Chapter 15

High-Resolution Fluorescence Microscopy to Study Transendothelial Migration

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Abstract

Immune system functions rely heavily on the ability of immune cells (i.e., blood leukocyte) to traffic throughout the body as they conduct immune surveillance and respond to pathogens. A monolayer of vascular endothelial cells (i.e., the "endothelium") provides a critical, selectively permeable barrier between two principal compartments of the body: the blood circulation and the tissue. Thus, knowledge of the basic mechanisms by which leukocytes migrate across the endothelium (i.e., undergo "transendothelial migration"; TEM) is critical for understanding immune system function. Cultured endothelial cell monolayers, used in combination with isolated blood leukocytes, provide a basis for highly useful in vitro models for study of TEM. When used in conjunction with high spatial and temporal resolution imaging approaches, such models have begun to reveal complex and dynamic cell behaviors in leukocytes and endothelial cells that ultimately determine TEM efficiency. In this chapter, we provide protocols for setting up a basic in vitro TEM system and for conducting high-resolution dynamic live-cell and three-dimensional fixed-cell imaging of TEM.

Key words: Fluorescence, Microscopy, Leukocyte, Endothelium, Diapedesis, Transmigration, GFP, Imaging, Antibody, Lymphocyte

1. Introduction

In order to satisfy their roles in immune surveillance and pathogen elimination, blood leukocytes (e.g., lymphocytes, monocytes, dendritic cells, and neutrophils) must continuously traffic throughout the body (1). Such trafficking can be broken into two major phases: (1) movement within the vascular and lymphatic circulation and (2) migration within tissues. The vascular and lymphatic circulatory systems are lined by an endothelial cell monolayer (the "endothelium") that grows on an abluminal layer

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of extracellular matrix (the basement membrane) and forms organized intercellular adherens, tight and gap junctions (2–4). In this way, the endothelium functions as a (selectively permeable) barrier between the circulation and the tissues. Thus, the movement of leukocytes into (intravasation) or out of (extravasation) the circulation requires the explicit crossing of the endothelial barrier (i.e., "diapedesis" or "transendothelial migration"; TEM). Thus, TEM is a critical and rate-limiting component of leukocyte trafficking and, in turn, of both normal immune system function and immune-mediated/inflammatory pathology. Understanding the regulatory mechanism of this process is, therefore, of great biomedical significance (1, 5).

TEM is important in diverse settings in vivo with a wide range of immediate purposes (1). For example, all leukocytes begin their life cycle by intravasation from the bone marrow, T and B lymphocytes constitutively enter and exit various lymphoid organs (including the thymus, lymph nodes, Peyer's patches, spleen, and tonsils) during maturation and immune surveillance processes, and diverse leukocytes (e.g., granulocytes, monocytes, memory/effector lymphocyte subsets, and NK cells) traffic into inflamed peripheral tissues in response to infection or injury.

Whereas TEM during intravasation has just begun to be studied (6), TEM during extravasation has been extensively characterized, revealing a discrete "five-step" cascade (Fig. 1). Extravasation begins with the accumulation of circulating leukocytes

