A Microphysiological System to Study Leukocyte-Endothelial Interaction during Inflammation
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2 A Microphysiological System to Study Leukocyte-Endothelial Interaction during Inflammation

3

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29 **KEYWORDS:**

30 microphysiological system, leukocyte-endothelial cell interaction, inflammation, adhesion,

31 migration, biomimetic microfluid assay

32

33 **SUMMARY:**

34 In this protocol, a biomimetic microfluid assay, which can reproduce a physiologically relevant

35 microvascular environment and reproduce the entire leukocyte adhesion/migration cascade, is

36 employed to study leukocyte-endothelial cell interactions in inflammatory disease.

37

38 **ABSTRACT:**

39 Leukocyte-endothelial cell interactions play an important role in inflammatory diseases such as

40 sepsis. During inflammation, excessive migration of activated leukocytes across the vascular

41 endothelium into key organs can lead to organ failure. A physiologically relevant biomimetic

42 microfluid assay (bMFA) has been developed and validated using several experimental and

43 computational techniques, which can reproduce the entire leukocyte rolling/adhesion/migration

44 cascade, to study leukocyte-endothelial cell interactions. Microvascular networks obtained from

45 *in vivo* images in rodents were digitized using a Geographic Information System (GIS) approach
46 and microfabricated with polydimethylsiloxane (PDMS) on a microscope slide. To study the effect
47 of shear rate and vascular topology on leukocyte-endothelial cells interactions, a Computational
48 Fluid Dynamics (CFD) model was developed to generate a corresponding map of shear rates and
49 velocities throughout the network. The bMFA enables the quantification of leukocyte-endothelial
50 cells interactions, including rolling velocity, number of adhered leukocytes in response to
51 different shear rates, number of migrated leukocytes, endothelial cell permeability, adhesion
52 molecule expression, and other important variables. Furthermore, by using human-related
53 samples, such as human endothelial cells and leukocytes, bMFA provides a tool for rapid
54 screening of potential therapeutics to increase their clinical translatability.
55
56 **INTRODUCTION:**
57 Inflammation is the host response to infection and injury and the endothelium plays an important
58 role in the inflammatory response[1–3]. Inflammatory dysregulation is the underlying cause of a
59 number of disease pathologies such as sepsis, cardiovascular diseases, asthma, inflammatory
60 bowel disease, cancer, and COVID 19. Leukocyte-endothelial cell interactions play a central role
61 in these inflammatory diseases. During inflammation, the release of PAMPS (pathogen-
62 associated molecular patterns) from pathogens or DAMPS (damage-associated molecular
63 patterns) from injured tissue activate immune cells to release cytokines/chemokines and other
64 proinflammatory mediators that lead to the activation of endothelium, resulting in alterations in
65 vascular endothelium barrier function and increased permeability[3,4]. Increased activation of
66 endothelial cells during inflammation results in enhanced leukocyte–endothelial cell interaction
67 leading to excessive migration of activated leukocytes across the vascular endothelium into key
68 organs[1,5–7].
69
70 The recruitment of leukocytes is initiated by chemically diverse chemoattractants composed of
71 bioactive lipids, cytokines, chemokines, and complement components[8,9]. Leukocyte recruitment
72 is a multi-step process that includes five discrete steps: 1) leukocyte margination and
73 capture/attachment, 2) rolling, 3) firm arrest, 4) spreading and crawling 5)
74 extravasation/migration (**Figure 1**), with each step of this process requiring crosstalk between
75 leukocytes and endothelial cells to orchestrate this dynamic phenomenon[1,9]. Ultimately, arrested
76 leukocytes extravasate to inflamed tissues across endothelium via a multi-step process
77 controlled by concurrent chemoattractant-dependent signals, adhesive events and
78 hemodynamic shear forces[1,9–12].
79
80 Given the central role of shear stress in regulating endothelial cell function and the significance
81 of the leukocyte-endothelium interactions[13], several *in vitro* models have been developed during
82 the last few decades to study various aspects of the leukocyte migration cascade in a more
83 controlled environment[14]. Traditional fluidic devices to study leukocyte-endothelium interactions
84 can be classified into two broad categories[14]: a) devices for studying leukocyte rolling, adhesion,
85 and adhesion molecule expression such as parallel plate flow chamber and b) devices for studying
86 leukocyte migration under static conditions such as transwell chambers. Systems like parallel
87 plate flow chambers have been used to study the roles of adhesion molecules and their ligands
88 in the adhesion cascade under shear forces[15]. However, a significant drawback is that these

89 simplistic, idealized devices (e.g., straight channel) are not able to reproduce the scale and
90 geometry of the *in vivo* microvasculature (e.g., successive vascular bifurcations, vascular
91 morphology), and the resulting flow conditions (e.g., converging or diverging flows at
92 bifurcations), and can only model adhesion but not transmigration. Transwell chambers can only
93 study transmigration under the static condition without considering the *in vivo* geometrical
94 features and flow conditions. Thus, these traditional models do not mimic the microenvironment
95 of living tissues or resolve adhesion and migration cascade in a single assay[6].
96
97 To address this limitation, we have developed and extensively validated a novel 3D biomimetic
98 microfluidic assay (bMFA) (**Figure** 2), which realistically reproduces *in vivo* microvascular
99 networks on a chip[16–18]. The protocol for microfabrication of this device has been published
100 previously[17] and is only briefly described here. The microvasculature of mouse cremaster muscle
101 was digitized using a modified Geographic Information System (GIS) approach[19]. Then the
102 synthetic microvascular network was generated on polydimethylsiloxane (PDMS) using soft-
103 lithography processes based on the digitized microvascular network[14,17,20-22]. Briefly, the digitized
104 network images were printed on Mylar film, which was then used as a mask to pattern a SU-8
105 positive photoresist on top of a silicon wafer to create the masters for fabrication.
106 Microfabricated pillars (10 μm diameter, 3 μm tall) were used to create the 3 μm high and 100
107 μm wide pores, an optimum size for leukocyte migration[23-25], connecting the vascular channels
108 and tissue compartments. PDMS was prepared according to the manufacturer's instructions and
109 poured over the developed masters. Further, the PDMS was degassed and allowed to cure
110 overnight in an oven (65 °C) to create complementary microchannels in PDMS. Subsequently, the
111 cured PDMS was peeled from the SU-8 master, followed by punching ports for inlet/outlet. Then
112 the PMDS was plasma bonded to a glass slide. The surface of the microfluidic device comprises
113 native glass and PDMS. In order to promote cell attachment, spreading, and proliferation, ECM
114 coating is required. The bMFA includes a microvascular network and a tissue compartment
115 connected via 3 μm high and 100 μm wide pores (**Figure** 2). This microfluidic system reproduces
116 the entire leukocyte adhesion/migration cascade in a physiologically relevant 3Denvironment of
117 a complete microvascular network with interconnecting vessels and bifurcations, including
118 circulation, margination, rolling, adhesion, and migration of leukocytes into the extra-vascular
119 tissue compartment in a single system[14,16,17,21,26].
120
121 It should be noted that even when the flow rate at the inlet of bMFA is fixed, the flow conditions
122 in the network vary at different locations and cannot be calculated by a simple mathematical
123 formula. A computational fluid dynamics (CFD)-based model was developed to calculate different
124 flow parameters (e.g., shear stress, shear rate, velocity) at different locations in the network. This
125 CFD model was used to simulate the dye perfusion patterns and flow parameters in the bMFA.
126 Cross-validation with experimental results suggested that the flow resistances across the
127 network are well predicted by the computational model (**Figure** 3)[17]. This CFD model was then
128 used to estimate velocity and shear rate profile in every vessel of bMFA (**Figure** 4), allowing
129 analysis of the effects of shear flow and geometry on leukocyte rolling, adhesion and migration[16].
130 Leukocytes preferentially adhere near bifurcations and in low shear regions *in vivo*, and this
131 spatial patterns of leukocyte adhesion were successfully demonstrated in bMFA using
132 neutrophils (**Figure** 5)[16]. This paper describes the protocol for preparing bMFA to study

leukocyte-endothelial cell interaction under inflammatory conditions using human lung microvascular endothelial cells (HLMVEC) and human neutrophils. Microphysiological systems, such as the bMFA, can be used to study endothelial cell interactions with different types of cells such as neutrophils, monocytes, lymphocytes, and tumor cells[18,27–30]. The bMFA can be seeded with primary endothelial cells from different organs (e.g., lung vs. brain) and different species (e.g., human vs. murine endothelial cells), as well as endothelial cell lines[21,27,31,32]. The bMFA can be used to study multiple cellular responses, cell-cell interactions, barrier function, drug delivery, and drug toxicity.

