration of the enzyme into the subendothelial layer of the mesenteric artery and dissociation of the intima from medial layer. Thus, we included the gentle scraping, i.e. the mechanical step at the cell collection step. We have also tested scraping step by varying the number of scrapes and found that two times scraping yields data as represented in this protocol, which detail has now been included in the revised manuscript (Line 137). The additional step with CD144 antibody can further increase the EC population but this will not exclude all other cells that interact with ECs due to the native cell-cell interaction. Nevertheless, although traditionally the other cell types would be regarded as "contamination" in EC isolation, they can actually provide useful information for cell-cell interactions in the tissue context with the enabling scRNA-seq and spatial profiling. In the subsequent scRNA-seq data analysis, ECs can be efficiently annotated using the EC markers and the cell-cell interactions can be inferred bioinformatically. Furthermore, the inclusion of these data can enable more effective integration with and deconvolution of the spatial profiling data, which are not at single-cell resolution (Rao et al. Nature 596, 211–220. 2021. https://doi.org/10.1038/s41586-021-03634-9).

We have taken the reviewer's suggestion to include such discussion in the revised manuscript (Lines 386-397 in "Discussion").

3. In Figure 2D, a CD31 gene expression on Visium should be shown to demonstrate the utility of the current method to capture EC information in a large vessel as this size.

We have visualized CD31 on Visium and are happy to show this to this reviewer (see figure to the right). We see CD31 expression partially localized to the inner lumen of the vessel. Intriguingly, we have also observed signal of CD31 (and other EC markers) in other layers of the vessel. We are currently investigating this in a separate study. We hope the reviewer understands and agrees with us not including this in the current protocol paper.



4. 3.6, "cooled Eppendorf", missed "tube here".

We have added "tube" in place of Eppendorf as the editor has asked us to remove Eppendorf due to it being commercial language.

Reviewer #2:

Manuscript Summary:

The paper entitled 'Isolation and profiling of human primary mesenteric arterial endothelial cells at the transcriptome level' describes a method of isolating native endothelial cells though two different methods for either cell culture or scRNA-seq. The authors highlight an important aspect of endothelial research, in that native endothelial cells change phenotype pretty rapidly in cell culture and describe various methods to address including methods for studying the endothelial cell gene expression in situ. Before I could recommend this paper for publication, there is a few minor comments I would like to raise.

We appreciate the excellent summary by the Reviewer and we have address the minor concerns as below.

Minor Concerns:

1. The authors state that the human mesenteric artery studied in the protocol was 6-8mm in diameter, and refer to it as a resistance artery. This artery is far too big to be labelled as this, as resistance arteries are < 400 µm in diameter. Is there a lower limit to the size of artery this method can be used on?

We agree with the reviewer that the artery at 6-8 mm in diameter should not be referred to as a resistant artery. Depending on the donor and isolation, we collect superior mesenteric artery (at 6-8mm in diameter) and/or attached branching resistance arteries from the arterial trunk. We have taken out "resistance artery" from the protocol. With regards to the lower limit of the size, for spatial transcriptomics, the protocol can be performed on 1 mm of vessel in total, regardless the diameter. For the dissociation protocol for cell culture and scRNA-seq, the lower limit would be if the vessel is too narrow (i.e. below 50 mm in diameter) to enable insertion of dissection scissors. This is an important point which we have included in the discussion (see Lines 370-376).

2. As this tissue is post-mortem, can the authors describe any time restraints between the time of death and the start of experiments? Can the authors discuss if they have any information on changes to gene expression of endothelial cells from post-mortem compared to arteries harvested in surgery or biopsies?

We typically process the post-mortem tissues between 8-14 hours after the time of death. We have tested scRNA-seq and Visium procedures with these tissues and obtained data with acceptable quality. However, we recommend beginning the isolation as soon as possible post extraction from patient and maintaining tissue on ice prior to isolation. We have added this point to the discussion (see Lines 362-369).

While the comparison between the post-mortem tissues vs those harvested in surgery/biopsies is interesting and important, we do not have any information on changes to gene expression of ECs in either condition.

3. In protocol section 1.3 the KCl concentration was 50 mM. This is high and I wonder if it is a typo and should be 5 mM. If not, please can you explain why? Also please add the pH required and what base is used to achieve this.