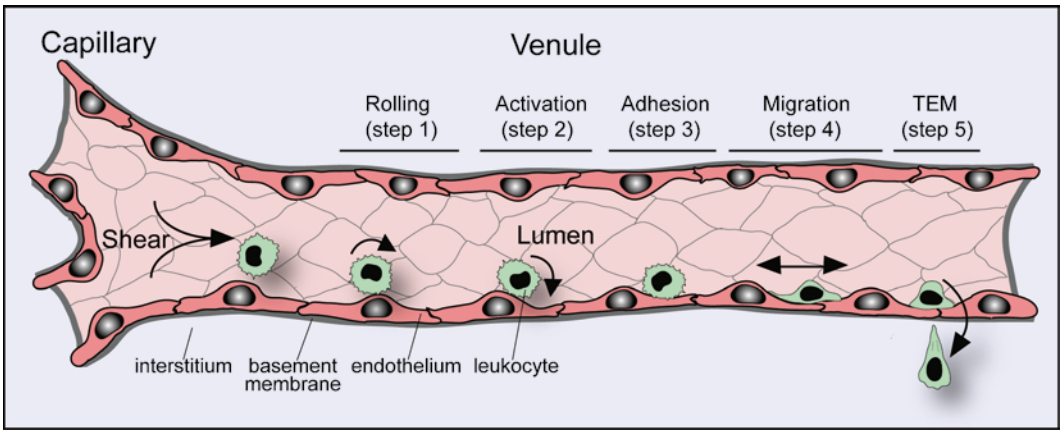

Fig. 1. The "Five-Step" Extravasation Cascade. Extravasation of leukocytes (*green*) across vascular structures (exemplified here as a postcapillary venule; *pink*) is a multistep process. In Step 1, leukocytes undergo transient rolling-type interactions with the endothelium that are mediated predominantly by selectins. This facilitates chemokine-dependent activation (Step 2) and firm adhesion or "arrest" (Step 3), which is mediated by the binding of leukocyte integrins (e.g., LFA-1, Mac1, and VLA4) to endothelial cell-adhesion molecules (e.g., ICAM-1, ICAM-2, and VCAM-1). Subsequently, leukocytes spread, polarize, and migrate laterally over the surface of the endothelium, probing for a site to penetrate the endothelium (Step 4). Finally, leukocytes cross the endothelial barrier (i.e., undergo transendothelial migration (TEM) or "diapedesis"; Step 5), either para- or trans-cellularly, and enter the interstitium.

on the luminal surface of the endothelium through a "classic" three-step adhesion and activation cascade (7–9). Initially, leukocytes undergo transient rolling interactions mediated by the selectin family of adhesion molecules (Step 1), which facilitates sensing of, and signaling responses to, chemokines presented on the surface of the endothelium (Step 2). This in turn triggers high-affinity interaction of lymphocyte integrin receptors (e.g., LFA-1, Mac1, and VLA-4) with their endothelial ligands (e.g., ICAM-1, ICAM-2, and VCAM-1), resulting in firm adhesion of leukocytes (Step 3) (10, 11). Subsequently, leukocytes undergo cytoskeleton-mediated spreading, polarization, and integrin-mediated lateral migration on the luminal surface of the endothelium (Step 4). This process confers leukocytes with the ability to search out sites permissive for endothelial barrier penetration (12, 13). Finally, leukocytes formally breach and transmigrate across the endothelium (Step 5), a process referred to specifically as "transendothelial migration" or "diapedesis" (though these terms are also often employed more loosely to refer to the entire five-step cascade).

Until recently, only one basic pathway, the "para-cellular" route, for diapedesis was widely recognized. Para-cellular diapedesis involves cooperative efforts on the part of both the leukocyte and the endothelium to disassemble the inter-endothelial junctions locally in order to form a para-cellular gap that will allow leukocyte transmigration (5, 14–17). In fact, however, their is a large body of literature (including some of the very first studies to investigate the route of diapedesis directly (18–20) and nearly 50 subsequent studies recently reviewed in detail (21)) that demonstrates the coexistence in vivo of the para-cellular route along with a second pathway termed that trans-cellular route, whereby leukocytes pass directly through individual endothelial cells via the formation of a trans-cellular pore. As a consequence of recent studies characterizing trans-cellular diapedesis in vitro for the first time, the mechanisms for this pathway have begun to be elucidated and its physiologic relevance more broadly appreciated (22). The relative roles, in distinct in vivo settings, of the para- and trans-cellular pathways remain important open questions.

Classic adhesion and TEM (e.g., transwell/Boyden chamber) assays coupled to various function perturbation approaches have been instrumental in identifying adhesion, chemoattractant, and signaling molecules that are important for TEM. However, a complete understanding of the cell biological mechanisms and orchestrated dynamics of TEM requires imaging approaches.

Dynamic live-cell imaging has been particularly instrumental in elucidating the dynamics of leukocyte lateral migration and initiation of diapedesis events. For example, recent studies of using fluorescent membrane markers in endothelial cells have uncovered a novel form for invadosome-like protrusive structures (23–25) formed dynamically by leukocytes that serve a role in

migratory pathfinding by effectively probing the endothelial surface mechanically (26–29).

High-resolution three-dimensional (3D) confocal imaging of fixed samples has been critical for defining adhesion receptor distribution within, and detailed topology of, leukocyte–endothelial interactions. For example, this approach was essential for the discovery and characterization of novel ICAM-1-, VCAM-1-, and actin-enriched endothelial structures termed "transmigratory cups" or "docking structures" (30–33). Studies in diverse in vitro settings that have included a range of leukocyte types, endothelial models (including HUVECs, as well as human dermal, lung, and brain microvascular endothelium and lymphatic endothelium), and activation stimuli have demonstrated that transmigratory cups play critical roles in adhesion and directional guidance during diapedesis (34–43). A key feature of these structures is their 3D architecture formed by vertical microvilli-like projections that surround adherent and transmigrating leukocytes (31–33).