PROTOCOL:

Heparinized human blood is obtained for neutrophil isolation from healthy adult donors (males and females, aged between 21 and 60 years old), following informed consent as approved by the Institutional Review Board of Temple University (Philadelphia, PA, USA).

1.   Priming and coating the device with human fibronectin

NOTE: The bMFA has two inlet ports and two outlet ports connected to the vascular compartment. It also has one port connected to the tissue compartment (**Figure** 2).

1.1   Insert tubing (O.D. of 0.06" and I.D. of 0.02") (**Table of Materials**) to all the ports except for one inlet port with fine-point forceps. Clamp the two outlet ports and the tissue compartment port with jaw clamps. Ensure that each tubing is ~1 inch in length.

1.2   Dilute human fibronectin to 100 µg/mL with PBS from fibronectin stock solution (1mg/mL). Load a 1 mL syringe with prepared fibronectin solution. Connect the syringe to a 24 G blunt needle and a ~4-inch long tubing.

NOTE: To prevent crosslinking, do not over mix/pipette human fibronectin. Other extracellular matrices (ECM) such as collagen may be used for different cell types.

1.3   Insert the tubing into the open inlet port, push the plunger until human fibronectin comes out of the other inlet port, then clamp it. Repeat this process for the rest of the ports until all the channels, tissue compartment and tubing are filled with the human fibronectin solution. Remove the needle but keep the 4-inch long tubing inserted and unclamped.

NOTE: Make sure the tubing is filled with fibronectin before it is clamped.

1.4   For degassing, connect the unclamped tubing to the Pneumatic Primer connected to a compressed nitrogen tank, adjust the pressure to ~5 psi and run for ~15 min.

1.5   After 15 min, check under the microscope to make sure there are no air bubbles trapped in the device. If there are air bubbles trapped in the channel or tissue compartment, reconnect the device to the Pneumatic Primer for degassing again, i.e., repeat step 1.4.

177

178 NOTE: Inverted microscope is used in all the steps involving microscopy in this protocol.

179

180 1.6   Remove the device from the Pneumatic Primer. Incubate the device at 37 °C for 1 h, then
181 flush all the channels and tissue compartment with pre-warmed HLMVEC culture media (**Table**
182 **of Materials**), similar to step 1.3. The bMFA is now ready for cell seeding.

183

184 NOTE: Before removing/inserting a tubing from port, first place a drop of water with a pipette
185 around the base of the inlet port tubing to prevent air from entering the device.

186

187 **2.   Seeding bMFA with HLMVEC**

188

189 2.1   Culture HLMVEC to 60%– 80% confluency according to the vendor's protocol in a T-25
190 flask.

191

192 2.2   Aspirate HLMVEC culture media from cell culture flask. Wash twice with PBS.

193

194 2.3   Add 2 mL of pre-warmed trypsin-EDTA solution (37 °C) in the T-25 flask. Incubate for 3–5
195 min in an incubator (37 °C, 5% CO_2) until most cells are detached from the flask.

196

197 NOTE: Incubation time may vary for different cell types and trypsin-EDTA concentration.

198

199 2.4   Add 2 mL of Trypsin Neutralization Solution (TNS, 37 °C) to the flask to neutralize trypsin-
200 EDTA.

201

202 2.5   Gently tap the flask to help dissociate the cells. Then transfer the cell suspension solution
203 to a 15 mL conical tube.

204

205 2.6   Centrifuge for 5 min at 150 x g to pellet the endothelial cells. Remove the supernatant
206 and resuspend cells in 3 mL of cell culture media. Count the number of cells with a
207 hemocytometer.

208

209 2.7   Centrifuge again for 5 min at 150 x g to pellet the cells. Remove the supernatant and
210 resuspend the cells to the desired seeding density of 20 x 10^6/mL.

211

212 NOTE: Depending on the cell types and confluency, about 2 x 10^6 endothelial cells can be
213 collected from two T-25 flasks, and with a cell concentration the seeding density of 20 x 10^6/mL,
214 100 µL of the cell suspension (2 x 10^6 endothelial cells), is sufficient to seed 5 devices.

215

216 2.8   Mount 1 mL syringe in the programmable syringe pump, attach tubing to the blunt
217 needle.

218

219 2.9   Draw ~20 µL of the cell suspension into the tubing, making sure the cell suspension is only
220 in the tubing, not in the syringe barrel.

221

222   2.10   Take clamp off an outlet port of the device. Connect the tubing to one inlet of the device.
223   Make sure no air bubbles are introduced into the channel.

224

225   2.11   Start the pump with a flow rate of 4–8 µL/min. Observe the device under a microscope.

226

227   2.12   When the channels are filled with cells, stop the pump, clamp the outlet, and cut the
228   inlet tubing.

229

230   NOTE: Take care not to introduce air bubbles into the device.

231

232   2.11   Put the device in the incubator for 4 h at 5% CO_2 and 37 °C.

233

234   2.12   After 4 h incubation, prepare another syringe filled with fresh cell culture media. Mount
235   the syringe in a syringe pump, connect the syringe to the inlet port, and remove the clamp from
236   an outlet port.

237

238   2.13   Run fresh media through the device for about 5 min at 4–8 µL/min to wash out
239   floating/unattached cells.

240

241   **3   Cell culture under flow for 48 h**

242

243   3.1   Prepare a syringe filled with fresh cell culture media. Mount the syringe in a syringe pump,
244   connect the syringe to one inlet port, and keep an outlet open. Put the device in the incubator
245   (5% CO_2, 37 °C).

246

247   3.2   Program the syringe pump for culturing endothelial cells under flow using the following
248   instructions mentioned in **Table 1.**

249

250   3.3   Check bMFA under a microscope after 48 h of culture under flow. HLMVEC should cover
251   vascular channels forming a complete lumen (**Figure** 6).

252

253   NOTE: Other flow regimes may be required for different cell types.

254

255   **4   Cytokine and/or therapeutic treatment**

256

257   NOTE: As an example, this section describes using bMFA to study the impact of treating the cells
258   with TNF-α and a novel anti-inflammatory inhibitor (PKCδ-TAT peptide inhibitor, PKCδ-i)[18,27,32-36].

259

260   4.1   Prepare three different bMFA devices using the protocol above (section 1–3).

261

262   4.2   Load three 1 mL syringe with cell culture media, or TNF-α (10 ng/mL), or TNF-α (10 ng/mL)
263   + PKCδ-i (5 µM), respectively.

264

265   NOTE: Cytokines are reconstituted in cell culture media.

266

267   <mark>4.3   Connect the three syringes to three bMFA devices. Treat HLMVEC with buffer TNF-α or</mark>
268   <mark>TNF-α + inhibitor for 4 h at 0.1 μL/min.</mark>

269

270   NOTE: Based on specific study requirements, other cytokines or cytokine cocktails (e.g., Tumor
271   Necrosis Factor-alpha (TNF-α) + Interleukin 1 beta (IL1-β) + Interferon-gamma (IFN-γ)), may be
272   used to treat endothelial cells.

273

274   **5   Human neutrophil isolation**

275

276   5.1   Use all reagents at room temperature (RT). Reagents include leukocyte isolation media ,
277   1x HEPES buffer with Ca^{2+}/Mg^{2+} (**Table 2**), 6% Dextran in normal saline (0.9% NaCl), 3.6% NaCl
278   solution, De-ionized water (DI) water.

279

280   5.2   Pipette 20 mL of leukocyte isolation media into a 50 mL conical tube and carefully layer
281   25 mL of blood over the Ficoll-Paque. Centrifuge for 40 min at 427 x *g* at RT.

282

283   NOTE: Perform this step slowly and carefully to avoid mixing the blood and leukocyte isolation
284   media.

285

286   5.3   Aspirate down to ~5 mL above red blood cells (RBC) layer. The white buffy layer on top
287   of the RBC is predominantly neutrophils.

288

289   5.4   Add HEPES buffer to double the current volume (existing volume: HEPES buffer = 1:1).

290

291   5.5   Add an amount of 6% Dextran, which will be 20% of the final volume. (current volume/4
292   = volume of added 6% Dextran).

293

294   5.6   Invert the tube gently a few times to mix well and let the RBC sediment (~25 min).

295

296   5.7   Carefully pipette the upper layer (neutrophil-rich layer) and transfer to a new 50 mL
297   tube, careful not to contaminate with sedimented RBC.

298

299   5.8   Centrifuge for 10 min at 315 x *g* at RT. Remove the supernatant and resuspend the
300   pellet in 8 mL of HEPES buffer.

301

302   5.9   To lyse the residual RBC, add 24 mL of DI water, gently mix for 50 seconds. Immediately
303   add 8 mL of 3.6% NaCl solution, mix, and centrifuge for 10 min at 315 x *g* at RT.

