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Isolation of Endothelial Cells from the Lumen of Mouse Carotid Arteries for Single-cell Multi-omics Experiments

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Corresponding Author:	Hanjoong Jo Emory University and Georgia Institute of Technology Atlanta, GA UNITED STATES
Corresponding Author's Institution:	Emory University and Georgia Institute of Technology
Corresponding Author E-Mail:	hjo@emory.edu
Order of Authors:	Sandeep Kumar
	Aitor Andueza
	Juyoung Kim
	Dong Kang
	Hanjoong Jo
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1 **TITLE:**
2 Isolation of Endothelial Cells from the Lumen of Mouse Carotid Arteries for Single-cell Multi-
3 omics Experiments
4
5 **AUTHORS AND AFFILIATIONS:**
6 Sandeep Kumar[1§], Aitor Andueza[1§], Juyoung Kim[1], Dong-Won Kang[1], Hanjoong Jo[1,2]
7
8 [1]Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and
9 Emory University, Atlanta, GA, USA
10 [2]Division of Cardiology, Georgia Institute of Technology and Emory University, Atlanta, GA, USA
11
12 [§] Equal contribution
13
14 **Email addresses of co-authors:**
15 Sandeep Kumar   (sandeepkumar@emory.edu)
16 Aitor Andueza   (aanduez@emory.edu)
17 Juyoung Kim   (juyoungkim@gmail.com)
18 Dong-Won Kang   (dkang9@emory.edu)
19
20 **Corresponding author:**
21 Hanjoong Jo   (hanjoong.jo@bme.gatech.edu)
22
23 **KEYWORDS:**
24 Artery cells, Endothelial cells, single-cell RNAseq, single-cell ATACseq, Atherosclerosis
25
26 **SUMMARY:**
27 We present a method for isolating endothelial cells and nuclei from the lumen of mouse carotid
28 arteries exposed to stable or disturbed flow conditions to perform single-cell omics experiments.
29
30 **ABSTRACT:**
31 Atherosclerosis is an inflammatory disease of the arterial regions exposed to disturbed blood
32 flow (*d-flow*). *D-flow* regulates the expression of genes in the endothelium at the transcriptomic
33 and epigenomic levels, resulting in proatherogenic responses. Recently, single-cell RNA
34 sequencing (scRNAseq) and single-cell Assay for Transposase Accessible Chromatin sequencing
35 (scATACseq) studies were performed to determine the transcriptomic and chromatin accessibility
36 changes at a single-cell resolution using the mouse partial carotid ligation (PCL) model. As
37 endothelial cells (ECs) represent a minor fraction of the total cell populations in the artery wall,
38 a luminal digestion method was used to obtain EC-enriched single-cell preparations. For this
39 study, mice were subjected to PCL surgery to induce *d-flow* in the left carotid artery (LCA) while
40 using the right carotid artery (RCA) as a control. The carotid arteries were dissected out two days
41 or two weeks post PCL surgery. The lumen of each carotid was subjected to collagenase digestion,
42 and endothelial-enriched single cells or single nuclei were obtained. These single-cell and single-
43 nuclei preparations were subsequently barcoded using a 10x Genomics microfluidic setup. The
44 barcoded single-cells and single-nuclei were then utilized for RNA preparation, library generation,

45   and sequencing on a high-throughput DNA sequencer. Post bioinformatics processing, the
46   scRNAseq and scATACseq datasets identified various cell types from the luminal digestion,
47   primarily consisting of ECs. Smooth muscle cells, fibroblasts, and immune cells were also present.
48   This EC-enrichment method aided in understanding the effect of blood flow on the endothelium,
49   which could have been difficult with the total artery digestion method. The EC-enriched single-
50   cell preparation method can be used to perform single-cell omics studies in EC-knockouts and
51   transgenic mice where the effect of blood flow on these genes has not been studied. Importantly,
52   this technique can be adapted to isolate EC-enriched single cells from human artery explants to
53   perform similar mechanistic studies.
54
55   **INTRODUCTION:**
56   This laboratory previously demonstrated that induction of *d-flow* leads to quick and rugged
57   atherosclerosis development in hyperlipidemic mice[1,2]. The novel mouse model of d-flow-
58   induced atherosclerosis was possible using partial carotid ligation (PCL) surgery [3]. PCL surgery
59   induces low and oscillatory blood flow condition or *d-flow* in the ligated left carotid artery (LCA).
60   In contrast, the contralateral right carotid artery (RCA) continues to face stable laminar flow (*s-
61   flow*). Previously, to understand the effect of *d-flow* on endothelial cells, the carotid arteries were
62   dissected out after partial ligation surgery and flushed with a phenol and guanidine
63   isothiocyanate-based lysing agent (luminal RNA/DNA flushing method)[2,4], which provided
64   endothelial-enriched "pooled bulk" RNAs or DNAs. These pooled bulk RNAs or DNAs were then
65   processed for transcriptomic studies or epigenomic DNA methylome studies, respectively[4-6].
66   These studies helped discover multiple flow-sensitive genes and microRNAs whose roles in
67   endothelial biology and atherosclerosis were extensively investigated[4,6,7].
68
69   However, despite endothelial enrichment, these bulk RNA/DNA studies could not distinguish the
70   specific role of each cell type in the artery wall in *d-flow*-induced atherosclerosis. Endothelial-
71   enriched single-cell isolation and single-cell (sc)RNA and scATAC sequencing studies were
72   performed to overcome this limitation[8]. For this, C57Bl6 mice were subjected to the PCL surgery
73   to induce *d-flow* in the LCA while using the *s-flow*-exposed RCA as control. Two days or two weeks
74   after the PCL surgery, the mice were sacrificed, and the carotids were dissected and cleaned up.
75   The lumen of both LCAs and RCAs were infused with collagenase, and the luminal collagenase
76   digests containing ECs as a significant fraction and other arterial cells were collected. The single-
77   cell suspension (scRNAseq) or single-nuclei suspension (scATACseq) were prepared and barcoded
78   with unique identifiers for each cell or nucleus using a 10x Genomics setup. The RNAs were
79   subjected to cDNA library preparation and sequenced.
80
81   The scRNAseq and scATACseq datasets were processed using the Cell Ranger Single-Cell Software
82   and further analyzed by Seurat and Signac R packages[9,10]. Each cell and nucleus was assigned a
83   cell type from these analyses and clustered into the cell type based on the marker genes and
84   unique gene expression patterns. The results of the scRNAseq and scATACseq demonstrated that
85   these single-cell preparations are enriched with ECs and also contain smooth muscle cells (SMCs),
86   fibroblasts, and immune cells.
87
88   Further analysis revealed that the EC population in the luminal digestion is highly divergent and

