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HHS Public Access

Author manuscript

Nat Protoc. Author manuscript; available in PMC 2017 July 13.

Published in final edited form as:

Nat Protoc. 2017 May ; 12(5): 865–880. doi:10.1038/nprot.2017.018.

On-chip human microvasculature assay for visualization and quantitation of tumor cell extravasation dynamics

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Abstract

Distant metastasis, which results in >90% of cancer related deaths, is enabled by hematogenous dissemination of tumor cells via the circulation. This requires the completion of a sequence of complex steps including transit, initial arrest, extravasation, survival and proliferation. Increased understanding of the cellular and molecular players enabling each of these steps is key in uncovering new opportunities for therapeutic intervention during early metastatic dissemination. Here, we describe an *in vitro* model of the human microcirculation with the potential to recapitulate discrete steps of early metastatic seeding, including arrest, transendothelial migration and early micrometastases formation. The microdevice features self-organized human microvascular networks formed over 4–5 days, after which tumor can be perfused and extravasation events easily tracked over 72 hours, via standard confocal microscopy. Contrary to most *in vivo and in vitro* extravasation assays, robust and rapid scoring of extravascular cells combined with high-resolution imaging can be easily achieved due to the confinement of the vascular network to one plane close to the surface of the device. This renders extravascular cells clearly distinct and allows tumor cells of interest to be identified quickly compared to those in thick tissues. The ability to generate large numbers of devices (~36) per experiment coupled with fast quantitation further allows for highly parametric studies, which is required when testing multiple genetic or pharmacological perturbations. This is coupled with the capability for live tracking of single cell extravasation events allowing both tumor and endothelial morphological

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Author contributions statement

M.B.C, J.A.W and R.D.K conceived the project and designed the experiments; M.B.C. and C.Y performed the experiments; J.F designed fluorescent cell lines; M.B.C analyzed the data; R.D.K supervised the project; Y.S designed the figure schematics, M.B.C and R.D.K wrote the paper.

Competing interests statement

The authors declare no competing financial interests.

dynamics to be observed in high detail with a moderate number of data points. This Protocol Extension describes an adaptation of an existing Protocol describing a microfluidic platform that offers additional applications.

INTRODUCTION

Despite the fact that metastasis is the leading cause of cancer related deaths, many of the cellular and molecular mechanisms that drive this process remain poorly understood. Metastasis is the sequence of events by which cancer spreads from the primary tumor via hematogenous or lymphatic dissemination, resulting in downstream colonization at remote, secondary sites, often the liver, lung, brain or bone marrow[1]. This complex cascade of events is initiated by the invasion of primary tumor cells into surrounding tissues, and entry into the vasculature, followed by transport and intravascular arrest at a distant site, extravasation, and finally, cell proliferation and formation of metastatic foci.

In particular, tumor cell extravasation is thought to be an essential and possibly rate-limiting step, as most metastases are found in the extravascular space rather than intraluminal. Thus, it is of utmost importance that we understand the effectors and signaling pathways that regulate the extravasation process. This can be greatly facilitated by assays that enable (1) visualization of single tumor cell extravasation dynamics at high spatiotemporal resolution combined with (2) rapid, highly parametric and robust quantitation of extravasation events. A fundamental understanding of these steps will likely lead to improved methods for screening of potential therapeutic agents targeted at inhibiting extravasation.

In this protocol, we describe an engineered *in vitro* microfluidic-based assay featuring perfusable microvascular networks (**μ**VN) for real time observation and quantitation of tumor cell extravasation [2]. Uniquely, the assay allows **μ**VNs to be perfused with tumor cells via lumens with openings that connect these networks to the device inlet ports. Via confocal microscopy at relatively low magnification (20X), robust and rapid quantitation of tumor cell extravasation kinetics and overall efficiency can be achieved. The assay can be used for the screening of therapeutic agents targeted towards, or genetic factors relevant to, the extravasation stage. By further employing higher magnification live imaging (e.g. 30–60X), one can also dissect the morphological dynamics of tumor cell arrest, invadopodia formation, endothelial breaching, basement membrane invasion, and early micrometastases formation, all using the same device. Importantly, the technique is highly accessible to standard biology and engineering labs as it depends only on standard soft lithography methods using poly(dimethylsiloxane) (PDMS), conventional labware, a confocal microscope and standard cell culture equipment.

EXPERIMENTAL DESIGN

Recapitulation and study of the critical steps in early metastatic seeding requires a system with several capabilities: 1) microvascular networks with dimensions and barrier functions comparable to the human microcirculation, 2) the ability to perfuse tumor cells into the network with the option to maintain intravascular flow for the remaining duration of the experiment, and 3) imaging of extravasation events with high spatiotemporal resolution. To

meet these requirements, we have developed a simple, easy to use *in vitro* microfluidic-based assay featuring perfusable **μ**VNs. The user can achieve rapid and robust quantitation of tumor cell extravasation efficiency, and if required, perform higher spatiotemporal resolution imaging of the morphological dynamics of tumor and endothelial cells during transmigration. The protocol consists of 5 sequential components: (1) device fabrication and sterilization, (2) injection of endothelial and fibroblast cell-laden fibrin gels, (3) device culture until formation of perfusable **μ**VNs, (4) perfusion of tumor cells, (5) live tracking of tumor cell extravasation and data analysis.

Device fabrication and μVN formation (Steps 1–12)

The device features three hydrogel regions (each 800 μm wide × 1300 μm long × 110 μm high) each separated by media channels that allow delivery and exchange of soluble factors and small particles. Upright micro-posts separated by 100 μm delineate the boundaries of each gel region, simultaneously allowing surface tension-assisted filling of cell-laden hydrogels and paracrine interactions between endothelial cells and flanking fibroblasts. Human umbilical vein endothelial cells (HUVECs) and human lung fibroblasts (FBs) suspended in fibrin gels are injected into the central and side gel regions, respectively (Figure 1a–c). During device culture, single HUVECs elongate, form vacuoles and connect with neighboring cells to form patent lumens within 4–5 days (Figure 2a). Paracrine signaling from fibroblasts in the flanking gel regions help to prevent the premature regression of μVNs (compared to HUVEC mono-culture)[3], such that the characterization of extravasation events is not affected by changes in network morphology. The number of devices seeded per experiment is up to the discretion of the user – we have robustly shown that 36 independent devices can be seeded in one sitting without compromising cell viability. While it is possible for human microvascular endothelial cells (HMVECs) to be used instead of HUVECs, we have observed that the current protocol with HMVECs yields significantly less perfusable and less interconnected vascular networks. Further optimization of the initial ECM composition and exogenous growth factors is required if HMVEC culture is desired.

Perfusion of tumor cells (Steps 13–19)

Vasculogenesis-like tube formation in hydrogels has long been demonstrated in a variety of assays, including gel droplets and endothelial cell spheroids [4,5]. However, while micro-vessels form, most systems remain un-perfusable to small particles due to the lack of accessible entry points into the vasculature. In our assay, the device is designed such that the **μ**VNs form open lumens that connect with the media channels via the space between micro posts (Figure 2b). Similar designs in microfluidics have been robustly employed to allow vascular network formation followed by perfusion to study endothelial barrier function[6–8]. In this set-up, tumor cells suspended in medium can be perfused into μVNs by applying a hydrostatic pressure drop (~ 4mm H_2O) across the HUVEC gel region, which results in physiologically relevant fluid shear stresses (~1 Pa). Details in the determination of average shear stress can be found in Supplementary Figure 1. During ~10 minutes of perfusion via a hydrostatic pressure drop, both single and aggregates of tumor cells physically lodge and/or adhere to the endothelium (Figure 2c). At times, tumor cells can lodge and dislodge multiple times throughout the network before permanently arresting, similar to *in vivo* observations [9]. In the case of tumor cells with diameters of ~15–20 μm, >80% of tumor cells arrested in the

networks are physically lodged, rather than purely adhered. At our seeding densities, the number of arrested tumor cells per device generally ranges between 40 to 150 cells. This depends on several factors including average vessel diameter, tumor cell diameter, duration of perfusion and degree of perfusability of the μVNs.

Rapid quantitation of extravasation efficiency (Steps 29–31)

Cells are scored as extravasated if the entire tumor cell body is found outside of the lumen. These can be clearly differentiated from mid-transmigrating and fully intravascular cells. The thin height of the hydrogel region causes **μ**VNs to be confined largely to a single plane, greatly increasing the speed of imaging and enabling robust extravasation scoring (Figure 3a, Supplementary Figure 2). Tumor cells are typically found to initiate the extravasation cascade within 2 to 6 hrs post-injection, and a fraction of these can complete transmigration anywhere between 4 to 48 hours depending on the tumor cell type and/or treatments applied (Figures 3b–c). Additionally, the extravasation behavior of different subpopulations of cells within a single device can be studied – for instance, the extravasation potential of single tumor cells versus tumor cells clusters can be differentiated (Figure 3d–e).