304

305   5.10   Remove the supernatant and wash the cell pellet with 15 mL of HEPES buffer, centrifuge
306   for 5 min at 315 x *g.* Resuspend the pellet in HEPES buffer.

307

308   **6   Neutrophil adhesion and migration experiment with bMFA**

309

310   6.1   Resuspend the isolated human neutrophils (15 million) in 999 µL of HEPES buffer.

311

312   6.2   Add 1 µL CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) dye stock solution (10
313   mM) to neutrophil suspension to obtain a working concentration of 10 µM CFDA-SE, incubate for
314   10 min at RT. Wash the cells twice with HEPES buffer by centrifuging for 5 min at 315 x *g*.

315

316   6.3   Count the neutrophils using a hemocytometer and resuspend at 2 million neutrophils/mL
317   with cell culture media, or TNF-α (10 ng/mL), or TNF-α (10 ng/mL) +PKCδ-I (5 µM); incubate for
318   15 min at RT before the start of the experiment.

319

320   6.4   Prepare a syringe filled with 1 µM N-formyl-met-leu-phe (fMLP), the chemoattractant for
321   the study, in cell culture media. Take bMFA and open one inlet port and one outlet port.

322

323   NOTE: These experimental conditions are used to mimic the conditions of sepsis that include a
324   systemic inflammatory response with high levels of circulating cytokine/chemokines.
325   Furthermore, fMLP, a Gram-negative bacteria-derived peptide, is used to model the presence
326   of bacteria in the lung (i.e., tissue compartment).

327

328   6.5   Remove the tissue compartment port tubing and insert the fMLP tubing. Inject ~20 µL of
329   fMLP into the tissue compartment for all bMFA except the one treated with cell culture media
330   alone. Then cut the tubing and clamp it.

331

332   6.6   Fill the syringe with ~200 µL of the neutrophil suspension and mount the syringe on the
333   syringe pump.

334

335   6.7   Put the device on the inverted microscope stage (**Table of Materials**).

336

337   6.8   Start the pump at a flow rate is 1 µL/min and wait until a small drop of the neutrophil
338   suspension comes out of the tubing. Insert the tubing into the inlet port. See **Figure 7** for the
339   setup.

340

341   **7   Acquire images**

342

343   7.1   Use the **Scan Large Image** function in the microscope image analysis software (**Table of
344   Materials**) to obtain the adhesion map in bMFA (**Figure 8**) 10 min after starting the experiment.
345   Most of the neutrophils enter bMFA during the first 10 min.

346

347   NOTE: Keep the fluorescence light off when not taking images to reduce photobleaching.

348
349 <mark>7.2   During the next hour, take timelapse images (1 image every 5 min) of the tissue</mark>
350 <mark>compartment (**Figure 8B,C**) for migration analysis to obtain the migration map.</mark>
351
352 **8   Digital Image analysis**
353
354 8.1   For the adhesion map, count the number of adhered neutrophils in each vessel. For each
355 bifurcation, create a circular region of interest (ROI) with a diameter equal to two times the vessel
356 diameter and count the number of adhered neutrophils.
357
358 8.2   Use manual count or the automated **Object Count** function to quantify the number of
359 adhering neutrophils in each vessel and bifurcation region.
360
361 8.3   Use the shear rate map of the network generated using CFD simulation[17] to determine
362 the shear rate in each vessel. Then plot the number of adhered neutrophils in a given vessel
363 against the shear rate in that vessel to create a shear rate map in bMFA as shown in **Figure 9A**.
364
365 8.4   Neutrophils are counted as migrated if they have crossed through the endothelium into
366 the tissue compartment. Count the number of migrated neutrophils at 10 min, 15 min, 30 min
367 and 60 min. Plot the number of migrated neutrophils versus time as shown in **Figure 9B**.
368
369 **REPRESENTATIVE RESULTS:**
370 After 48 h of culture under shear flow in bMFA, endothelial cells covered the surface of the
371 vascular channels in bMFA and aligned in the direction of flow (**Figure 6**). Confocal microscopy
372 indicated that all surfaces of the vascular channels were covered by endothelial cells, forming a
373 complete 3D lumen in bMFA[18].
374
375 Using this protocol, a neutrophil adhesion map can be acquired, showing that there is significant
376 adhesion of neutrophils to endothelial cell in bMFA (**Figure 8**). Correlating the spatial distribution
377 of neutrophils in this adhesion map with the shear rate map generated from the CFD model,
378 shows that neutrophil adhesion is shear dependent, and neutrophils preferentially adhere at a
379 low shear rate and bifurcation regions (**Figure 9A**). The adhesion of neutrophils to TNF-activated
380 endothelial cells increased significantly. Analyzing timelapse images indicated that TNF activation
381 of endothelial cells increases neutrophil migration in response to the chemoattractant fMLP
382 (**Figure 9B**). As stated above, bMFA can be used to rapidly test the efficacy of a novel therapeutic
383 for treating inflammatory disease. For example, treatment of endothelial cells and neutrophils
384 with PKCδ-i significantly reduced neutrophil adhesion and migration (**Figure 9A,B**)[27].
385
386 **FIGURE AND TABLE LEGENDS:**
387 **Figure 1: Endothelial cell activation during inflammation.** (**A**) Under normal conditions, the
388 vascular endothelium is covered by the glycocalyx and forms a tight barrier that regulates barrier
389 permeability, leukocyte migration, and anti-inflammatory defenses. (**B**) During inflammation,
390 leukocytes and endothelial cells are activated by PAMPS and DAMPS to produce cytokines and
391 chemoattractants, leading to the elevated expression of surface molecules on both leukocytes

392 and endothelial cells, enhancing the interactions. Interactions of E/P-selectin on endothelial cells
393 and their ligand (e.g., PSGL-1) on leukocytes were involved in rolling, which slows down the
394 neutrophil. Firm adhesion is regulated by endothelium-expressed adhesion molecules, including
395 ICAM-1, ICAM-2, and VCAM-1, and their l ligands, β2 integrins. In response to chemoattractant
396 gradients, adhered leukocytes migrate through endothelial junctions regulated by PECAM-1 and
397 JAMs. Activated leukocytes migrate to tissue, where they release cytokines, reactive
398 oxygen/nitrogen species and proteases to fight against infection. However, dysregulated
399 leukocyte migration can damage organ tissue, resulting in organ failure. During inflammation, the
400 glycocalyx is degraded, endothelial cell tight junctions are damaged and there is increased
401 endothelial cell apoptosis, leading to damaged barrier function and increased permeability. This
402 figure has been modified from Yang et al.[2].

404 **Figure 2: Overview of the bMFA. (A)** Images of the mouse cremaster muscle are obtained and
405 (**B**) digitized using the GIS system to fabricate the network on PDMS. (**C**) Bright-field image of
406 bMFA network shows that in bMFA, vascular channels are connected to the tissue compartment
407 through a 3 μm barrier (the schematic insert in Panel C). Flow direction of the vascular channels
408 is indicated. (**D**) A bMFA chip, consisting of a PDMS layer bonded to a microscope slide, is on
409 microscope stage. Tubing is inserted into two inlet ports, two outlet ports and one tissue
410 compartment port. (C: scale bar = 500 μm)

412 **Figure 3: Transient perfusion studies comparing experimental and simulation results in bMFA**.
413 (**A**) The network of bMFA showing the inlet, outlet and flow direction. (**B**) Top panel: CFD
414 perfusion profiles. Bottom panel: Experimental perfusion profiles. Images are pseudo-colored to
415 show gradients. The scale is normalized with blue (no perfusion; 0) and magenta (complete
416 perfusion; 1).

418 **Figure 4: Velocity and shear rates profile in bMFA from CFD simulations. (A)** Distribution of
419 velocity magnitudes (Vmag) across the network. (**B**) Distribution of shear rate indicates
420 heterogeneous shear rates in the network.

422 **Figure 5: Neutrophil adhesion patterns in bMFA is similar to neutrophil adhesion patterns *in***
423 ***vivo*.** The distributions of the number of adhered neutrophils *in vivo* and the number of
424 neutrophils in bMFA are both skewed to the left, indicating that neutrophils preferentially adhere
425 near bifurcations with the peak occurring at one vessel or channel diameter from the nearest
426 bifurcation (mean ± SEM; N = 3). The distance of adhered neutrophils to the nearest bifurcation
427 was normalized by the following: normalized distance = distance to the center of the nearest
428 bifurcation/diameter of the channel. This figure has been modified from Lamberti et al.[16]
429 (https://pubs.acs.org/doi/10.1021/ac5018716, further permissions related to the material
430 excerpted should be directed to the American Chemical Society).