89 plastic (8 different EC clusters) and responsive to blood flow. Most importantly, these results
90 demonstrated that *d-flow* reprograms ECs from an athero-protected anti-inflammatory
91 phenotype to pro-atherogenic phenotypes, including pro-inflammatory, endothelial-to-
92 mesenchymal transition, endothelial stem/progenitor cell transition, and most surprisingly,
93 endothelial-to-immune cell-like transition. In addition, scATACseq data reveal novel flow-
94 dependent chromatin accessibility changes and transcription factor binding sites in a genome-
95 wide manner, which form the basis of several new hypotheses. The methodology and protocol
96 for preparing single endothelial cells for single-cell multi-omics studies from the mouse carotid
97 arteries are detailed below.

98
99 **PROTOCOL:**

100
101 All animal procedures described below were approved by the Institutional Animal Care and Use
102 Committee at Emory University. Non-hypercholesterolemic, age- and sex-matched C57BL/6 mice
103 were used to mitigate sex-dependent variation and offset any complication of
104 hypercholesterolemic conditions*.*

105
106 **1.   Partial carotid ligation (PCL) surgery**

107
108 NOTE: Partial carotid artery ligation of LCA was carried out as previously described and
109 demonstrated[3].

110
111 1.1.   Anesthetize the mouse by isoflurane inhalation (5% isofluorane in oxygen for induction
112 and 1.5% after that for maintenance) throughout the procedure using an anesthetic vaporizer.

113
114 1.2.   Apply an ocular ointment to prevent exposure keratitis, and confirm the depth of
115 anesthesia by the absence of toe pinch response.

116
117 1.3.   Inject buprenorphine 0.05 mg/kg subcutaneously to manage pain preemptively. Disinfect
118 the epilated area by applying and cleaning with betadine and isopropanol solution three times.
119 Ensure that betadine is the last step to sterilize the surgery area, starting from the center and
120 finishing at the sides.

121
122 1.4.   Using aseptic techniques, make a ventral midline incision (~1 cm) in the neck region, and
123 expose the LCA branch point by blunt dissection.

124
125 1.5.   Ligate the left internal carotid, external carotid, and occipital arteries with 6-0 silk suture;
126 leave the superior thyroid artery untouched.

127
128 1.6.   Approximate the skin and close the incision with tissue glue and/or sutures.

129
130 1.7.   Transfer the mouse to a pre-warmed recovery cage (maintained at 37 °C) and place it on
131 a clean towel for up to 1 h to avoid post-surgery hypothermia.

132

133 NOTE: If performed correctly, there is no mortality associated with this procedure, and
134 postoperative stress to the mouse is minimal.

136 1.8.   Prepare the perfusion setup.

138 1.8.1.   Use 0.9% NaCl (normal saline) containing 10 U/mL heparin in an IV bag. Hook the IV bag
139 244–274 cm (8–9 feet) from the ground or approximately 122–152 cm (4–5 feet) higher than the
140 dissection board. Attach the butterfly needle to the end of the saline line and flush any air
141 bubbles out of the IV line.

143 **2.   Isolation of carotid arteries post sacrifice**

145 2.1.   Sacrifice the mice by CO_2 inhalation following the institutional IACUC protocol.

147 2.2.   Spray 70% ethanol on the mouse skin and place it in the supine position on the dissection
148 board containing adsorbent paper towels. Secure the paws of the mouse to the adsorbent towels
149 on the dissection board using adhesive tape or a 21 G needle.

151 2.3.   Using a pair of sterile scissors, remove the skin of the mouse starting from the abdomen
152 and all the way to the top of the thorax.

154 2.4.   Use a pair of scissors to open the abdominal wall below the ribcage by blunt dissection.

156 2.5.   Using the scissors, carefully make an incision along the length of the diaphragm, and
157 continue through the ribs on both sides of the thorax until the sternum can be lifted away. Gently
158 lift away the sternum with a pair of forceps; after that, remove the ribcage to expose the heart.

160 2.6.   Carefully remove the thymus and any connective tissue over the heart to visualize the
161 major vessels.

163 2.7.   Sever the vena cava with scissors to allow blood to exit from closed circulation.

165 2.8.   Insert a 21 G butterfly needle connected to the IV line through the apex of the heart into
166 the left ventricle and allow retrograde perfusion for 2–3 min with normal saline at room
167 temperature. Ensure that a constant flow rate of normal saline is maintained by keeping the
168 saline bag at the height of 8–9 feet from the ground.

170 NOTE: The lungs and liver become pale, indicating optimal perfusion. Approximately 20 mL of
171 perfusion buffer is used for each mouse.

173 2.9.   Remove the skin from the neck region and remove all the fat, muscles, and connective
174 tissues to expose the carotid arteries (**Figure 1A**).

176 NOTE: The rest of the procedure is carried out under the dissecting microscope.

177

2.10.   Adjust the field of view to locate the ligation sites. Using the 10 mm micro-dissection scissors, make a small incision below the ligation site in the LCA to allow perfusion.

2.11.   Perfuse again for an additional 1 min via the left ventricle and ensure that the LCA is well perfused and free from any visible traces of blood.

2.12.   Use fine-tip forceps and small spring scissors to carefully remove the peri-adventitial tissues surrounding the carotids while the carotid is attached to the body.