If desired, the user can impose constant flow of media through the μVNs to simulate the flow condition *in vivo,* throughout the entire extravasation process. This can be achieved via hydrostatic pressures generated from integrated reservoirs (typically for <6 hours before significant equilibration), or for longer durations using syringe pumps. Extravasation efficiency can be assessed at a specific end-point or over time via live confocal microscopy. At 20X magnification, imaging of an entire device typically takes 6 to 12 minutes, depending on the imaging modality and settings (Supplementary Table 1). Images are later reconstructed in 3D using standard software packages such as Imaris Bitplane (Belfast, UK) for scoring of extravasation.

Tracking extravasation kinetics (Steps 20 and 32)

If desired, kinetic data from single or clusters of extravasating cells can be quantified in a moderate throughput manner via time-lapse imaging. The user can observe and differentiate between detailed morphological states such as initial tumor cell protrusion past the endothelium versus complete transmigration (Figure 4, Supplementary Movie 1). Such information can provide valuable insight into differences in extravasation kinetics, which is not possible to obtain with end-point measurements. Generally, a magnification of ~20X is sufficient to visualize these details, with time intervals as small as 5 minutes, depending on the number of cells to be imaged. Such imaging is facilitated by the short distance (between 170 to 210 μm) between the outer bottom surface of the device and the plane of the **μ**VNs. In addition, temporal and spatial organization of intracellular or extracellular proteins can be resolved, provided that proteins of interest are fluorescently labeled prior to imaging. Depending on the desired level of discrimination between discrete elements, imaging of some cellular proteins (e.g. F-actin, VE-cadherin, focal adhesion proteins) may require higher magnification (>30X), at the cost of increased imaging duration (Figure 5).

Immunofluorescent staining (Steps 21–28)

Devices can be fixed and processed for immunofluorescent staining at any desired time point, in a manner similar to tissues. One key advantage is the low volume of reagents required (<30 μl) of diluted antibody solutions per device, rendering it an economical choice, especially when a large number of targets or samples are involved and/or when antibodies are particularly costly or rare. High spatial resolution can be achieved – for example, the position of transmigrating tumor cells relative to the vascular basement membrane can be assessed via staining for proteins such as collagen IV and laminin (Figure 6). The ability to generate these data may be valuable, as it has recently been found that protease-localizing invadopodia can mediate extravasation, suggesting that basement membrane degradation may be required for complete transmigration.

Tracking tumor cell proliferation and early micrometastasis formation

Tumor cells can also be tracked after extravasation by continued culture of the endothelial-tumor cell system for periods up to 5–6 days post tumor seeding. Paracrine factors secreted by supporting FBs maintain stable **μ**VNs longer compared to HUVEC monoculture where vessel regression can be evident as little as 3 days post endothelial seeding[3]. Via time-lapse microscopy or time point imaging, the number of tumor cells can be quantified when tumor cell nuclei are labeled with fluorescent tags (e.g, mCherry H2B) or when the tumor cell cytoplasm is labeled with nuclear excluded markers. Cell nuclei can be counted with conventional imaging software such as ImageJ or Imaris Bitplane (Belfast, UK) to assess the level of tumor cell proliferation in both extravasated and intravascular subpopulations.

ALTERNATIVE EXTRAVASATION ASSAYS

Conventional in vivo extravasation assays

Current *in vivo* and *ex vivo* models for visualizing extravasation include mouse [10–14] zebrafish [15] and chorioallantoic membrane (CAM) assays[16–18]. While murine models remain the gold standard for recapitulating metastatic seeding, the organs in which tumor cells arrest are generally not very optically accessible to allow live single cell imaging, rendering it difficult to assess extravasation rates; these are more suitable for end point *ex vivo* analyses[13,14]. CAM and zebrafish assays have previously been demonstrated to be much more optically accessible partly due the “planar” nature of the capillary beds, allowing intravascular and extravasated cells to be more clearly differentiated. However, the ability to resolve the dynamics of tumor cell protrusion kinetics with a large number of data points is still limited, likely due to the technical challenges associated with tumor cell injection and live imaging in intact organisms. Quantification of extravasation events in murine systems is often subjective due to the tortuosity of *in vivo* vasculature (particularly in the lung), while precise measurements relies on high resolution imaging (60X), greatly increasing data collection times and rendering them generally unsuitable for highly parametric studies. One heavily employed method to score extravasation is by counting how many tumor cells are retained in the lung after a certain time after tail-vein injection, with the assumption that extravasated cells will remain detectable while non-extravasated cells are lost due to shear flow or immune factors. However, recent analysis of mouse lung tissues fixed at 24 and 48 hours reveal a non-negligible proportion of “retained” tumor cells to be intravascular, so

these assumptions are questionable [19]. Furthermore, all of the above systems feature non-human host cells, which may be problematic if specific human tumor-human host cell interactions are not conserved, particularly in the case of various types of immune cells. Lastly, intravenous injection of tumor cells in most of these systems often require significant technical expertise, which can limit the number of qualified users and even compromise the reproducibility of results. Moreover, procedures such as intra-cardiac injection of tumor cells often result in very few number of tumor cells found in the sites of dissemination, rendering it difficult to gather a sufficient amount of data points.

Conventional in vitro monolayer-based extravasation assays

In vitro tools have proven invaluable for the investigation of cellular interactions as they allow observation of discrete events from a complex cascade, tight control of experimental parameters, and exclusive usage of human cell lines. The most commonly used model to study extravasation is the Boyden chamber/Transwell assay, where an endothelial cell monolayer is grown to confluence on a porous insert, followed by the seeding of tumor cells on top of the layer. The number of cells transmigrated can be quantified by collecting the cells that migrate to the underside of the membrane [20,21]. More recently, similar assays incorporating a 3D ECM matrix beneath the endothelial monolayer have increased the physiological relevance by recapitulating the subendothelial ECM into which tumor cells transmigrate [22]. However, in many of these assays, extravasation cannot be observed in real-time or in high resolution, and quantitative assessment is limited to end-point measurements. Microfluidic models of the human vasculature have emerged as a potentially useful tool for studying tumor cell-endothelial cell interactions. For instance, “networks” of vessels can be formed by lining lithography-based ECM templates (formed via pin-pull out [23], sacrificial gels[24] or 3D printing[25]) with an endothelial monolayer. Alternatively, endothelial monolayers can be grown in a microfluidic channel to allow application of relevant fluid shear stresses, while tumor cell adhesion and transmigration are monitored dynamically [26,27]. In one approach, an endothelium can be grown perpendicular to the plane of view on the surface of ECM hydrogels held in place via surface tension [28–33]. This type of assay is particularly amenable to imaging of tumor cell morphological dynamics, as migration occurs in the plane of view; however the details of the endothelium during transmigration are compromised (such as morphological dynamics of endothelial junctions). A unique advantage to these platforms is their ability to incorporate chemical gradients orthogonal to the endothelial barrier, which may be useful to test hypotheses involving the dependence of transmigration on chemotactic agents. However, in these assays, the number of observable transmigration events is often limited due to the small surface areas between posts, frequent sprouting of ECs into the gel, as well as difficulties in ensuring a continuous monolayer at the junction of PDMS posts and the hydrogel surface. Confirming the barrier integrity at each of these points via diffusive permeability measurements would be laborious but an essential step if the assay were to be used for extravasation studies. Conversely, dextran perfusion in our microvasular network assays show that vessels are nearly always free of such artifactual focal leaks, suggesting patent lumens at nearly all possible transmigration points.

Moreover, the majority of monolayer extravasation systems face challenges, including an artifactual dependence of transmigration rates on the degree of endothelial confluency and geometrical over-simplification as extravasation occurs in complex 3D **μ**VNs with vessel segments of 5–30 μm in diameter instead of >100 μm [24] or an "infinite" flat plane of endothelial cells [28,30]. Additionally, in many monolayer systems (especially Transwell filters), nearly all tumor cells seeded at t=0 will translocate across a monolayer over a 24–48 hour time frame[34]. When compared to much lower extravasation rates of 40–60% at 24 hours that are typically found in mouse[19,35] and zebrafish [36] models for a variety of cell lines, this suggests that endothelial monolayers exhibit much greater permissiveness to tumor cells compared to *in vivo* microvasculature. We note however, that this value can vary widely depending on the cell type and *in vivo* model used. Conversely, we have found that the present assay exhibits decreased permissiveness to tumor cells (such as MDA-MB-231) compared to many endothelial monolayer assays, and exhibit values similar to those found in various mouse models (also for MDA-MB-231) [19,35]. Furthermore, our measurements of diffusive permeability to 70 kDa dextran reveal a generally higher barrier function than those reported in numerous monolayers studies (8×10^{-7} to 1.2×10^{-6} cm/s [2,37] versus 4 to 8×10^{-6} cm/s[23,24,38]), which could contribute to the lower and more realistic permissiveness to tumor cells. Lastly, while monolayer assays have adequately modeled many microvascular behaviors, they do not readily allow simultaneous application of lumenal flow with a 3D tubular organization.