432 **Figure 6: Endothelial cells form a complete 3D lumen in the bMFA. (A)** Phase-contrast images
433 show that endothelial cells are lined up in the direction of flow. (**B**) Fluorescence images show
434 endothelial cells cover the vascular channel; F-actin is labeled green using phalloidin and nuclei
435 is labeled red using Draq5. (A: scale bar = 100 μm, B: scale bar = 50 μm).

437 **Figure 7: A computer-controlled inverted fluorescence microscope is used to study neutrophil-**
438 **endothelial interactions in bMFA.** A computer-controlled stage with a warmer is used to keep
439 the bMFA at 37 °C. The bMFA is connected to a programmable syringe pump through the tubing.
440 The inverted fluorescence microscope is equipped with a video camera to take images/video for
441 further offline analysis on the computer.

442

443 **Figure 8: Representative images of neutrophil adhesion and migration map.** (**A**) Neutrophil
444 adhesion map in bMFA. The image was taken 10 min after the start of the experiment using "Scan
445 large map" function of Nikon software. The white dots in the map are fluorescently labeled
446 neutrophils with CFDA-SE. Vascular channel boundary is digitally labeled (scale bar = 100 µm). (**B**)
447 Neutrophil migration map in bMFA without TNF-α activation. The image was taken 60 min after
448 the start of the experiment. (**C**) Neutrophil migration map in bMFA with TNF-α activation. The
449 image was taken 60 min after the start of the experiment.

450

451 **Figure 9: Patterns of neutrophil adhesion and migration in bMFA during inflammation.** (**A**) TNF-
452 α treatment significantly increased human neutrophil adhesion to human pulmonary
453 microvascular endothelial cells, which was inhibited following treatment with a PKCδ inhibitor.
454 Neutrophil adhesion occurred preferentially in vessels with low shear rate and near bifurcations
455 in bMFA. (**B**) In response to fMLP, human neutrophil migration across TNF
456 -α activated endothelial cells was significantly increased compared to untreated cells. Treatment
457 with the PKCδ inhibitor reduced migration to untreated levels. n = 4, Mean ± SEM, one-way
458 ANOVA, ** $p < 0.01$, and *** $p < 0.001$). The figure has been modified from Soroush et al.[27].

459

460 **Table 1: Syringe pump program**

461

462 **Table 2: HEPES buffer composition.**

463

464 **DISCUSSION:**
465 The bMFA reproduces the topography and flow conditions of the *in vivo* microvascular networks
466 and can be used to study leukocyte-endothelial cell interaction and endothelial function *in vitro*
467 under physiologically realistic conditions. In the microvasculature of either mouse or human, the
468 geometry of the microvascular networks are self-similar and fractal, and the Reynolds number
469 << 1, indicating that vascular geometry does not significantly impact flow patterns. Therefore the
470 bFMA can be used to study leukocyte-endothelial interactions for different species, including
471 humans, though the microvasculature of mouse cremaster muscle was mapped to manufacture
472 bMFA.

473

474 bMFA allows for the real-time assessment of individual steps of the neutrophil migration cascade
475 in a single assay, including rolling, firm adhesion, spreading, and migration of neutrophils into the
476 tissue compartment. Primary human cells and clinically relevant samples can be used in bMFA to
477 increase translatability and to rapidly screen potential therapeutics. Compared to traditional
478 fluidic systems such as parallel plate flow chambers, bMFA has the advantage of using less than
479 95% of reagents[37] due to its small vessel sizes and the fact that it can study the whole neutrophil

480   migration cascade in one single assay. However, this small size also requires users to work with a
481   very small volume of reagents, which can be challenging and requires extensive practice. The
482   most common problem with this protocol is the presence of air bubbles in bMFA, which can block
483   the channel and alter the flow patterns. Therefore, great care should be taken when removing
484   or inserting tubing into the ports, and a drop of water must be placed at the base of the tubing
485   at the ports to prevent air from entering the channel whenever tubing is removed from bMFA.
486
487   In addition to the application of bMFA for studying neutrophil-endothelial interactions, the bMFA
488   has also been used to study the integrity of endothelium during inflammation by measuring
489   variables such as permeability and transendothelial endothelial resistance (TEER)[27]. Furthermore,
490   by functionalizing microparticles with specific antibodies, bMFA can be used to study the
491   expression of adhesion molecules on endothelial cells[27]. It was demonstrated that the
492   inflammatory response could also be upregulated by other stimuli in the bMFA, such as ionizing
493   radiation and bMFA has been used to study radiation-induced endothelial cell damage and
494   neutrophil-endothelial interactions[29]. Another advantage of this device is that endothelial cells,
495   neutrophils and/or other blood cells or drug-carrying particles can be isolated from microvascular
496   channels and/or tissue compartment for further analysis. Furthermore, cells/particles that did
497   not interact with the endothelial cells in the device can be isolated from the effluent exiting the
498   device. Pericytes and the corresponding tissue types can also be cultured in the device to better
499   represent the *in vivo* conditions. In conclusion, the protocol presented here provides a
500   physiologically relevant environment in bMFA to study leukocyte-endothelial cell interactions
501   during inflammation.
502