NOTE: Be extremely careful not to squeeze or stretch the carotid arteries during this cleaning step, as this can increase the number of non-viable cells. If performed correctly, the cell viability in the final cell suspension is usually >93%.

3.   Endothelial-cell-enriched single-cell isolation from mice carotids

NOTE: The reagents described below can be prepared in advance and stored at 4 °C until use: 1x and 0.1x single-nuclei lysis buffers with the reagents listed in **Table 1**; single-nuclei wash buffer with the reagents listed in **Table 1**; single-nuclei Nuclei Buffer recipe with the reagents listed in **Table 1**. The working stocks of these buffers are to be prepared following the manufacturer's protocol. **Digestion buffer composition:** Collagenase Type II 600 U/mL and DNase I 60 U/mL in 0.5% fetal bovine serum (FBS)-containing phosphate-buffered saline (PBS).

3.1.   While the carotids are still attached, wash the exterior of the carotid arteries with normal saline solution to flush away any traces of blood.

3.2.   Using an insulin syringe fitted with a 29 G needle, inject ~ 50 µL of the digestion buffer into the lumen of the distal end of the left carotid artery. As the digestion buffer starts to fill the carotid artery, clamp the proximal end of the carotid with a micro-clip (**Figure 1B**). Introduce an additional 15–20 µL of digestion buffer into the lumen and clip the distal end to avoid the release of the digestion buffer.

3.3.   Carefully explant the carotid arteries from the mouse (**Figure 1B,C**) and place them in 35 mm dishes containing warm (at 37 °C) HEPES-buffered saline solution.

NOTE: Make sure that both the clamps are securely placed. If the clamp becomes loose, the digestion solution can leak out of the lumen and affect the obtained cell count. If this happens, add additional enzymatic solution using an insulin syringe fitted with a 29 G needle from the open end of the carotid and secure the clamp again (**Figure 1D**). If the enzyme buffer solution leaks again, it is likely that the carotid is severed during the explantation process. In this case, discard the carotid and exclude the sample from the study. Repeated rough handling of the carotid will decrease the cell viability and the quality of single-cell preparation. **Take care to identify and correctly label the left and right carotid arteries.**

221   3.4.   Incubate the explanted carotids for 45 min at 37 °C with intermittent rocking.
222

223   **4.**   **Flushing the carotid arteries**
224

225   4.1.   After completing the luminal enzymatic digestion, remove the carotid artery together
226   with the clamps from the 35 mm dish. Carefully remove each clamp, taking care that the digestion
227   buffer should not leak.
228

229   4.2.   Gently hold one end of the carotid artery with fine forceps on top of a 1.5 mL
230   microcentrifuge tube. Insert a 29 G needle fitted with an insulin syringe containing 100 µL of
231   warm digestion buffer (37 °C) into the lumen with the other hand (**Figure 1D**).
232

233   4.3.   Quickly flush the lumen of the carotid into the microcentrifuge tube. Block the enzymatic
234   reaction by adding 0.3 mL of FBS into the 1.5 mL tube. Place the tube on ice.
235

236   NOTE: The flushing contains the cells from the luminal enzymatic digestion. Adding FBS and
237   reducing the temperature stops the enzymatic digestion process. If the number of cells in the
238   preparation is high and contains debris or fat tissue, the use of a 50 or 70 µm cell strainer is highly
239   recommended to filter out unwanted tissues debris. To increase the single-cell count, flushing
240   multiple carotids is recommended. Here, to obtain at least 5,000 cells, 10–12 carotids were
241   flushed and pooled as one sample.
242

243   4.4.   Centrifuge the cells at 500 × g for 5 min at 4 °C using a centrifuge equipped with a fixed-
244   angle rotor (see the **Table of Materials**).
245

246   4.5.   Discard the supernatant and resuspend the cell pellet in digestion buffer containing cell
247   dissociation reagent (see the **Table of Materials**) for 5 min at 37 °C to separate all the cells into
248   single cells.
249

250   NOTE: If the number of cells in the preparation is low, increase the centrifuge speed up to 2,000-
251   3,000 × g to spin down the cells. Moreover, using 0.5 mL centrifuge tubes facilitates better
252   visualization of the cell pellet at the bottom of the tube.
253

254   4.6.   Block the enzymatic reaction by adding 0.15 mL of FBS into the 0.5 mL tube.
255

256   4.7.   Centrifuge the single-cell suspension at 500 × g for 5 min at 4 °C using a centrifuge
257   equipped with a fixed-angle rotor, as in step 4.4.
258

259   4.8.   Discard the supernatant and resuspend the cells in 100 µL of ice-cold 1% bovine serum
260   albumin (BSA) solution in PBS in a 0.2 mL microcentrifuge tube.
261

262   NOTE: Using 0.2 mL centrifuge tubes enhances the visualization of the single-cell-pellet at the
263   bottom of the tube.
264

265 **5.   Single-cell and single-nucleus analyses**

267 5.1.   Resuspend and submit the single-cell preparation for scRNAseq.

269 5.1.1.   Resuspend the single-cell pellet with 100 µL of ice-cold 1% BSA in PBS in a 0.2 mL tube.

271 5.1.2.   After resuspending the cells for single-cell encapsulation, immediately proceed for single-
272 cell encapsulation and barcoding using a microfluidics-based single-cell partitioning and
273 barcoding system (see the **Table of Materials**).

275 5.1.3.   Before submitting the samples to the Genomics Core, count and inspect the single-cell
276 preparations; ensure the absence of cell aggregates.

278 NOTE: The aggregation of single cells can be further minimized by increasing the BSA amount up
279 to 2%.

281 5.2.   Resuspend and submit the single-cell preparation for scRNAseq.

283 5.2.1.   Resuspend the cell pellet in 100 µL of 0.04% BSA in ice-cold 1x PBS and centrifuge the
284 single-cell suspension at 500 × g at 4 °C.