ADVANTAGES AND LIMITATIONS OF THE ON-CHIP MICROVASCULATURE EXTRAVASATION ASSAY

The key advantageous features of this device can be summarized as follows:

- Arrest of tumor cells and extravasation from small diameter (ranging from 7–100 micron) vessels represents greater physiological relevance than conventional monolayer based assays.
- Human cells (endothelial, tumor, stromal, immune) can be exclusively used in this model, decreasing the uncertainty of "species incompatibility."
- Extravasated or mid-extravasating cells are clearly distinct from intravascular cells and extravasation efficiency can be evaluated at lower power magnifications (20X), enabling both greater speed of data acquisition and robustness of extravasation scoring.
- Regions of interest with tumor cells are easily identified due to the fact that the **μ**VN lie largely in the same plane of focus.
- This feature also enables tumor/endothelial cell morphological dynamics to be resolved with relatively high spatiotemporal resolution and throughput (~30 cells per time-lapse at 30X–60X, taken at 10 minutes intervals) due to the applicability of high magnification objectives (which have shorter working distances). This provides novel metrics on extravasation kinetics such as protrusion initiation rate and speed of complete transmigration.

- Tumor cells interact and invade past a HUVEC -deposited basement membrane and into 3D ECM, all of which can be visualized in considerable detail via standard immunofluorescent techniques.
- The technical expertise and equipment required to form microvascular networks is low compared to techniques such as pin pull-out, sacrificial gels or 3D scaffold printing techniques.
- Tumor cell injection into **μ**VNs only requires basic pipetting maneuvers instead of acquired animal intravenous injection skills.
- The material cost of each device is low (<$0.40 per device) compared to most *in vivo* alternatives, and is easily fabricated via standard soft lithography techniques that are now prevalent amongst most bioengineering laboratories.
- Required reagent volumes and cell numbers can be orders of magnitude lower than most other extravasation assays (< 4000 tumor cells/device and <80 μL of media/device) due to miniaturized features and low dead volume, greatly reducing costs associated with expensive or rare cells (e.g. patient samples), drugs, or antibodies.

One of the limitations is the general inability of *in vitro* systems to fully recapitulate the *in vivo* scenario. In reality, the early metastatic seeding and extravasation microenvironment is characterized by a range of host cells and factors such as platelets and leukocytes, with which tumor cells have been found to interact during transit *in vivo* [9,39,40]. However, the devices are flexible enough to accommodate increased complexity of the microenvironment in order to achieve greater realism. For instance, tumor-immune cell interactions can be studied via isolation of platelets or leukocytes from whole human blood followed by perfusion in conjunction with tumor cells, which we have provided a preliminary demonstration of (Figure 7). In fact, the reductionist nature of such an *in vitro* system can be highly beneficial when one is required to parse out specific interactions independent of the effect of other cell types; this would be more difficult *in vivo* as it requires either complete ablation or loss of function of endogenous cells. Another limitation is that while soft lithography techniques are widespread amongst bioengineering labs, there are fewer biology/cancer research labs that preform soft lithography on a routine basis. However, protocols of PDMS-based device fabrication are now well established and robust, and only require several pieces of easily accessible equipment such as a degasser, 80°C oven and plasma etcher. The photo-mask of the device design is accessible through this article (Supplementary CAD File) and fabrication of molds can be easily outsourced to companies (e.g. FlowGEM, etc.) if in-house fabrication is not available.

MATERIALS

Device Fabrication

- Photomask with microdevice design (available for download in Supplementary Data)

- Dow Corning Sylgard 184 Silicone Elastomer base and curing agent (Ellesworth, Cat. no. 184 SIL ELAST KIT 0.5KG)
- Biopsy punches (Ted Pella, Cat. no. 15110-40)
- Razor blades (Ted Pella, Cat. no. 121-32)
- Scalpel
- No.1 22X40 mm glass coverslips (VWR, Cat. no. 48393-048)
- Scotch tape

Cell seeding

- Human umbilical vein endothelial cells (p8 or lower, Lonza, Cat. no. CC-2519) and normal human lung fibroblasts (p15 or lower, Lonza, Cat. no. CC-2512)

  CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- Fibrinogen (Sigma, Cat. no. F8630-1G)
- Thrombin (Sigma, Cat. no. T9549)
- Endothelial growth media (EGM-MV BulletKit™) (Lonza, Cat. no. CC-3202)
- Fibroblast growth media (FGM-2 BulletKit™) (Lonza, Cat. no. CC-3132)
- 0.05% trypsin (wt/vol) (ThermoFischer, Cat. no. 25300054)
- Hemocytometer or other cell counting tools
- Dulbecco's PBS (D-PBS, pH 7.2–7.4; Wisent, Cat. no. 311-010-CL)
- Sterile dH_2O
- DMEM GlutaMAX (ThermoFischer, Cat. no. 10566-016)
- FBS (ThermoFischer, Cat. no. 10437-010)
- Gel loading pipette tips (VWR, Cat. no. 37001-152)
- Pair of surgical scissors
- Humidity chambers (e.g. empty P1000 pipette tip boxes with rack, filled halfway from the bottom with sterile water) (e.g. VWR, Cat. No. 10034-060)

Equipment

- Vacuum desiccator (Cole Parmer, Cat. no. WU-06514-30)
- Drying oven (60 – 80°C)
- Benchtop centrifuge for use with 15 mL tubes
- Incubator at 37°C and 5% CO_2
- Autoclave

- Plasma cleaner (Harrick Plasma, Cat. no. PDC-001)
- Water bath at 37°C
- Confocal microscope with environmental chamber (maintained at 37°C and 5% CO_2) and motorized stage
- 3D imaging processing software (we recommend Imaris Bitplane (Belfast, UK))

REAGENT SET UP

Fibrinogen solution, 6 mg/mL—Dissolve 15 mg of bovine fibrinogen in 2.5 mL of sterile PBS (−/−) in a 15 mL tube by incubating the mixture in a 37°C water bath for 3 hours. Do not vortex. Sterile filter the solution (0.2 μm filter) and aliquot the dissolved fibrinogen mixture in 1 mL eppendorf tubes and store at 4°C until usage.

■ **PAUSE POINT** It is recommended that fibrinogen solutions be made on the day of or the day prior to expected use. Long-term storage of fibrinogen solutions (>1 week) is not recommended.

Thrombin solution, 100 U/mL—resuspend thrombin with sterile PBS (−/−) to a final concentration of 100 U/mL). Aliquot and store at −20°C (no longer than 6 months) until use.

HUVEC and FB culture—Thaw HUVECs and FBs until the required amount for the number of devices to be seeded (at least 2×10^6 HUVECs and 1×10^6 HLFs are required for a total of 12 devices). Cell numbers can be scaled accordingly if more or fewer devices are to be seeded. Use lower passage HUVECs (<P8) for best **μ**VN formation results, as endothelial cells become more senescent as passage number increases. Take care as to not allow HUVECs to become over-confluent prior to trypsinization.

Re-suspension media—Add 40 μL of thrombin stock solution to 1mL EGM-2MV to make a 4U/mL thrombin solution. Make this solution immediately prior to device seeding and do not reuse.

EQUIPMENT SETUP

Preparation of humidity chambers—Prepare humidity chambers for device seeding by filling pipette tip boxes (with a removable rack) with water and autoclaving the whole box for 20 minutes. Set aside to cool, then transfer to a 37 °C incubator until time of device seeding.

PROCEDURE

PDMS device fabrication ● TIMING 24 hrs

1 Fabricate the micro device master using the photo mask pattern given in this protocol (Supplementary CAD file). Wafers can be made via SU-8 micro patterning methods that are established and detailed elsewhere [41] or via outsourcing. A channel height between 110–150 microns is optimal for the volumes and densities in this given protocol. Silanize wafer overnight in a vacuum desiccator.

2 Fabricate and sterilize PDMS devices using the procedure outlined in our previous protocol (Steps 9–26 in Shin et al, Nature Protocols, 2012)[42]. We recommend punching 3 mm diameter holes for the media reservoirs and 1 mm diameter holes for gel injection ports (see Figure 1c). Incubate bonded devices at 80°C for >24 before seeding to ensure that hydrophobicity is restored.

■ **PAUSE POINT** Make sure to keep devices in sterile conditions until opening the device container under a sterile cell culture hood.

Seeding of HUVECs and FBs in microdevices for μVN formation ● TIMING 2 hr for seeding and 4–5 days for culture

3 Make sure prepared devices are at room temperature (20–23°C).

4 Aliquot 8 **μ**L of pre-made fibrinogen solution into 0.5mL eppendorf tubes. The number of aliquots made should be 2X the number of devices that will be seeded. For each device to be seeded, one aliquot will be designated for HUVEC injection, while the other is designated for FB injection. Keep aliquots on ice.

▲ **CRITICAL STEP** Separate aliquots of fibrinogen should be made for each device because once thrombin is added to fibrinogen, gelation can occur in < 30 seconds.

5 Trypsinize HUVECs and FBs with 0.05% trypsin for 3 minutes, neutralize with EGM-2MV and centrifuge for 5 min at 200 g.

6 Aspirate media from the cell pellets and re-suspend the cells at 12×10^6/mL (HUVECs) and 6×10^6/mL (FBs) in re-suspension media.

■ **PAUSE POINT** Keep resuspended cells on ice during the remaining procedure and not longer than 1 hour.

■ **CRITICAL STEP** Make sure to aspirate as much of the supernatant as possible (e.g. by waiting a few seconds and aspirating the residual media), since the error in resuspended cell density can be easily increased and propagated when working with small volumes.