507

508   **DISCLOSURES:**
509   The authors declare no conflict of interest.
510

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513   inflammation. *Nature Reviews Immunology.* **13** (3), 159–175 (2013).
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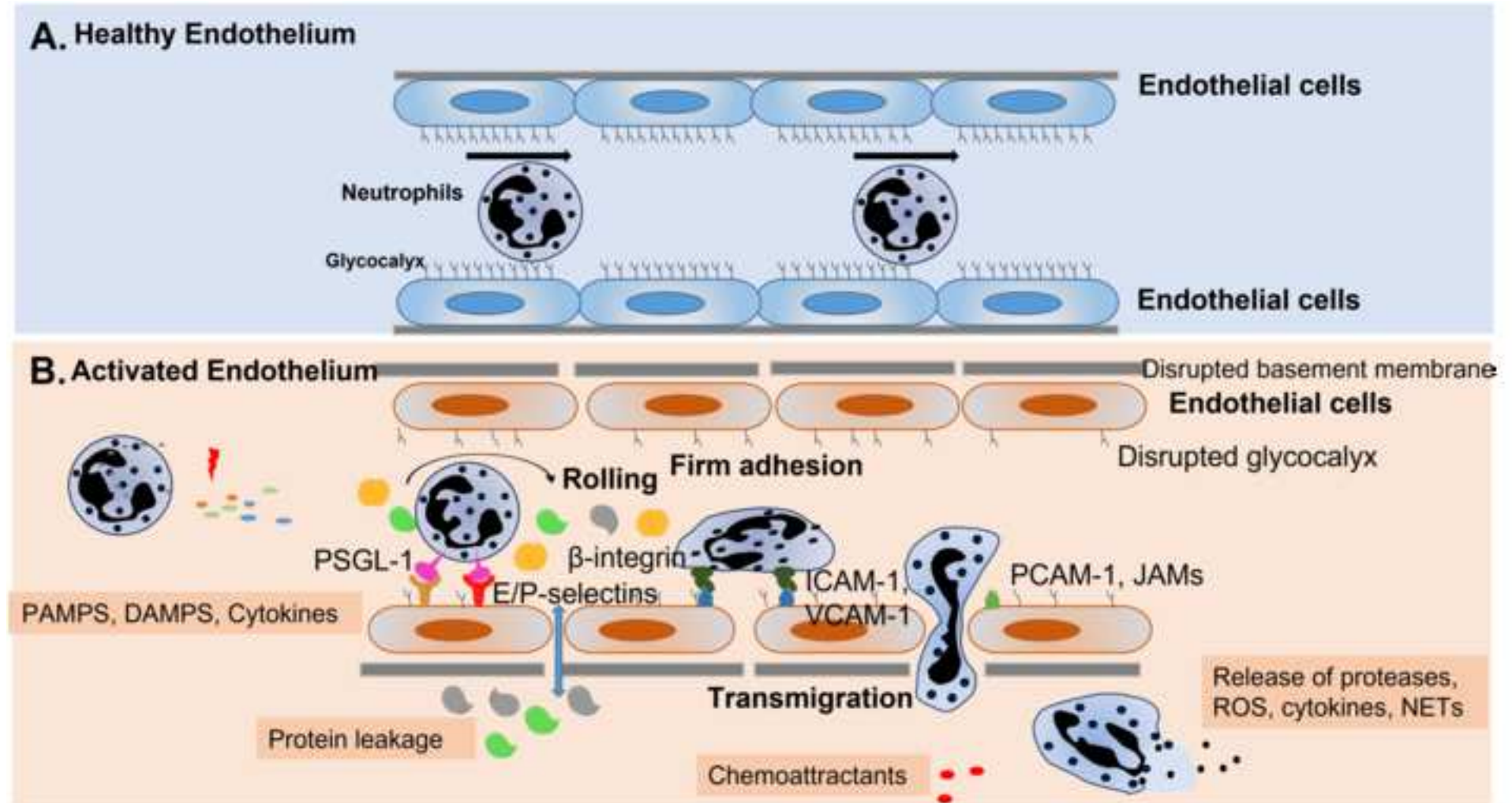
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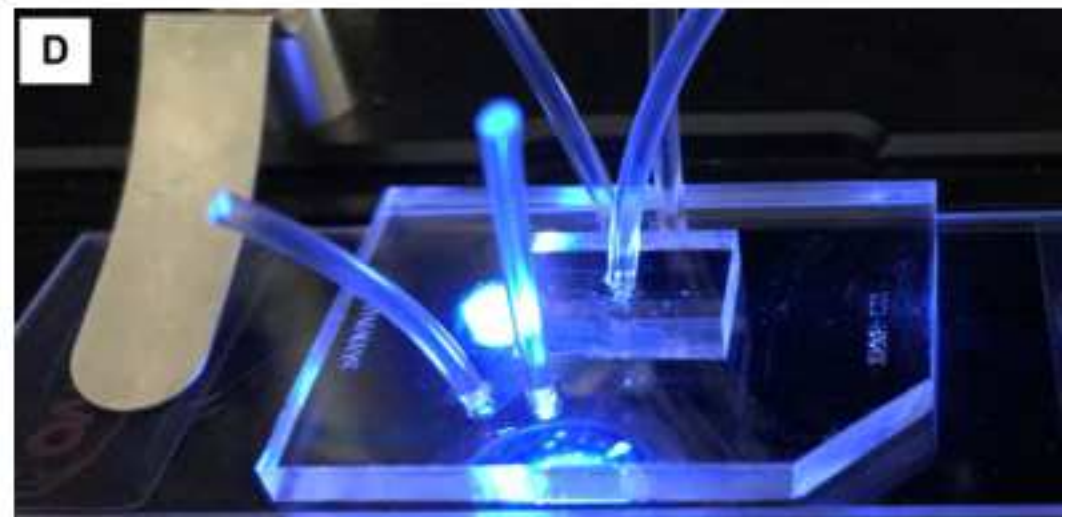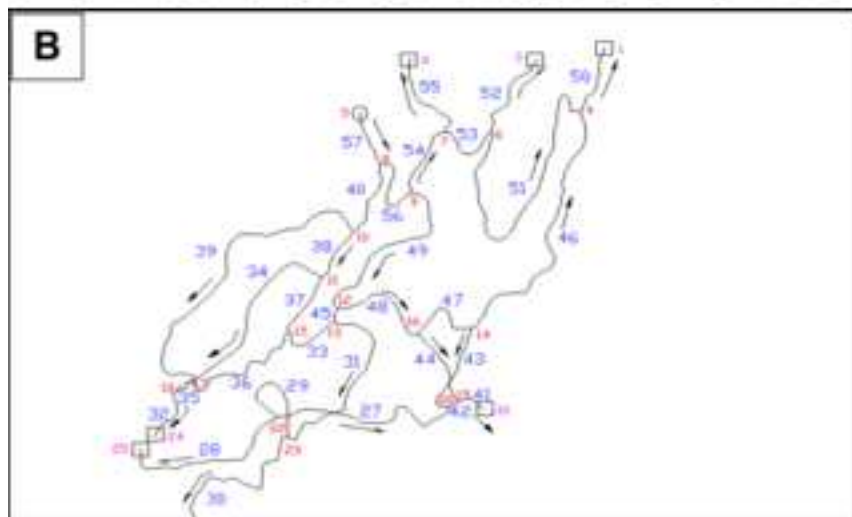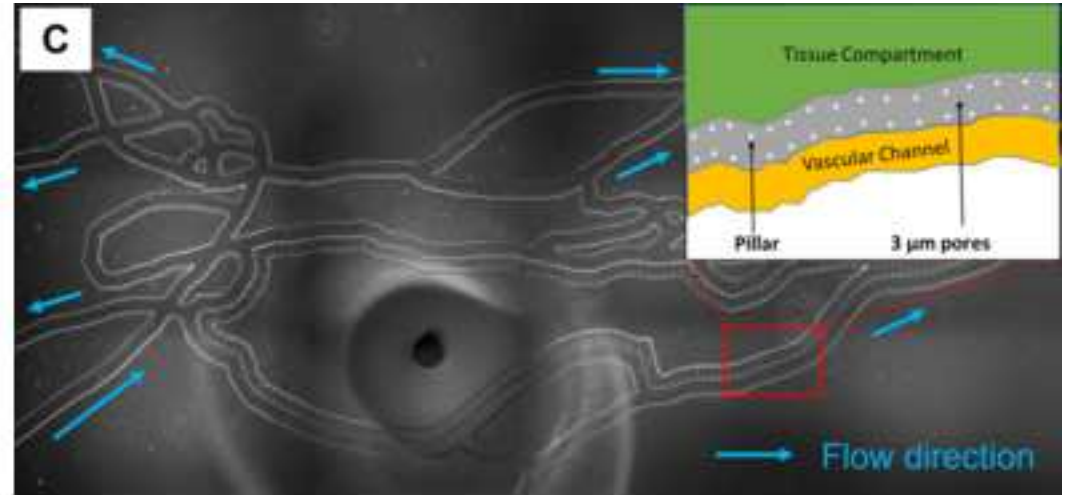596   SynVivo synthetic microvascular networks. *Journal of Visualized Experiments: JoVE*. **87**, e51025
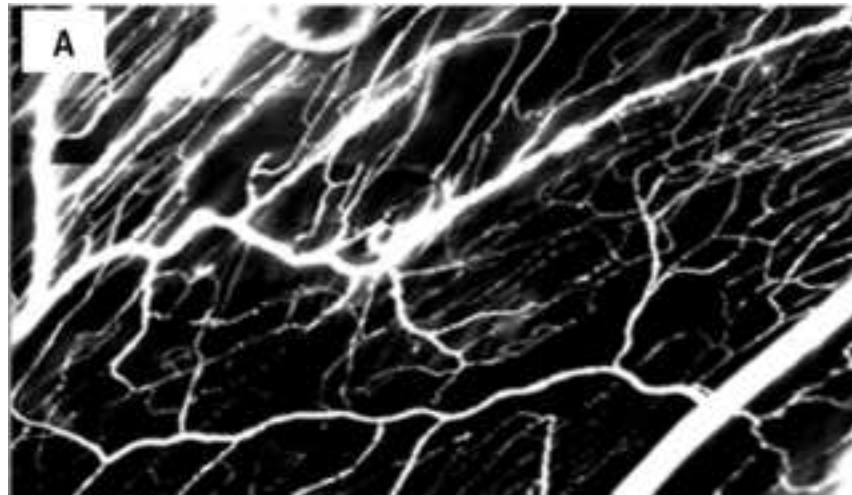597   (2014).