286 5.2.2.   Lyse the single-cell suspension with ice-cold 0.1x lysis buffer (**Table 1**) and incubate for 5
287 min on ice.

289 5.2.3.   Mix the lysate 10 times with a P-20 pipette and incubate for an additional 10 min.

291 5.2.4.   Add 500 µL of chilled wash buffer (**Table 1**) to the lysed cells and mix 5 times using a
292 pipette.

294 NOTE: Use a 70 µm cell strainer to filter any debris from the cell suspension and transfer them
295 into a 2 mL tube.

297 5.2.5.   Centrifuge the lysate at 500 × g for 5 min at 4 °C. Discard the supernatant and resuspend
298 the nuclei-pellet in the diluted nuclei buffer (150 µL) (see the **Table of Materials** and **Table 1**).
299 Count and inspect the single-nuclei preparations using a hemocytometer[11].

301 5.2.6.   Submit the single-nuclei sample to the Genomics Core for single-nuclei transposition,
302 nuclei partitioning, library preparation, and sequencing.

304 NOTE: If the initial cell number is low, it is common to observe debris along the nuclei. Therefore,
305 it is recommended to start with a high cell number.

307 **REPRESENTATIVE RESULTS:**
308 Partial carotid ligation surgeries were performed on 44 mice, and the onset of *d-flow* in the LCA

309 was validated by performing ultrasonography one day post partial ligation surgery. Successful
310 partial ligation surgery causes reduced blood flow velocity and reverses blood flow (disturbed
311 flow) in the LCA[3]. The carotid arteries were dissected out either at two days or at two weeks post
312 ligation. The lumen of each carotid was subjected to collagenase digestion, and endothelial-
313 enriched single-cells or single-nuclei suspensions were prepared. Single-cell suspensions were
314 pooled from 10 RCAs and LCAs to increase the cell yield. The cells/nuclei prepared were
315 subsequently barcoded and sequenced. For the sc-RNAseq study, the number of single cells
316 obtained was ~9,700. The distribution of cells from 4 samples is shown in **Table 2**.

318 Likewise, for the single-nuclei ATAC sequencing study, single cells were isolated from 12 RCAs
319 and LCAs that were prepared, subjected to transposase treatment, barcoded, and sequenced.
320 Sequencing was performed for 18,324 single nuclei, which were pooled from 1,291 (2-day RCA
321 [2D-R]), 5,351 (2-day LCA [2D-L]), 5,826 (2-week RCA [2W-R]), and 5,856 (2-week LCA [2W-L])
322 (**Table 2**). Representative single-cell and single-nuclei preparations as visualized by brightfield
323 microscopy and phase-contrast microscopy are shown in **Figure 2A,B**. The efficiency of single-
324 nuclei preparation from single-cell suspension is shown in **Figure 2C**.

326 The nuclei (~7,000 each) from 2D (RCAs and LCAs) and 2W (RCAs and LCAs) samples were
327 incubated with a Transposition Mix (Tn5 transposase enzyme and buffer) see the **Table of**
328 **Materials**) for 60 min at 37 °C following the manufacturer's protocol. Based on the
329 manufacturer's recommendation, a mild detergent condition helped keep the nuclei intact
330 during tagmentation. A master mix, consisting of a Barcoding Reagent, Reducing Agent B, and
331 Barcoding Enzyme, was then loaded onto a microfluidic cell/nuclei encapsulation platform to
332 prepare single-nuclei gel emulsions with barcoding according to the manufacturer's instructions.

334 Post sequencing, the Cell Ranger Single-Cell Software suite was used for demultiplexing, barcode
335 processing, alignment, and initial clustering of the raw scATACseq and scRNaseq profiles. For the
336 scRNAseq study, the distribution of genes per cell, unique molecular identifier (UMI) per cell,
337 mitochondrial reads per cell, and sequencing saturation information are shown in **Figure 3-C**.
338 Likewise, for the scATACseq study, quality control metrics showing the insert size distribution
339 (nucleosome banding pattern) and normalized TSS enrichment score are shown in **Figure 3D–F**.
340 Additionally, the percent fragment reads in the peaks, peak region fragments, TSS enrichment
341 score, ratio of reads in blacklist genomic sites, and nucleosome signal ratio are shown in **Figure**
342 **3G–K**.

344 Endothelial enrichment was quantitated by comparing this method to that of enzymatic digestion
345 of the whole carotid artery[12]. The endothelial cell count from the complete carotid artery
346 digestion was 3–5% of the total cells obtained, whereas this method allowed enrichment of
347 endothelial cells to >50% [8]. Similarly, another single-cell study that used the whole mouse aorta
348 showed that endothelial fraction was <7% of the total cell count. For an in-depth single-cell
349 RNAseq and single-cell ATACseq analysis, readers are requested to refer to [8].

351 **FIGURE AND TABLE LEGENDS:**
352

Figure 1: Isolation of carotid arteries for single-cell preparation. (**A**) Anatomical view of the carotid arteries in mice. Red arrows and inset show the isolated left carotid artery after clean up of periadventitial fat. (**B**) Image shows the location of micro-clips after filling the carotid artery with the digestion buffer. (**C**) Explanted carotid artery containing digestion buffer. Scale: distance between black lines = 1 mm. (**D**) A 29 G needle in the lumen of the mouse carotid artery. This step helps replenish the carotid artery with digestion buffer if needed.

Figure 2: Single-cell and single-nuclei preparation from luminal enzymatic digestion of mouse carotid artery. (**A**) Representative single-cell and single-nuclei preparations. Scale bars = 0.25 mm (**B**) Representative phase-contrast and Gel-Red images of single-nuclei preps. Scale bars = 0.25 mm. (**C**) Efficiency of single-nuclei preparation in the left column shows the number of single cells at the start while the right column shows the number of single nuclei after processing with nuclei isolation buffer in different steps.