7 Mix 8 **μ**L of HUVEC suspension with 8 **μ**L of a fibrinogen aliquot by pipetting up and down 4–5 times inside the 0.5 mL eppendorf tube. Withdraw 10 **μ**L and immediately inject the HUVEC-gel mixture into the center gel region by slowly pipetting it (using a P10 pipette) via one injection port until the solution reaches the start of the narrower downstream channel. Retract the pipette tip slowly without releasing the injector. Repeat this process with the FBs by injecting the FB-fibrinogen mixture into the two flanking channels.

▲ **CRITICAL STEP** The cell-gel mixture must be injected slowly in order to prevent leakage into the media channels or gel fiber alignment.

■ **PAUSE POINT** Do not re-use the leftover fibrinogen-thrombin mixture in the aliquot for a subsequent device. Even if leftover the aliquot is still “liquid,”

injection of this leftover solution may result in gel fiber alignment in the device because the solution has already begun to polymerize.

?TROUBLESHOOTING

8 View the device in a sterile petri dish under a phase contrast or bright field microscope to ensure gels are intact and cells are homogeneously seeded.

▲ **CRITICAL STEP** Ensure that gels are not "under-filled." The interface formed between the inter-post region and the media channel must be flat, otherwise subsequent introduction of media into media channel will result in bubble formation.

?TROUBLESHOOTING

9 Place the finished device into the humidity chamber and allow gelation for 15 minutes at room temperature (our room temperature ranges between 20–23°C). While waiting, repeat steps 7–9 with the rest of the devices.

▲ **CRITICAL STEP** Ensure that the cell solution is well mixed before withdrawing 8 **μ**L each time as cells will sink to the bottom of the tube quickly resulting in inhomogeneous seeding densities.

10 After gel polymerization, remove the device from the humidity chamber and wipe the condensation from the glass bottom with a paper towel misted with 70% ethanol. Place the device into a petri dish and fill the media channels with EGM-2MV by forming a tight seal around the media port with the pipette tip. Depending on the size of the reservoir punched, the pipette tip may have to be cut to size in order to form a seal. Inject media until all reservoirs are filled. Repeat with the remaining devices.

?TROUBLESHOOTING

11 Culture the devices at 37°C and 5% CO_2.

?TROUBLESHOOTING

12 Change the media in the devices every 24 hours by aspirating each media reservoir with a fine tipped gel-loading tip. Then fill all reservoirs on one side with media, wait until the media has flowed down the channel causing the media levels to rise in the opposing reservoir. Top off the reservoirs with more media until they are full. **μ**VNs will be ready to perfuse after 4 to 5 days of culture.

Perfusion of tumor cells ● TIMING 20 min to 1.5 h (if using cell tracker)

13 Briefly check the vessel openings at the inter-post regions to assess whether the vascular networks have formed connections with the media channel. Examples of connections (and lack of connections) can be found in Supplementary Figure 3, as well as the how perfusability is scored, and sample data on the typical number of perfusable devices that should be generated per experiment.

? TROUBLESHOOTING

14 Replace all media in the channels of the device with 0.1% BSA in PBS and incubate for 20 minutes to minimize unspecific adhesion of tumor cells to the glass during tumor perfusion. Replace with fresh EGM-2MV.

15 If live imaging will later be performed to track extravasation, HUVECs should be fluorescently labeled to robustly differentiate between intravascular and extravascular cells. It is recommended that HUVECs be transduced to stably express cytoplasmic or membrane fluorescence. The protocol used to generate our stable fluorescent HUVECs is provided in Supplementary Methods. Alternatively, the device can be incubated with cell-tracker or DiI solution in EGM-2MV for 1 hour at 37°C and 5% CO_2. Apply a hydrostatic pressure drop across the HUVEC gel region to help the dye penetrate the entire **μ**VN. A pressure drop of 4mm H_2O (equivalent to ~38 μL of solution in both reservoirs on one side of the gel region, for a 3 mm diameter reservoir) can be applied to promote convection and thus penetration of the dye throughout the vascular bed. After incubation, wash the device twice with EGM-2MV before proceeding.

16 We recommend the use of tumor cells that stably express a fluorescent cytoplasmic or membrane marker. If tumor cells are non-fluorescent, label cells by adding cell tracker™ or DiI solution directly to adhered cells after washing once with PBS, and following the manufacturer's instructions.

17 Aspirate the dye and wash adherent tumor cells twice with PBS. Trypsinize and re-suspend tumor cells to a final concentration of 5×10^5 cells/mL in EGM-2MV and store on ice until perfusion.

18 If perfusing platelets and/or neutrophils with tumor cells, mix the cells together to achieve the desired ratio.

19 With a gel-loading tip connected to an aspiration line, withdraw media from all 4 reservoirs directly flanking the HUVEC gel region. Mix the tumor cell suspension well, and fill one reservoir with 20 **μ**L of cell suspension. Wait 5 seconds for the cells to flow down the medial channel (parallel to the gel). Aspirate all reservoirs again and apply 38 μL of cell suspension to both reservoirs on one side (this is creates a pressure drop of ~4mm H_2O across the vascular bed). The volume should be adjusted if the reservoir diameter is different than 3 mm. Cells should now flow across the HUVEC gel region within the lumens due to the pressure drop. Check the device using bright field or phase contrast microscopy to ensure that cells are flowing through the **μ**VNs (Supplementary Movie 2). Flowing cells can be clearly seen even at 10X magnification. Cells should gradually arrest in microvasculature due to size restriction. If vessel openings are small and few tumor cells flow through the capillary bed, use a fine pipette tip on an aspiration line to *gently* aspirate media from a downstream media port for 1–2 seconds.

? TROUBLESHOOTING

▲ **CRITICAL STEP** Aspirate gently and with a fine tip as to not form a tight seal with the media ports, which may cause the media channel to be fully

aspirated (rendering the device prone to bubble formation), or cause vessels to rupture.

20 If you only wish to determine extravasation rates at a desired time point, proceed with option A. For live imaging of the process of transendothelial migration, proceed to option B.

A) Termination of tumor cell perfusion

i. After ~10 minutes of tumor cell perfusion via the hydrostatic pressure drop, fill all the reservoirs on one end of the device with EGM-2MV and allow media to flow through the media channel (parallel to the gel region) to wash away remaining tumor cells. If constant perfusion of media is not required for the rest of the extravasation experiment, top off the media reservoirs with fresh EGM-2MV to prevent media evaporation. Devices can be maintained in an incubator until any desired time point (<5 days), and extravasated cells can be imaged live or post-fixation.

(B) Live imaging of transendothelial migration ● TIMING 4 to 72 hours

i. Prepare the environmental chamber on the confocal microscope beforehand to assure that the chamber reaches a steady 37°C and 5% CO_2 by the time of tumor cell perfusion into the devices.

ii. Immediately after step 19, transfer the device(s) in a sterile petri dish and secure the device onto the specimen holder in the environmental chamber.

▲ **CRITICAL STEP** Ensure that the chamber is well humidified and sealed for the entire duration of the time-lapse to prevent evaporation and cell death.

Parafilm can be used to seal crevices and help create a well-contained and humidified chamber.

iii. Allow the device to rest on the specimen holder in the primed environmental chamber for at least 10 minutes before imaging to allow for thermal drift.

iv. While waiting for equilibration of the device, set up the time-lapse parameters. For quantification of extravasation and/or visualization of tumor cell protrusions during extravasation, use at least a 20X objective (we use NA=0.75 resolution=800 × 800) or higher and at least 2 μm z-slice thicknesses. To track protrusion formation, image each region at least once every 20 minutes or faster, depending on the overall speed of protrusion onset and total translocation time for a particular cell type or condition (Figure 4). Set region locations if there are multiple areas of interest in the device. For quantification of extravasation efficiency of one device, select enough regions of interest to span the entire device – this typically yields approximately 40 to 150 tumor cells per sample. For tracking of tumor cell proliferation, acquire images at least once every 5 hours for > 24 hours.

?TROUBLESHOOTING

v. Ensure the chamber is protected from other sources of light and begin the time lapse.

■ **PAUSE POINT** Avoid high laser intensities as this can cause photo toxicity and cell death during imaging.

?TROUBLESHOOTING

(Optional) Immunostaining and fluorescent visualization of cellular proteins and basement membrane ● TIMING 24 hours (includes overnight incubation)

21 Devices can be fixed and stored for further imaging or immunofluorescent staining after a time-lapse, or directly after incubation to a desired time point. Aspirate all media reservoirs and fill all reservoirs on one side with 4% paraformaldehyde (~50 microliters per device). Allow the fluid to flow through the channels 2–3 times by aspirating the downstream reservoirs as they begin to fill. Incubate devices at RT in the dark for 10 minutes, then remove PFA from all reservoirs and replace with 1X PBS.

■ **PAUSE POINT** Fixed devices can now be stored for 2–3 weeks in a well sealed petri dish to prevent evaporation. Devices can later be used for imaging and/or immunofluorescent staining.

■ **PAUSE POINT** From this step, introduction of new solution will follow the same technique as step 21.