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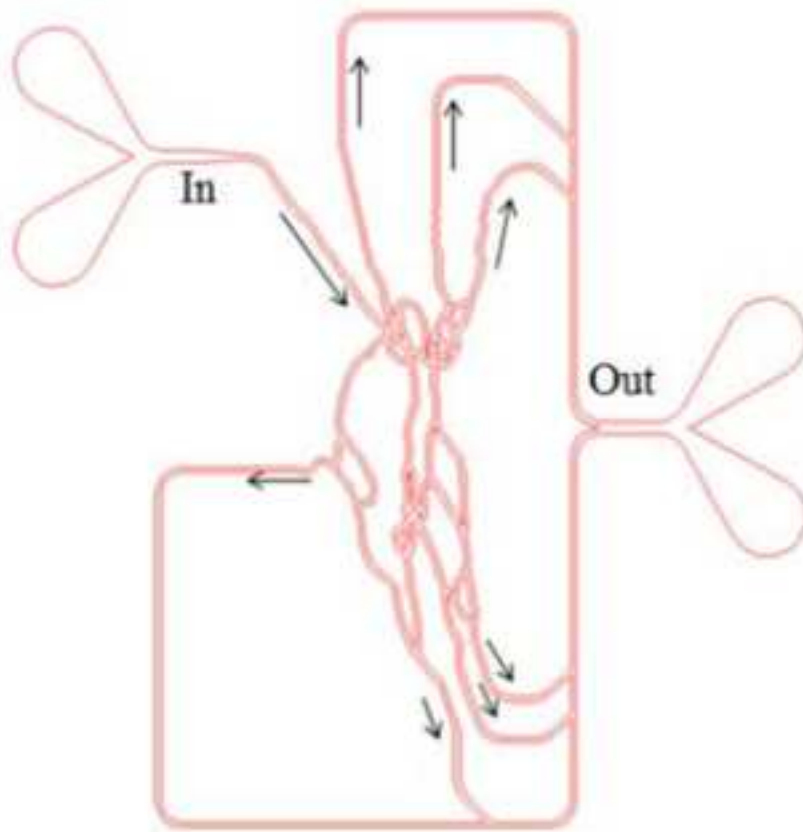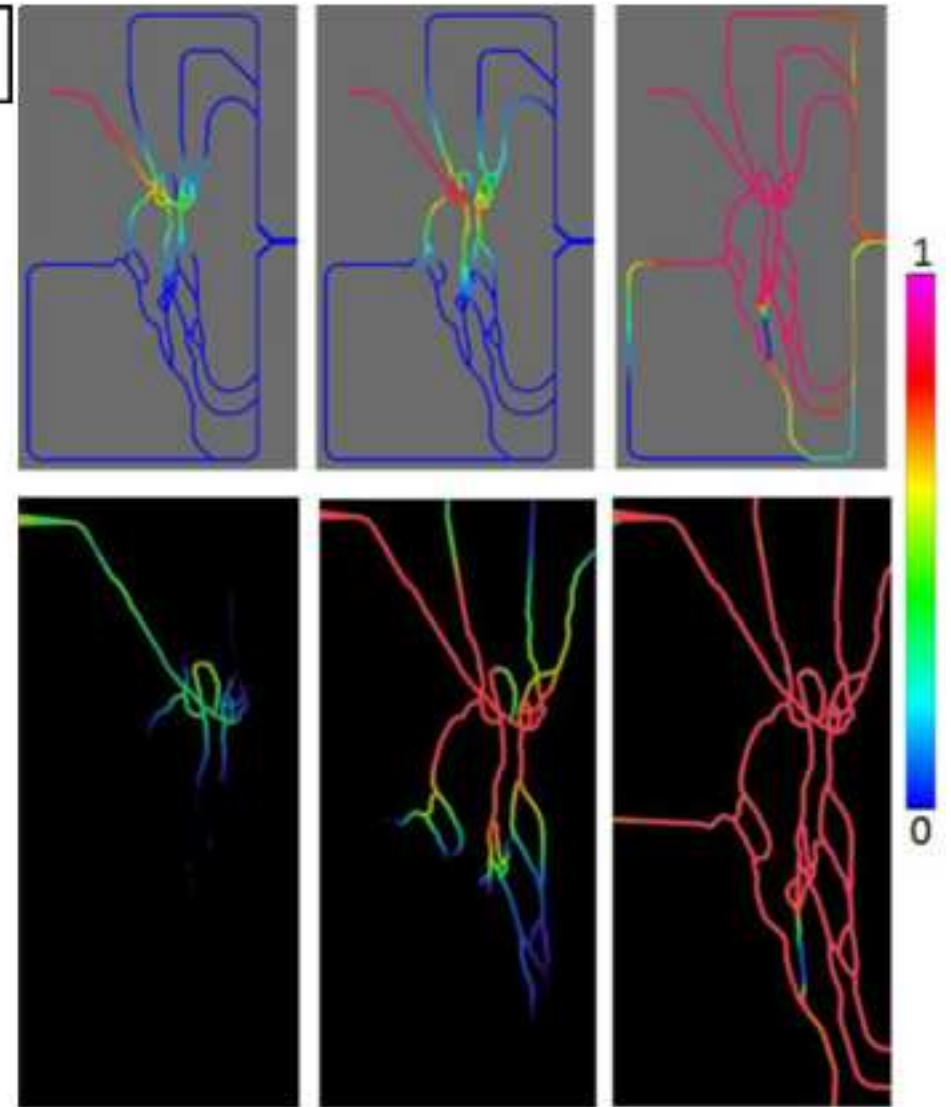
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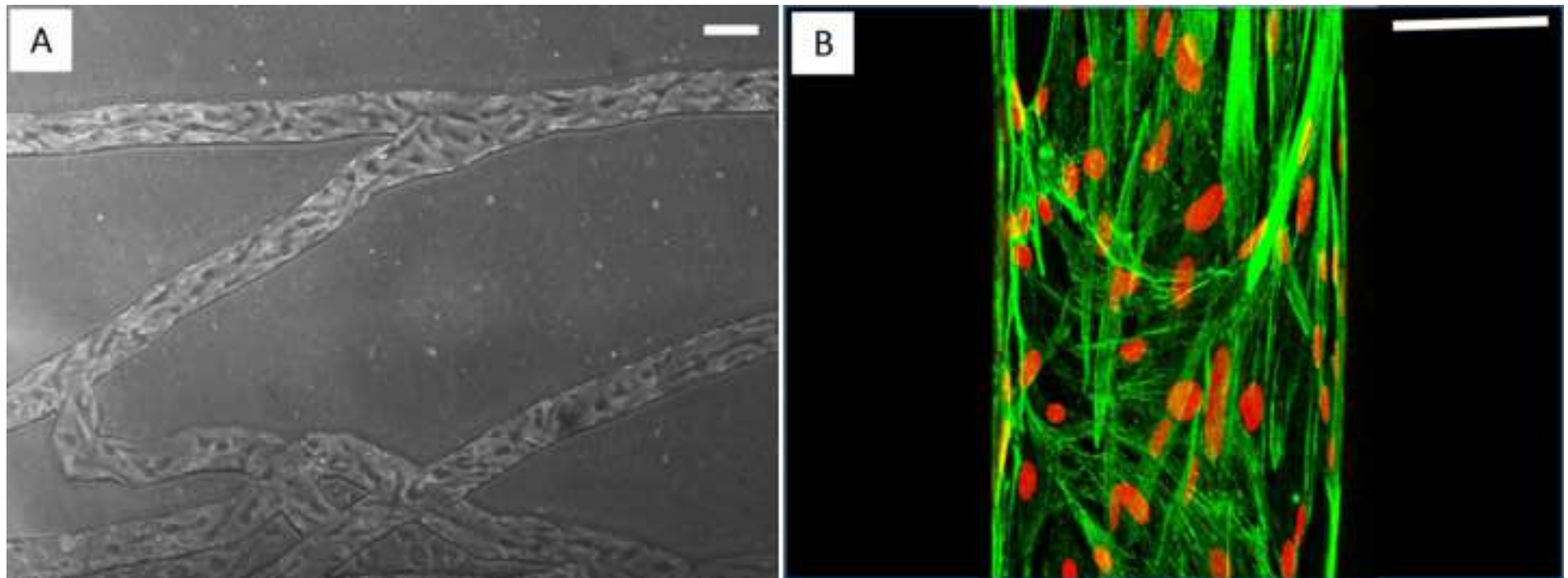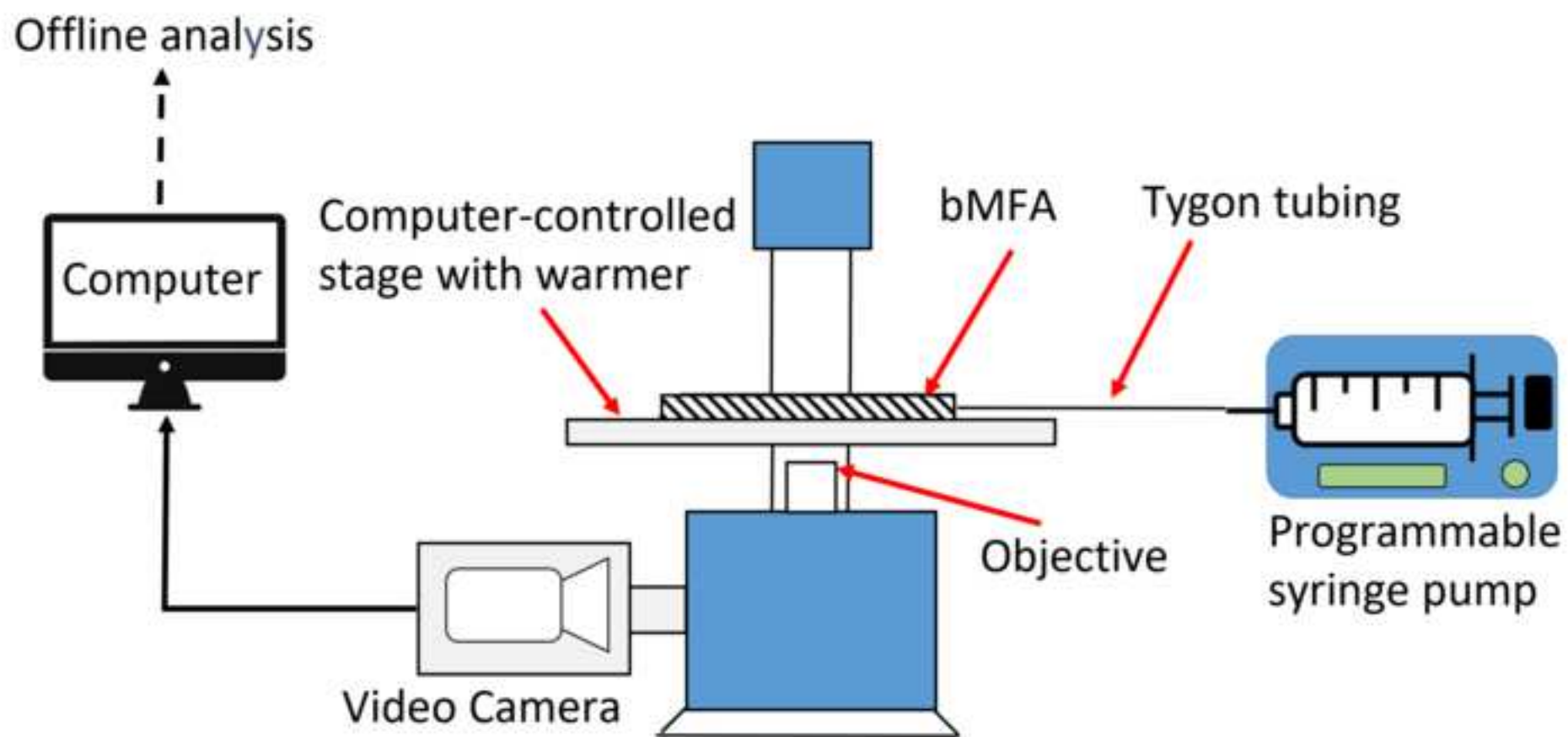Click here to access/download;Figure;Figure 5.tif ⬇