Figure 3: Standard QC metrics for the scRNAseq and scATACseq study. Violin plots show (**A**) the distribution of genes per cell (nFeature RNA), (**B**) UMI per cell (nCount_RNA), (**C**) mitochondrial reads per cell (percent mt) for the scRNAseq data. (**D** and **E**) show the nucleosome banding pattern for the scATACseq study. The histogram of DNA fragment sizes exhibits a strong nucleosome banding pattern corresponding to the length of DNA wrapped around a single nucleosome. (**F**) Normalized TSS enrichment score at each position relative to the TSS. The scatter/violin plots show (**G**) percent fragment reads in the peaks, a measure of sequencing depth, (**H**) peak region fragments showing the number of fragments overlapping peaks, (**I**) TSS enrichment score, a ratio between the aggregate distribution of reads centered on TSSs and that flanking the corresponding TSS, (**J**) blacklist ratio, a ratio of reads in blacklist genomic sites, and (**K**) nucleosome signal, a ratio of mononucleosomal to nucleosome-free fragments. Abbreviations: scRNAseq = single-cell RNA sequencing; scATAseq = single-cell Assay for Transposase Accessible Chromatin sequencing; QC = quality control; UMI = unique molecular identifier; TSS = transcription start site.

Table 1: Composition of single-nuclei lysis and wash buffers.

Table 2: Single-cell and single-nuclei count from mouse carotid artery luminal digestion. The table also shows means reads/cell and number of genes/cell for scRNAseq data. For the scATACseq data, mean fragments/cell and total reads obtained per sample are shown[8]. Abbreviations: sc-RNAseq = single-cell RNA sequencing; scATA seq = single-cell Assay for Transposase Accessible Chromatin sequencing; 2D = 2-day; 2W = 2-week; R/RCA = right carotid artery; L/LCA = left carotid artery.

DISCUSSION:
This paper provides a detailed protocol to isolate single-cell preparations from the mouse carotid arteries. The influence of *d-flow* on the endothelial cells can be accurately studied if the PCL surgery is performed correctly. It is crucial to correctly identify the branches of the common carotid, such as the external carotid, internal carotid, occipital artery, and superior thyroid artery. Validation of flow patterns by ultrasonography further validates the successful onset of *d-flow*

397 conditions. Although PCL surgery can be performed on mice irrespective of their age, the
398 preferred age is 10 ± 2 weeks. Older mice and mice with hypercholesterolemic backgrounds
399 generally tend to have more periadventitial fat and stiffer skin.
400
401 It is essential to carefully dissect the carotid artery endothelial-enriched single-cell preparations
402 free of the surrounding tissue and periadventitial fat. The lumen of the carotids must be perfused
403 thoroughly to avoid contaminating blood cells in the single-cell preparations. Improper tissue
404 identification and labeling can result in high standard deviation. Careful planning and time
405 management are required for this protocol. A skilled surgeon and operator can take 15–20 min
406 to perform one PCL surgery. Sacrificing, carotid isolation, and luminal enzymatic digestion from
407 each mouse would take an additional 35–40 min. The hands-on processing time from endothelial
408 cell flushing to single-cell/single-nuclei prep is an additional 2 h. Meticulous planning and
409 teamwork are highly recommended.
410
411 As with any experimental protocol, some disadvantages and limitations should be considered.
412 The current protocol does not incorporate steps to avoid artifactual activation of immediate early
413 response during the luminal tissue dissociation. It has been reported in the literature that
414 enzymatic digestion can lead to stress signaling events, such as inflammation and apoptosis. This
415 can be addressed by incorporating appropriate control groups in the experimental design. In
416 addition, wet laboratory methods that can minimize the artifactual activation of immediate
417 response during enzymatic digestion, such as cold-active proteases, should be considered[13,14].
418
419 This protocol can be used for any mouse strain irrespective of its genetic background. However,
420 the endothelial-enriched preparations can best answer research questions pertaining to the
421 changes in endothelium and are therefore well suited for EC-specific knockouts and EC-specific
422 transgenic mice. This single-cell isolation method can be adapted to perform single-cell multi-
423 omics studies and other flow cytometry-based assays such as Imaging Flow Cytometry. The
424 luminal digestion approach could be used for freshly obtained mouse aortas, arteries from large
425 animals (rabbits and pigs), as well as for freshly obtained human vascular explants. Collectively,
426 this method would allow us to fully understand the precise role of blood flow on endothelial
427 function and reprogramming at a single-cell resolution.
428
437
438 **DISCLOSURES:**
439 HJ is the founder of FloKines Pharma. Other authors have no conflicts of interest to declare.
440