22 To stain for intracellular proteins, permeabilize cells with 0.1% Triton X and incubate for 15 minutes. Wash twice with PBS, incubating 5 minutes per wash. For staining of basement membrane proteins, skip this step.

23 Block the device with 5% BSA and 4% goat serum in PBS for 3 hours at RT.

24 Dilute primary antibody of choice in the recommended dilution in blocking buffer (~30 microliters/device) and incubate it with the device overnight at 4°C in the dark.

25 Wash the devices with 0.1% BSA in PBS 5 times, at 15 minutes each.

26 Dilute secondary antibody of choice at recommended dilutions (we typically use 1:200) in washing buffer and incubate at RT in the dark for 3 h.

27 Remove secondary antibody from reservoirs and wash 3 times with washing buffer, incubating 15 minutes per wash.

28 Image the device on a confocal microscope or store the device at 4°C. For visualization of basement membrane proteins such as collagen IV and/or laminin relative to extravasating tumor cells, acquire images at 60X for best results (Figure 6).

?TROUBLESHOOTING

Quantification and analysis of extravasation ● TIMING 6–10 minutes per device

29 For quantification of extravasation for a single condition, images should either be acquired for the entire device (roughly equivalent to 5 regions of interest per device at 20X magnification) or the same number of arbitrary regions per device. Take images at a 2 **μ**m z-slice size or thinner, to obtain adequate resolution during 3D image reconstruction for extravasation determination. Optimal microscope settings may depend on the fluorescent intensity of cultured cells and model of the microscope. See Supplementary Table 1 for confocal settings we use on an Olympus FV1000.

30 Using Imaris or other 3D rendering software such as the Image J "orthogonal views" function, score each individual cell as intravascular, mid-extravasation or extravascular (See Figure 3a for examples of scoring).

?TROUBLESHOOTING

31 Calculate extravasation rate as the number of completely extravascular cells divided by the total number of cells in a single device.

Ensure that a sufficient number of total cells have been imaged per device before making extravasation calculations. Total number of imaged cells should ideally be in the range of 50 – 150 cells per device, given a perfusion concentration of ~0.5 million/mL. Total cell counts that are too low results in large deviations in the extravasation rates between devices, and may not be very informative. However, perfusion of too many tumor cells per device (>200) should also be avoided since over-crowding of tumor cells will render it difficult to analyze, or affect extravasation behaviors.

To quantify extravasation rates for a single condition, we advise to make the quantification on at least 3 independent devices per experiment, and preferably over at least 2 independent experiments (i.e. a total of n>6 devices per condition).

32 To calculate rate of protrusion formation from time-lapse experiments, analyze the status of the protrusion for individual tumor cells at multiple time frames (e.g. 20 minute frame intervals). The first time frame where a cell exhibits protrusion formation past the endothelium can be analyzed as the "time for protrusion onset" (Figure 4a). The same tumor cell can be followed until the cell has fully transmigrated past the endothelium. The time interval between full body transmigration and the initiation of protrusion can be analyzed as the "total time required for transmigration."

TIMING

Step 1–2: PDMS device fabrication – 24 hrs

Step 3–12: Cell seeding and **μ**VN formation – 4 days

Step 13–19: Perfusion of tumor cells – 2 hrs

Step 20: Live imaging of extravasation – 4 to 72 hrs

(Optional) Step 21–28: Immunofluorescent staining – 24 hrs

Step 29–32: Quantification of extravasation – 10 minutes per device

TROUBLESHOOTING

See Table 1 for troubleshooting guidance.

ANTICIPATED RESULTS

This protocol has been used to conduct robust and highly parametric studies of extravasation efficiencies subject to perturbations in either tumor or endothelial cells using various pharmacological agents or genetic perturbations (Figure 3c). This is especially useful when sample numbers greatly exceed those that are realistic in an *in vivo* setting. While the microvascular network assay cannot replace *in vivo* extravasation models, it can serve as a rapid and cost-efficient preliminary test of hypotheses, which can later be validated in an *in vivo* assay. For instance, we have previously assessed the roles of various tumor cell integrins on extravasation efficiency in a panel of tumor cells lines. Via both function-blocking antibodies and siRNA knockdown of integrins alpha 1, 2, 3, 4, 5, 6, v and beta-1, 3 and 4, we found that tumor cell adhesion to subendothelial laminin via alpha 3 and alpha 6-beta-1 integrins were critical for efficient extravasation. These findings were then validated in a mouse model to confirm attenuation of metastasis formation. Furthermore, higher resolution confocal microscopy revealed that extravasation is characterized by the recruitment of activated beta-1 integrin and F-actin to the tips of tumor cell protrusions extending past the endothelium into the surrounding matrix [35]. Combined, we demonstrate the ability of achieving both moderate throughput quantitation as well as resolving detailed morphological dynamics of extravasation from a single assay.

By increasing the level of complexity of the microdevice, one can further augment the realism of the extravasation model. Recently, much attention has been directed to the role of “pre-metastatic” niches, which consist of complex tumor-host cell interactions during circulation, extravasation and re-colonization. For instance, circulating immune cells including platelets and leukocytes have been shown to interact with tumor cells, and can act to enhance metastasis [9,40,43]. We demonstrate that human platelets and/or leukocytes isolated from whole human blood can be perfused with tumor cells and immune-tumor interactions and the effects on extravasation can be monitored in the micro devices (Figure 7a–b). Additionally, the contribution of stromal cells such as pericytes can also be investigated by seeding these cells together with HUVECs in the same gel region (Figure 7c). There is also increasing evidence favoring the “seed and soil” theory for metastatic cancer cells, which suggest that some cancer cell types display increased propensity to “home” to and/or subsequently colonize certain organs. While the current device cannot be used to model homing processes such as selective arrest (since tumor cells flow into and are physically trapped in the vasculature), it is possible to use the assay to understand how different “organ-microenvironments” influence extravasation and subsequently tumor cell proliferation, beyond extravasation. Such organ-mimicking microenvironments may be

achieved by adding relevant cell types to the matrix, allowing either direct physical contact or paracrine communication, depending on the choice of cell arrangement in the various micro-channels. For instance, bone- or muscle-mimicking extravasation microenvironments were recapitulated in our model by co-culturing HUVECs with osteo-differentiated primary hBM-MSCs or C2C12 (myoblasts), respectively. Extravasation rates were significantly different in co-culture and mono-culture environments, suggesting key roles for organ-specific host stromal cells in modulating the factors controlling extravasation [37]. These micro devices can be very useful tools for studying organ specific microenvironments and metastatic "niches"; they thus complement *in vivo* studies by providing relatively higher throughput and kinetic cell migration data.

Lastly, the assay has the potential to be employed for the study of micrometastases formation via prolonged culture of microdevices (e.g. >24 hours). We have found that **μ**VNs can remain relatively stable between days 1–6 post tumor cell seeding, such that the morphological changes of the **μ**VNs (i.e. vessel diameter, interconnectivity) are small enough that the same vessel segments can be recognized and tracked as culture progresses[3]. In this time frame, various tumor cell lines can be seen proliferate extravascularly (Figure 8). However, more work is required to understand the roles of the stromal microenvironment such the presence of fibroblasts, pericytes, stromal macrophages and other cell types, which may be implicated in the proliferation and survival of tumor cells in the perivascular space. Taken together, this assay has the potential to further the understanding of the underlying mechanisms of extravasation, which is critical for the development of therapeutic opportunities at distinct stages of cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S. Chung for scientific discussions and A. Boussommier-Calleja for critical reading of the manuscript. We thank B. Bista, R. Hynes and J. Massague for sharing cell lines. M.B.C and R.D.K acknowledge support from the National Cancer Institute (CA202177). RD.K and J.A.W thank the support of the National Science Foundation (CBET-0939511).

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EDITORIAL SUMMARY

This protocol describes how to set up and use an in vitro model of the human microcirculation with the capability to recapitulate discrete steps of early metastatic seeding, including tumor cell arrest and transendothelial migration.