Figure 5

Figure 6

Figure 7

Figure 7

Figure 8_combined_R2

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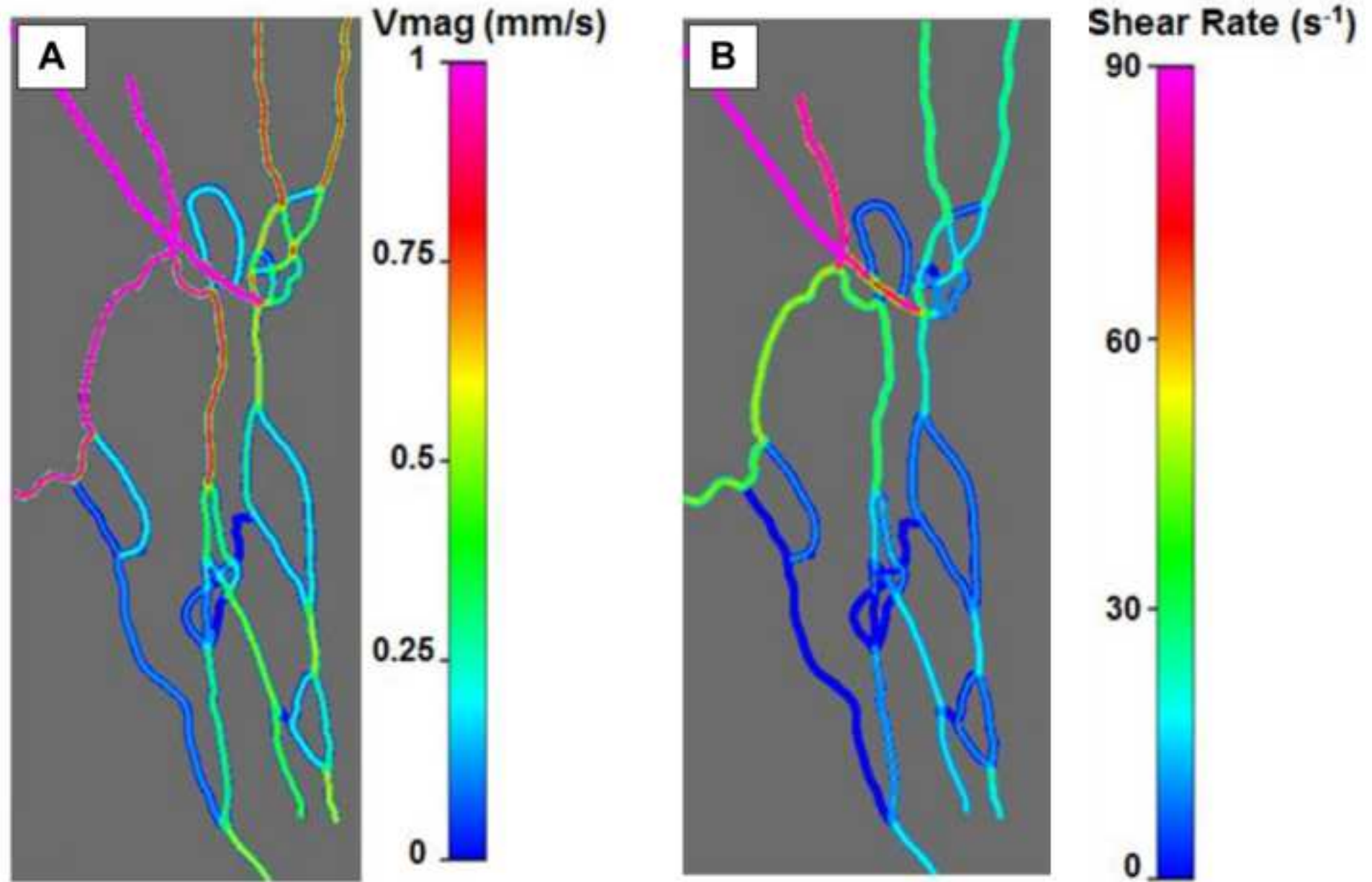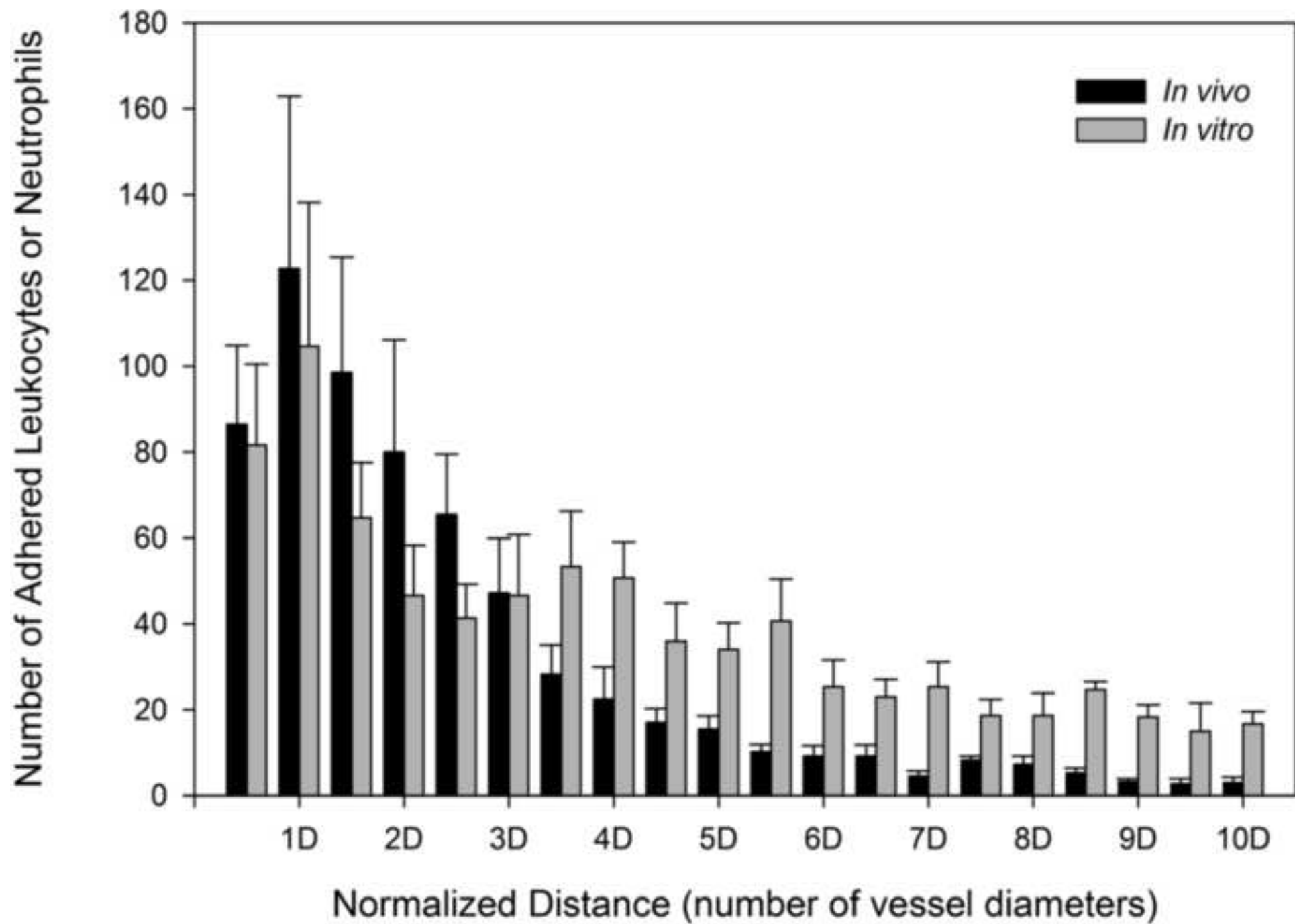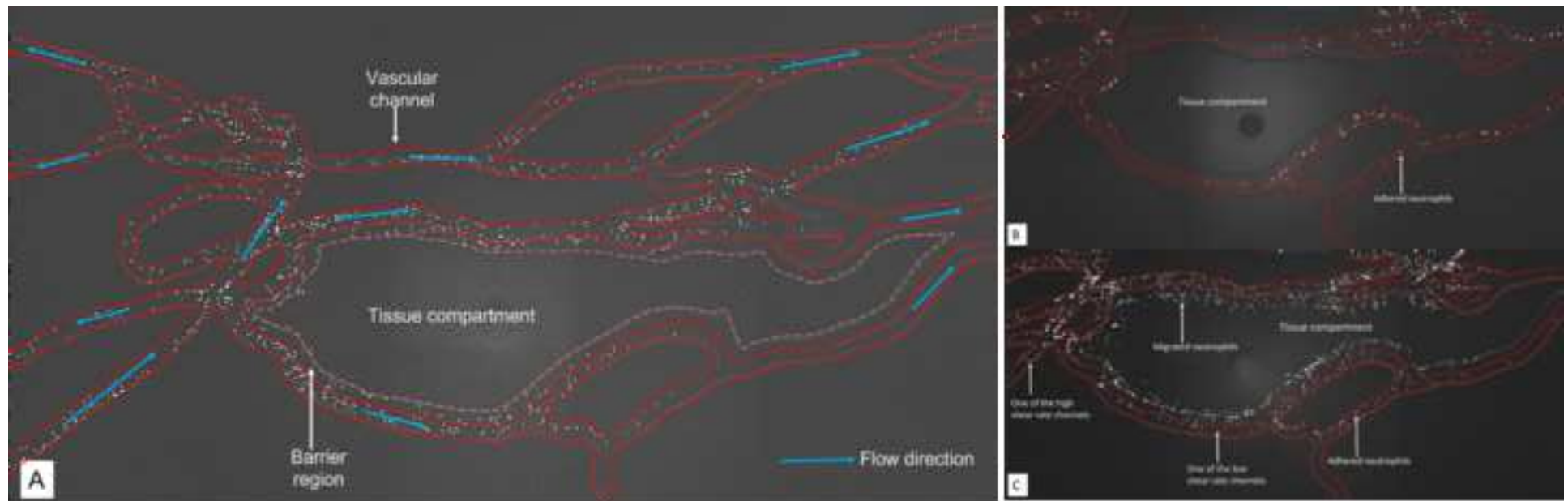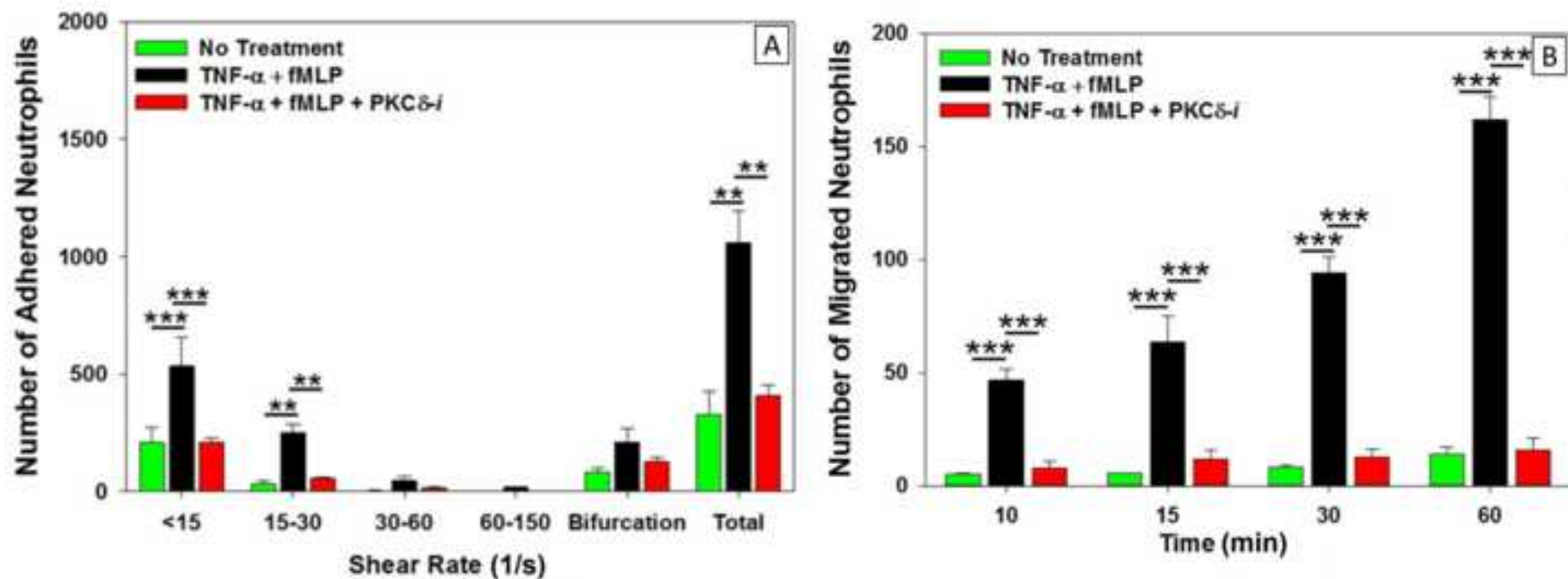
Table 1