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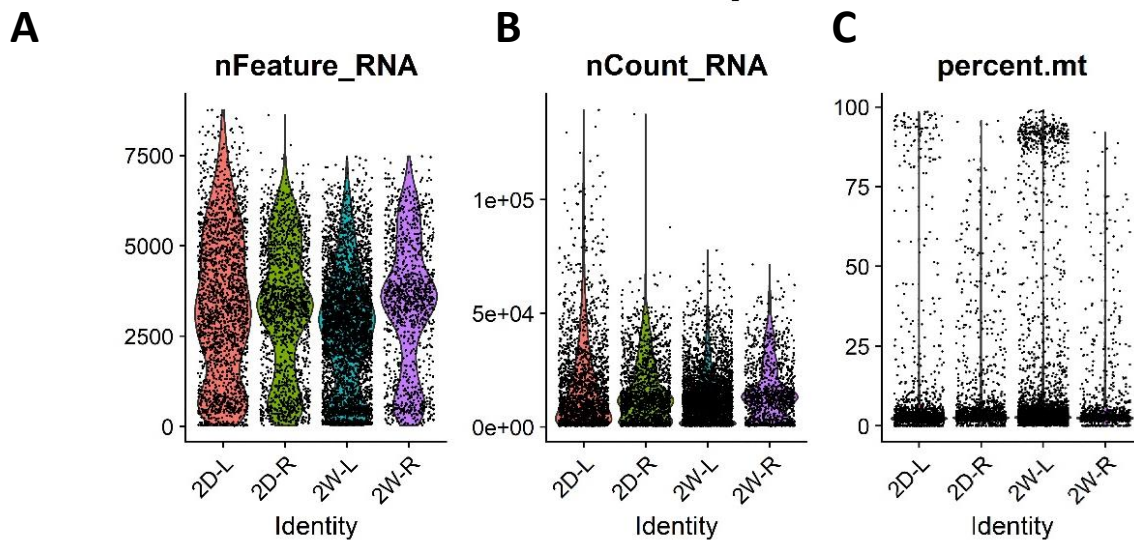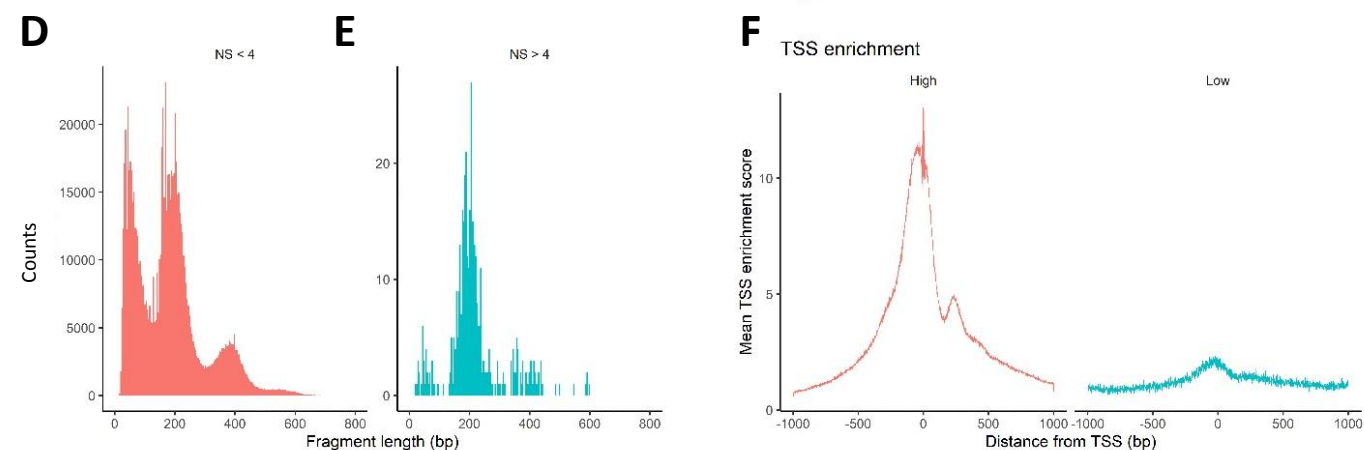
Figure 1

A.

B.

C.

D.