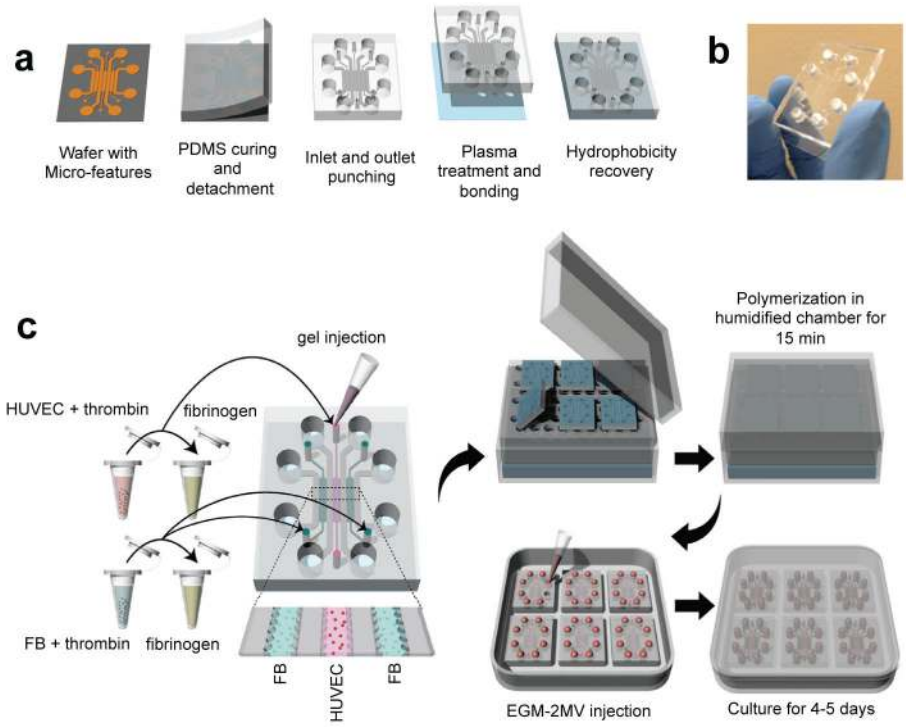

Figure 1. Schematics of device fabrication and cell-fibrin gel seeding protocol

(A) SU-8 master with micro features are fabricated and PDMS devices are made via standard soft lithography techniques. Inlet and outlet ports are cored via biopsy punches and PDMS slabs are bonded to glass coverslips as previously described[42]. **(B)** Photograph of finished microdevice prior to cell-fibrin seeding. **(C)** HUVECs and FBs are separately suspended in fibrin gels and injected into parallel gel regions. Upon gel polymerization, media channels are filled with EGM-2MV growth media and cultured for 4–5 days to allow for perfusable lumen formation.

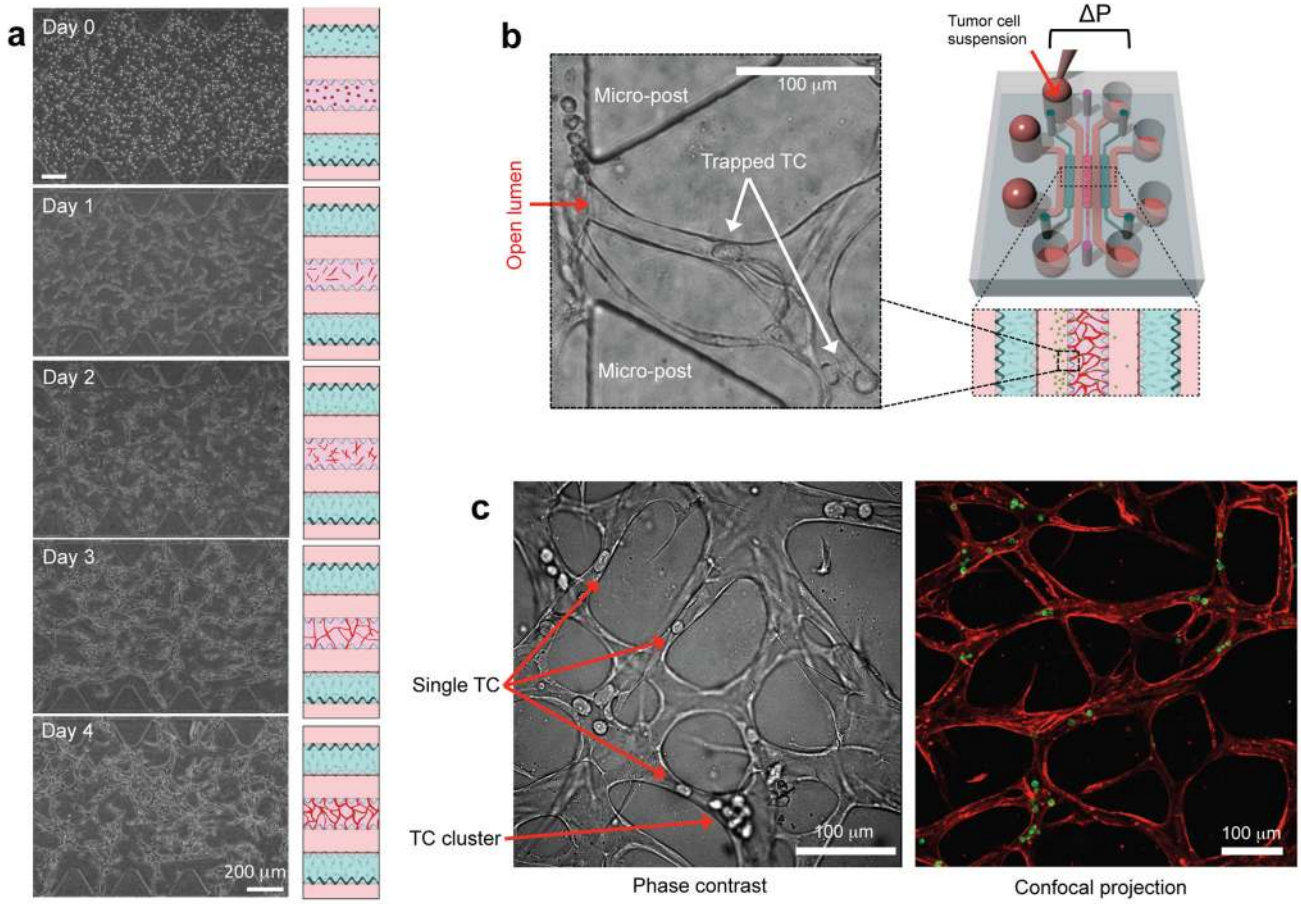

Figure 2. Microvessel bed formation and tumor cell perfusion procedure

(A) HUVECS elongate, form vacuoles and subsequently self-assemble into interconnected perfusable vascular networks over the course of 4–5 days. **(B)** Triangular PDMS micro posts spaced 100 μm apart allow cell-laden fibrin gels to be spatially contained, and also serve as a connection between the cell-laden hydrogels and media sources. Uniquely, lumens form openings that connect the microvascular network to the media ports, allowing tumor cells to be perfused via a hydrostatic pressure drop. **(C)** Arrested single or clusters of tumor cells can be clearly identified via phase contrast and fluorescence microscopy.

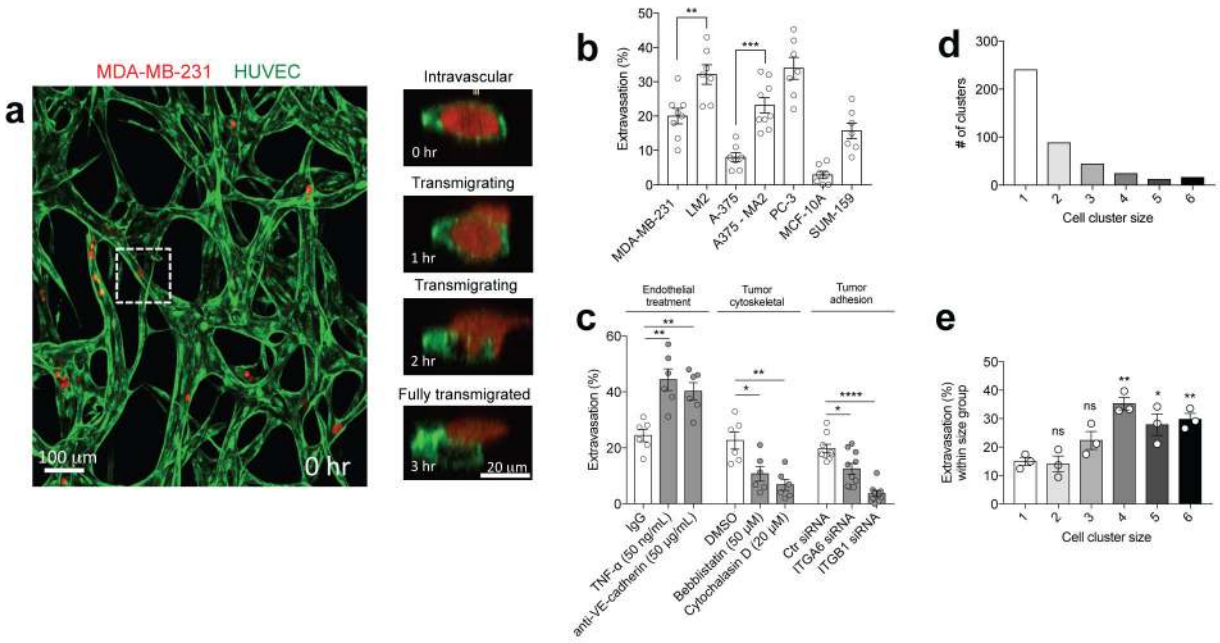

Figure 3. Scoring and quantification of extravasation efficiency

(A) Confocal projection of a representative region of tumor (red) perfused microvascular network (green) at 20X. Representative cross-sectional views of a single transmigrating tumor cell (in dotted white box) and example of extravasation scoring**. (B)** Extravasation efficiencies of a panel of tumor cell lines with varying extravasation potentials (*n=7* (A375, MCF10A, SUM-159, MDA-MB-231_LM2, PC-3), *n=8* (MDA-MB-231), *n=9* (A375-MA2)). **(C)** Effects of various treatments on extravasation efficiencies of MDA-MB-231 cells (*n=9* (Ctr siRNA and ITGB1 siRNA), *n=10* (ITGA6 siRNA), *n=6* (for all other conditions)). In (b–c), each point represents one device. **(D)** Frequency of tumor cluster sizes (1–6 cells) found arrested in microvascular networks (summed over 5 independent devices in 1 experiment)**. (E)** Extravasation efficiencies of tumor cells within each cluster size subpopulation (*n=3* per cluster size, where each data point represents the pooled value from 5 devices in 1 experiment). In all experiments, percentage extravasation was determined at an endpoint of 8 hrs post-injection, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, error bars = SEM.