Table 1

Step 1: Constant rate		Comments
Mode	Infuse	
Flow rate	1 µL/min	
Time	10 min	
Step 2: Delay (no flow)		
Mode	Delay	
Time	4 h	
Step 3: Repeat		
Mode	Repeat	Step 1 and Step 2 will be repeated 6 times before going to Step 4.
Repeat from	Step 1	
Repeat	6 times	
Step 4: Constant flow rate		
Mode	Infuse	
Flow rate	0.1 µL/min	
Time	48 h	
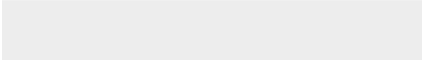
Table 2

Table 1

Formula	Name	MW	Add	Concentration
NaCl	Sodium Chloride	58.44	87.66 g	1.5 M
KOH	Potassium Hydroxide	56.11	2.81 g	50 mM
Hepes	Hepes	238.3	23.83 g	100 mM
$MgCl_2$	Magnesium Chloride	203.31	2.44 g	12 mM
$CaCl_2$	Calcium Chloride	147.62	1.90 g	12.9 mM

10x HEPES buffer (Add DI water to 1000 mL and adjust pH to 7.3. Dilute to 1x before use)

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Table of Materials

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1. Please note that the manuscript has been formatted to fit the journal standard. Please retain the changes. Comments to be addressed are included in the manuscript. Please review and revise accordingly.

Response: The manuscript has been revised accordingly.

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Response: The manuscript has been revised accordingly.

3. The syringe pump program is revised into a Table (Table 1) Please check. Please add more details in the Repeat mode for clarity (What is repeated, etc.)

Response: Table 1 has been revised. These steps are specifically for the PhD Ultra Syringe Pump.

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"Note: Inverted microscope is used in all the steps involving microscopy in this protocol."

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6. Figure 8: Please combine figures 8A, 8B, and 8C to form a multipanel image.

Response: Corrected.

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Response: Corrected.

8. We cannot have buffer compositions in the Table of Materials. It is now included as a separate Table (Table 2). Please check Table 2 attached.

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Response: This comment has been addressed on line 425 to 427 in the revised manuscript.

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