A

Single-cells   Single-nuclei

B

Phase contrast   Gel red staining
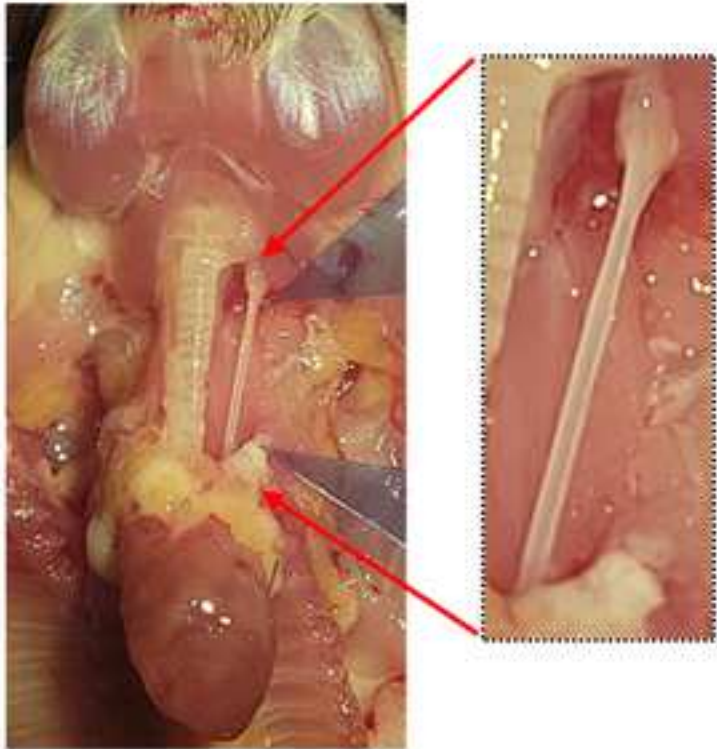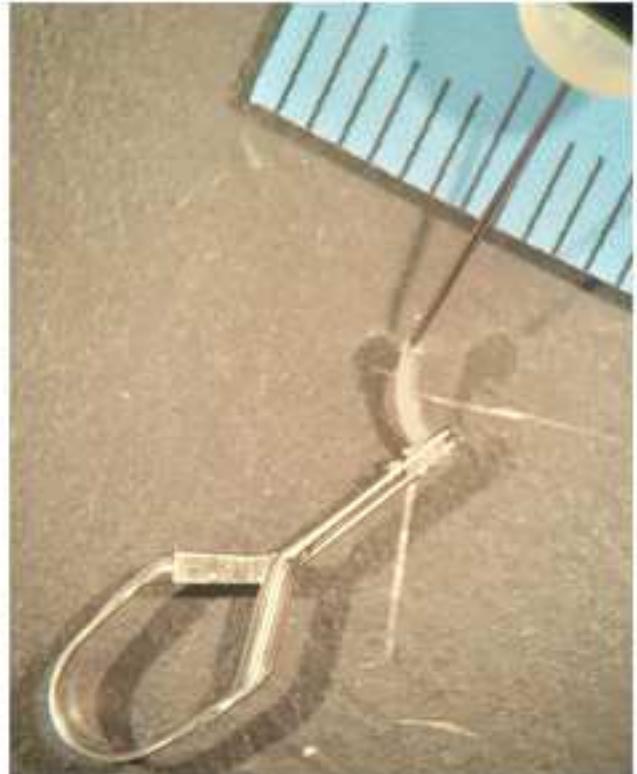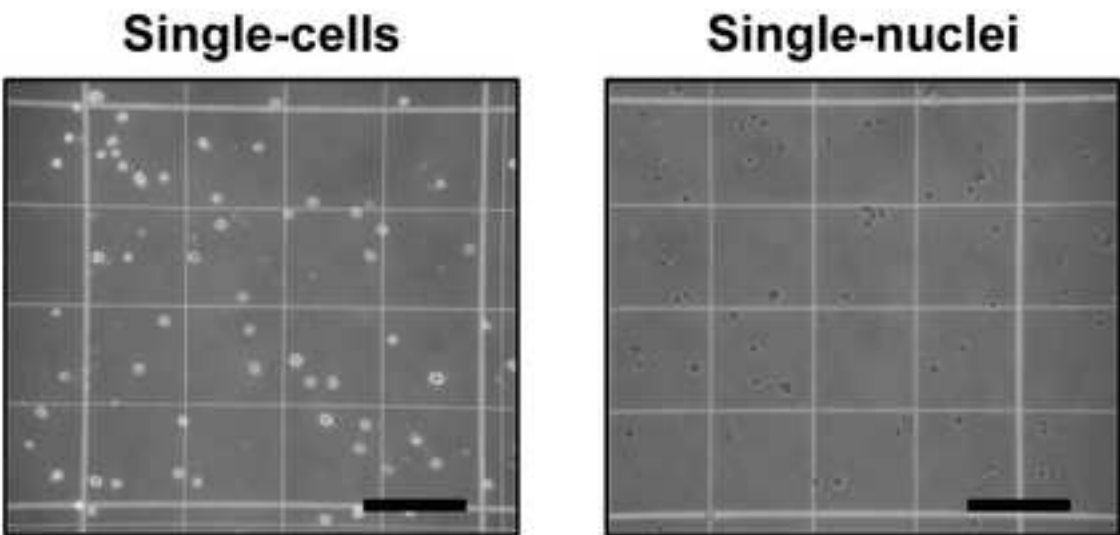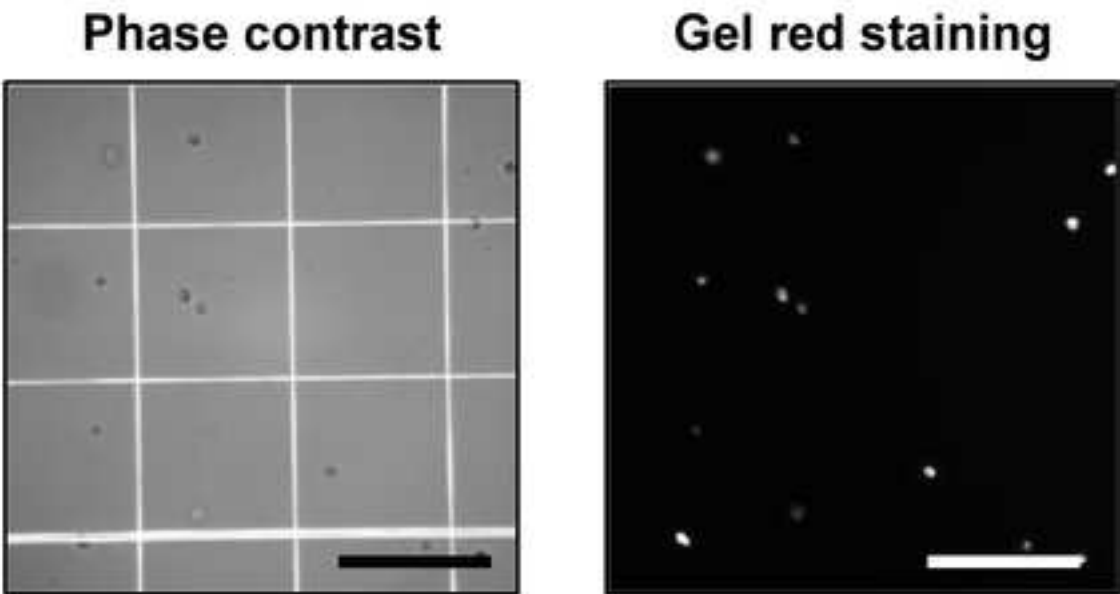
C

Efficiency	
Single cells	Single Nuclei
10,000	4,800
50,000	28,000
100,000	43,000
200,000	102,000