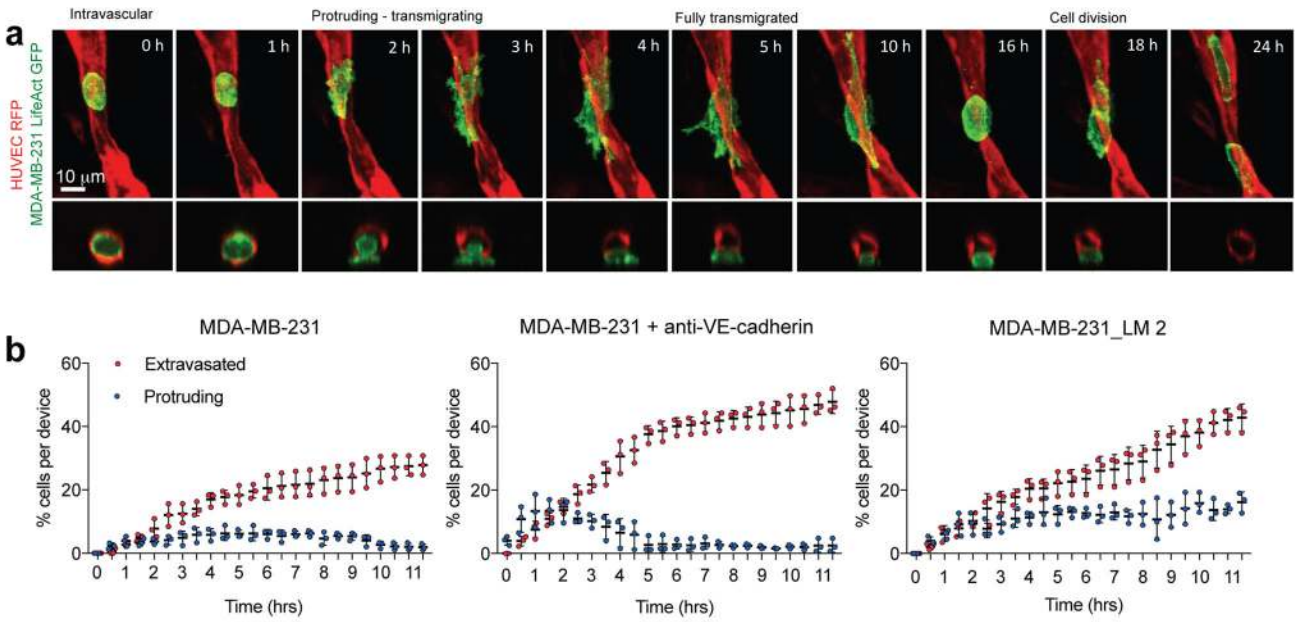

Figure 4. Tracking and quantification of extravasation kinetics

(A) Time lapse sequence of an arrested tumor cell extravasating from a lumen and subsequently undergoing cell division over 24 hrs. **(B)** Kinetics of tumor cell protrusion formation and complete transmigration can be quantified by following individual cells over time. Sample data of the percentage of tumor cells in either a "protruding" (mid-transmigrated) state (blue) or "fully extravasated" state (red) is presented for MDA-MB-231 with and without the presence of a VE-cadherin antibody, and MDA-MB-231_LM2 cells (highly metastatic variant of MDA-MB-231) (*n=3* per condition, each data point represents a single device, error bars = standard deviation).

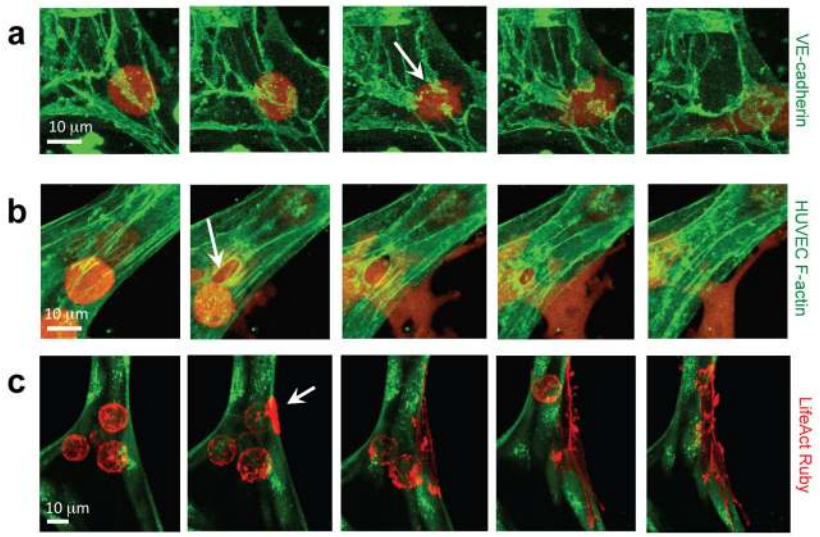

Figure 5. Resolving temporal-spatial organization of endothelial and tumor cell proteins on a single cell level

Time-lapse sequences depicting the dynamics of **(A)** HUVEC VE-cadherin (arrow depicts junctional disruption), **(B)** endothelial f-actin (arrow depicts HUVEC disruption) and **(C)** tumor f-actin (arrow depicts actin-rich tumor cell protrusion past endothelium) during the process of extravasation. Time interval between each image is 1 hour.

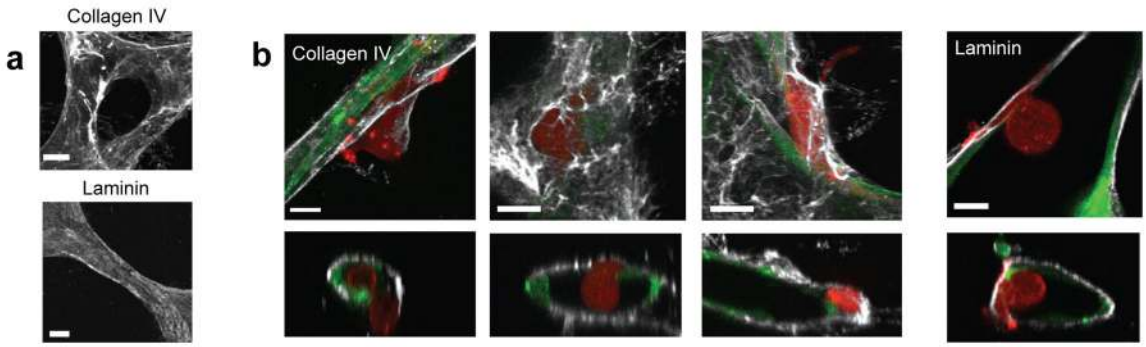

Figure 6. Beyond the endothelium: the basement membrane

(A) *In vitro* endothelial networks deposit collagen IV (white) and laminin (white) on the abluminal side, constituting part of the vascular basement membrane. (**B**) Fixed immunostaining at various time points for human collagen IV or laminin followed by confocal microscopy at 60X reveal the close association of extravasating tumor cells to the basement membrane. All scale bars are 10 μm.