We appreciate the reviewers' meticulousness. We indeed use 50mM, but not 5 mM KCl. This is based off a method we originally developed in collaboration with our colleague Dr. Qiong Annabel Wang at City of Hope, who was a co-author on (Asterholm et al. *Cell Metab.* 2014. 20(1):103-18. doi: 10.1016/j.cmet.2014.05.005). We have adapted this method to isolate murine ECs from adipose tissues (Tang et al. *Circulation.* 2020. 142(4):365-379. doi: 10.1161/CIRCULATIONAHA.119.041231) and extended the use to human EC isolation. The pH required is 7.0 and does not require adjustment.

Reviewer #3:

Manuscript Summary:

The manuscript described the workflow to analyze the endothelial cells from the mesenteric

artery. The manuscript revealed a complex methodology to investigate the endothelial cells not only after cell isolation, but also by in situ techniques.

Major Concerns:
There are many assays in the current manuscript and I am afraid that they cannot be well illustrated all together. First, the authors describe the dissociation of the mesenteric artery in two ways, each one for a specific type of analysis. Then, the authors described the analysis on sections, including IHC and spatial transcriptomic. All these techniques have their tricks and they are not well explained in the text. The work should explain the techniques in detail in order to be done by young researchers without experience, not to refer to other protocols.

The reviewer's concerns are well taken. Accordingly, we have streamlined our protocol to focus on only the two methods for EC dissociation and spatial transcriptome profiling using cryopreserved tissue sections. We have removed FISH/ISH, IHC, and snRNA- and scATAC-seq from the protocol, which we do not provide enough technical details (see revised Fig. 2). Moreover, we have provided more details to each remaining technique to facilitate the readership of people without experience in these procedures.

Minor Concerns:
Please mention from the beginning the equipment needed for these assays.

We now mention the necessary equipment from the beginning.

Reviewer #4:

Manuscript Summary:

The authors of this study propose a method of endothelial cell (EC) isolation from donor mesenteric arteries. The aim of this method is to capture endothelial cells in their native state, through bypassing culturing steps. The authors' provide strong justification for the need of this protocol. The protocol is well thought out and provides many details. A more in depth introduction to spatial transcriptome profiling should be made, especially considering the authors' careful consideration of utilizing ECs in the intimal layer alone. Additionally, the length of time it takes to complete the protocol needs to be kept into consideration. The authors' should be careful not to state that this method better emulates ECs in their native state without comparative analyses. Altogether, if published, the authors would contribute something meaningful to the field.

We appreciate the reviewer's positive and insightful feedback. We have provided more in-depth introduction to spatial transcriptome profiling. We have included the timeline by adding the estimated time on the sub-headings of the methods. We agree fully that we should not state that this method is "better" to emulate ECs in their native state without comparative analyses and thus have removed any remarks reflecting this.

Major Concerns:
Line 390-391: A better introduction to spatial transcriptome profiling in the introduction of this paper is warranted, especially given their careful consideration of utilizing ECs in the intimal layer alone.

We have improved the introduction according to the reviewer's suggestion. See Lines 94-105 in Introduction.

Minor Concerns:
Line 61-75: This paragraph does a great job at capturing the complexity of ECs. Perhaps consider giving specific examples of EC metabolism and EndMT.

Thank you for this comment. We are still analyzing the data in more depth and would prefer to opt out of including such data to keep the article focused on the method. We will look into markers of EC metabolism and EndMT as reviewer suggested.

Line 366-367: This is a bit of a strong statement. How long does it take to perform physical dissociation, preparation for RNA-seq studies, and spatial profiling using 10x Genomics Visum?

We have toned down this statement. We have added the estimated time for each step at the beginning of each sub-section.

Line 369-388: Justification to use the intimal layer alone is logical here.

The reasons that we use the intimal layer alone are as follows: 1) in the published scRNA-seq datasets collected from whole blood vessels, without enrichment, ECs would only constitute a small percentage (<7%) of cell clusters (Hu, H., et al. *ATVB.* **41** (4), 1408–1427. 2021. doi:10.1161/ATVBAHA.120.315373; Wirka, R.C. et al. Nat Med 25, 1280–1289 (2019). https://doi.org/10.1038/s41591-019-0512-5); 2) use of the intimal layer allows for an enrichment of ECs without the need of harsh tissue digestion and enhance cell viability for scRNA-seq or subsequent culture (Rao et al. *Nature* 596, 211–220. 2021. https://doi.org/10.1038/s41586-021-03634-9. We have incorporated this point in the Discussion (Lines 378-397).