Figure 3 updated

Table 1
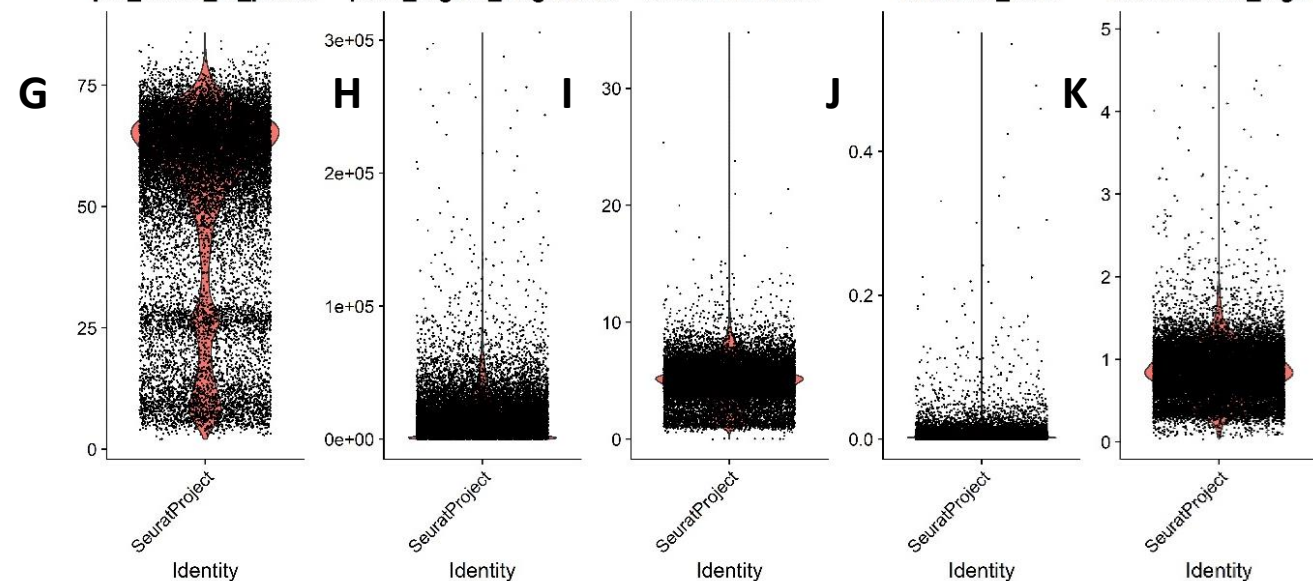
1x single-nuclei lysis buffer

1x Lysis buffer	Stock	Final	2 mL
Tris-HCl (pH 7.4)	1 M	10 mM	20 μL
NaCl	5 M	10 mM	4 μL
MgCl$_2$	1 M	3 mM	6 μL
Tween-20	10%	0.10%	20 μL
Nonidet P40	10%	0.10%	20 μL
Digitonin	5%	0.01%	4 μL
BSA	10%	1%	200 μL
Nuclease-free water	-	-	1.732 mL

0.1x single-nuclei lysis buffer

0.1x Lysis buffer	Stock	Final	2 mL
Tris-HCl (pH 7.4)	1x	0.1x	200 μL
Nuclease-free water	-	-	1.8 mL

Single-nuclei wash buffer

Wash Buffer	Stock	Final	2 mL
Tris-HCl (pH 7.4)	1 M	10 mM	20 μL
NaCl	5 M	10 mM	4 μL
MgCl2	1 M	3 mM	6 μL
BSA	10%	1%	200 μL
Tween-20	10%	0.10%	20 μL
Nuclease-free water	-	-	1.75 mL

Single-nuclei Nuclei Buffer

Diluted Nuclei Buffer	Stock	Final	1 mL
Nuclei Buffer (20x) see the **Table of Materials**	20x	1x	50 μL
NaCl	5 M	10 mM	4 μL

Table 2

Table 5.

	Sample	Mice (n)	Study	Single cells	Mean reads/ cell
scRNAseq	2D-R	10	2 day, RCA	1,867	55,593
	2D-L		2 day, LCA	2,119	76,050
	2W-R		2 wk, RCA	1,263	88,631
	2W-L		2 wk, LCA	4,460	34,142
	Sample	**Mice (n)**	**Study**	**Single cells**	**Mean fragments/ cell**
scATACseq	2D-R	12	2 day, RCA	1,291	24,459
	2D-L		2 day, LCA	5,351	22,201
	2W-R		2 wk, RCA	5,826	19,690
	2W-L		2 wk, LCA	5,856	22,398

Genes/ cell
3,376
3,284
3,537
2,770
Total reads
304,487,130
372,162,757
336,611,489
370,557,062
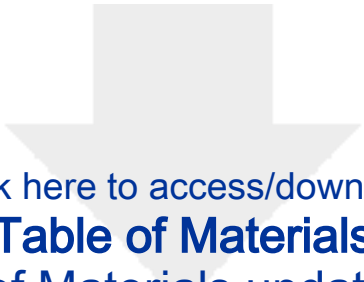
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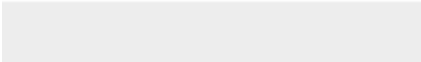
September 14, 2021

Kind Attention:

Vidhya Iyer, Ph.D.
Review Editor
JoVE
vidhya.iyer@jove.com

Re: JoVE63128R1 "Endothelial-enriched Single-cell Preparation from Mouse Carotid Artery Lumen for Single-Cell Multi-omics Studies"

Dear Dr. Iyer,

Thank you for reviewing and editing our manuscript and providing our valuable feedback.

Based on your comments and suggestions, we have updated the manuscript.

Our edits are made as a track-changed version while retaining your edits and comments for future reference.

We agree with consolidating Table 1-4. The Table numbers for the rest of the tables have been updated accordingly. The commercial names for equipment/reagents have been moved to the updated Table of Materials.

We have updated the Figure #3 to include links to images with appropriate labels. The Representative Results section and the figure legend for Figure #3 has been updated accordingly.

We also paraphrased the text in the Title, Abstract, Introduction, Discussion and Acknowledgement section to further reduce the overlap.

We have also highlighted the specific text in the Protocol that identifies essential steps for the video.

Thanks and regards,

Hanjoong Jo, Ph.D.
Wallace H. Coulter Distinguished Faculty Chair Professor
Associate Chair for Emory BME
Wallace H. Coulter Department of Biomedical Engineering
Emory University and Georgia Tech
1760 Haygood Drive
Health Sciences Research Bldg E170
Atlanta, GA 30322

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Dear Dr. Jo,

Your manuscript JoVE63128R1 "Endothelial-enriched Single-cell Preparation from Mouse
Carotid Artery Lumen for Single-Cell Multi-omics Studies" has been editorially reviewed and
formatted to meet JoVE's requirements (see **63128_R1_edit** uploaded in this submission). Please
go through the comments inserted in **63128_R1_edit** and address them, tracking your changes
and retaining mine. Some commercial names had to be removed from the text; please add these
to the Table of Materials. Also, please check if changes made (such as consolidation of tables 1
to 4) are OK, and renumber the referenced tables if you're OK with these changes. Please refer to
the attached iThenticate report to see if you can paraphrase a few sentences and reduce overlap.

Your revision is due by **Sep 15, 2021**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your
submission under the heading **Submission Needing Revision**.

Best,

Vidhya Iyer, Ph.D.
Review Editor
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vidhya.iyer@jove.com
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