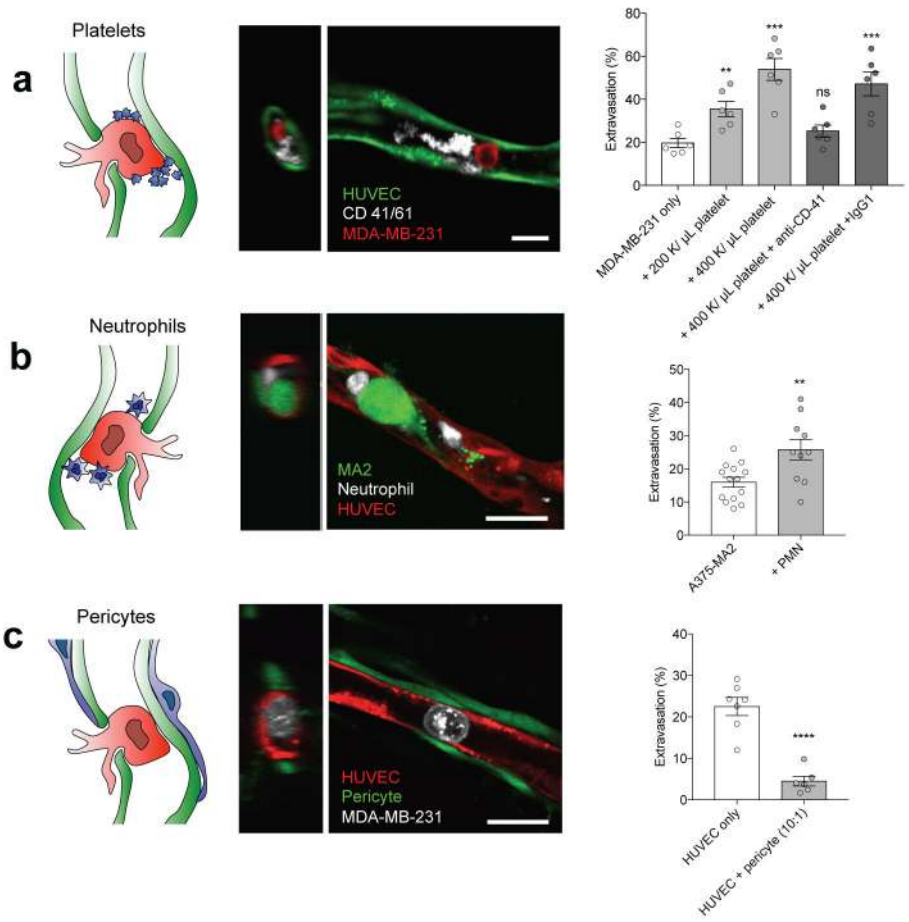

Figure 7. Addition of non-cancer host cells

(A) Simultaneous perfusion of platelets (immunostained with anti-CD 41/61, white) with MDA-MB-231 (red) results in tumor-platelet aggregates lodged in capillaries. Quantification of extravasation rates with or without addition of platelets at 8 hrs post-injection (*n=6* for all conditions). **(B)** Human neutrophils (PMNs) isolated from whole blood (stained with Cell Tracker far red, white) are co-perfused with A375-MA2 cells. Quantification of extravasation rates with or without the presence of neutrophils at 6 hrs post-injection (*n=12* (MA2 only), *n=9* (+PMN)). **(C)** Human umbilical vein pericytes are co-cultured with HUVECs to form pericyte-covered lumens. Extravasation rates of MDA-MB-231 (stained with Cell Tracker far red, white) from pericyte-covered vasculature are significantly altered at 8 hrs post-injection (*n=7* (HUVEC only), *n=6* (HUVEC + pericyte)). All scale bars = 20 µm. For all experiments, each point represents one device, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, error bars = SEM.

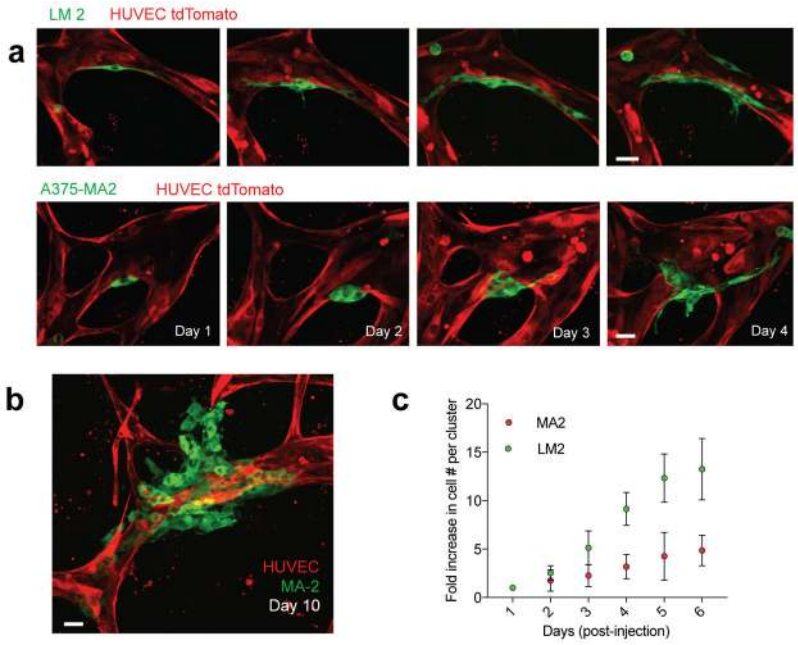

Figure 8. Tracking tumor cell proliferation post-extravasation

(A) Representative time-lapse images of single extravasated LM2 and A375-MA2 cells on Day 1 proliferating in close association to the abluminal side of the lumen over 4 days after transmigration. **(B)** Example of an extravascular A375-MA2 cluster 10 days after tumor cell perfusion. **(C)** Quantification of growth of extravasated tumor cells over 6 days (*n=15* clusters over 4 devices per condition, error bars = standard deviation). All scale bars = 20 μm.

TABLE 1

TROUBLESHOOTING

Step	Problem	Possible cause	Potential solution
7	Gel does not eject from pipette into microdevice	Fibrin has gelled before injection	Pipette only 4–5 times when mixing aliquots to prevent premature gelation Gels should be injected within 30 s of mixing fibrinogen with thrombin
		Pipette tip did not form a tight seal with injection port	Ensure that the pipette tip forms a tight seal with the injection port (i.e. an interference fit)
7–9	Cell distribution is not homogeneous between devices	Cell solution was not mixed well enough prior to combining with fibrinogen	Mix the12M/ml HUVEC solution well (with a P200 pipette) **each time** prior to withdrawing and mixing with a fibrinogen aliquot.
7–9	Gel does not form a flat gel-media interface at the inter-post regions	Gel is underfilled	Inject with slightly more pressure, particularly giving a slight increase in injection speed when reaching the end of the gel channel.
		Gel has started to evaporate	Make sure the device is kept in a sealed humid chamber immediately after seeding, and remains there until media injection.
7–9	Gel bursts into the media channel	Injection pressure is too high	Decrease the speed of injection into the gel region.
		Device was not well bonded	Ensure that microposts are well bonded to the glass coverslip and that dust or debris is not caught between the PDMS and glass preventing bonding.
		Defect in master wafer at posts	Check to see if the master wafer is missing any post features, which can break off.
10	Bubbles form at the interpost regions after media filling	Gel was likely underfilled or was not properly humidified. This results in concavity at the gel-media interface, which traps bubbles when media is filled. This will result in cell death within the hydrogel.	Ensure that gels are not “under-filled” by injecting slightly more gel before retracting the pipette tip. Make sure a seeded device is immediately placed into the humid chamber after seeding.
11	Microvascular networks do not form over 4–5 days	Cell seeding density may be too low	Ensure HUVECs are seeded at a final concentration of 6M/mL. Ensure that the cell suspension is mixed well prior to mixing with a single fibrinogen aliquot since cells can settle quickly in the re-suspension.
		Cells begin to die because media has evaporated from the reservoirs and/or was not refreshed every 24 hours	Media should completely fill the reservoir. The user may choose to fill extra media so it forms a “dome” over the reservoirs. This is helpful if the reservoir diameters are smaller than 4mm.
		Batch to batch variability of HUVECs or NHLF	Acquire a new batch of cells (i.e. different Lot. #), and choose pooled populations when possible
11	Microvascular networks regress/die over 4–5 days	Improper media exchange	Ensure that both reservoirs are emptied and that fresh media is allowed to flow at least twice across the media channel before topping off the reservoirs.
		Incorrect temperature and/or CO_2 levels	Devices must be kept at 37°C and 5% CO_2 at all times.
		Media has dried out	The amount of media in the channel alone is less than 10 μL, thus make sure reservoirs contain an additional >50 μL of media at all times.
13	Networks do not form openings to the media channel	Cell seeding density is not high enough	Ensure HUVEC seeding density is around 6M/mL (final concentration)

Step	Problem	Possible cause	Potential solution
		Lumens have not yet opened up to the media channel to connect to the media ports	Check to see whether the HUVECs at the interpost region have invaded slightly onto the glass surface in the media channel. This behavior usually indicates that lumens openings have formed. If this is not the case, continue to culture the devices for 1–2 more days to allow sufficient time for lumens to open up to the media channel.
19	Tumor cells are not flowing through the network	Not enough of a pressure drop across the gel region	Use a pressure drop of around 4 mm to achieve an adequate flow rate through the network. This can be achieved by pipetting ~38 **μ**L of cell suspension in both reservoirs on one side of the gel region (for a 3 mm diameter port). If lumen openings are particularly narrow, attach a gel loading pipette tip to a vacuum aspirator and gently aspirate the media channel downstream of the microvascular bed.
20B	Cells are fluorescent but are fuzzy or unclear	May be condensation on the objective lens or the glass surface of the device	Decrease the humidification level of the environmental chamber
		Underside of glass coverslip is dirty	Remove sample and clean the underside of the coverslip with a tissue wetted with 70% ethanol
		Poor sample placement	Check to see that the sample is placed correctly on the specimen holder
20B	Cells are dying over imaging time	Environmental chamber is not properly maintained	Check to see if there are leaks resulting in less than optimal CO_2 and temperature conditions
		Photo toxicity	Decrease the laser intensity or the frequency of imaging
28	Unspecific staining	Not enough washing after incubation of primary or secondary antibody	Make sure when washing that solutions are completely displaced down the media channel and removed. Let the wash buffer incubate for >10 minutes between washes to facilitate diffusion.
30	Reconstructed cross sectional view of lumens on image processing software is “fuzzy” or not well-defined enough for robust quantification	Step size setting during image acquisition was too large	See SI Table 1 for sample microscope settings used to acquire typical data.