It remains clear that many additional mechanistically important features of leukocyte–endothelial interactions remain to be elucidated in order to refine our understanding of TEM. The following protocols describe the details for setting up one specific in vitro model system (designed to recapitulate effector lymphocyte homing to sites of inflammation in the microvasculature) and using this system to conduct both dynamic wide-field and fixed-sample confocal imaging of TEM. As pointed out in the extensive notes provided under Subheading 4, these basic approaches can be readily modified to incorporate diverse endothelium, leukocytes, and migratory stimuli, effectively allowing modeling of wide-ranging leukocyte–endothelial interactions.

2. Materials

2.1. Preparation of Endothelium

1. Phosphate-buffered saline (PBS).
2. Hanks balanced salt solution (HBSS).
3. EGM-2 MV, endothelial cell culture medium: Prepare complete EGM-2 MV media (Invitrogen) by thawing and adding all components of the "Bullet kit" (see Note 1).
4. Fibronectin (FN) stock solution (50×): Prepare a 1 mg/ml stock solution by dissolving 5 mg of sterile lyophilized FN (Invitrogen) in 5 ml sterile PBS (adjusted to pH 6.5 with ~50 ml 1 M sodium citrate, pH 3.0). Aliquot 100 ml to 15-ml conical tubes and store at −80°C.
5. Trypsin solution.

6. Trypsin inhibitor.
7. Falcon (Fisher, Hampton, NH) T25, T75, 6-well and 24-well cell culture plates (see Note 2).
8. 12-mm No. 1 thickness circular coverslips.
9. Bioptechs Delta-T cell-culture dishes (Bioptechs, Butler, PA).
10. Amaxa HMVEC-L Nucleofector kit and electroporator (Invitrogen).

2.2. Preparation of Effector Lymphocytes

1. Citrate solution: Dissolve 25 g sodium citrate and 8 g citric acid in 500 ml PBS. Sterile filter (0.2 μm) and store at room temperature for not more than 6 months.
2. Dextran solution: Dissolve 20 g dextran-500 into 500 ml MilliQ-H_2O (6% w/v). Sterile filter (0.2 μm) and store at room temperature for not more than 6 months.
3. Histopaque 1077.
4. Gelatin solution: Prepare fresh by dissolving 1 g gelatin into 50 ml PBS. Incubate at 37°C for ~30 min with occasional vortexing. Sterile filter (0.2 μm).
5. PBS with Ca/Mg: To 50 ml of PBS, add 50 ml of 1 M $CaCl_2$ and 50 ml of 1 M $MgCl_2$. Use a 60-ml syringe with 0.2-μm filter to sterilize.
6. RPMI base media: RPMI, 10% FCS, 1% glutamine, and 1% penicillin/streptomycin (see Note 1).
7. RPMI-phytohemagglutin (PHA) media: RPMI base media with 1 μg/ml PHA (see Note 1).
8. RPMI-interleukin-2 (IL-2) media: RPMI base media with 10 ng/ml IL-2 (R & D Systems, Minneapolis, MN) (see Note 1).

2.3. Live-Sample Dynamic Imaging of TEM

1. Live-cell imaging buffer (Buffer-A): Prepare fresh phenol red-free HBSS (see Note 3), supplemented with 20 mM HEPES, pH 7.4, and 0.5% (v/v) human serum albumin (HSA). Pre-warm to 37°C.
2. Fixative solution (3.7% formaldehyde): Combine 2.5 ml of 37% formaldehyde solution with 22.5 ml PBS (see Notes 4 and 5).
3. Tumor necrosis factor-α (TNF-α) (Invitrogen): Prepare a 100 μg/ml stock solution by dissolving 10 μg of TNF-α in 100 μl sterile filtered 5 mM Tris–HCl, pH 8.0. Aliquot single use volumes (~2–4 μl) in sterile PCR tubes and store at −80°C.
4. Disposable 5-ml transfer pipettes.
5. Delta-T live-cell imaging culture dishes (Bioptechs).

2.4. Immunofluorescence Staining and Confocal Imaging of TEM

1. Fixative solution (3.7% formaldehyde): Combine 2.5 ml of 37% formaldehyde solution with 22.5 ml PBS (see Notes 4 and 5).
2. Block solution (5% w/v NFDM): Dissolve 2.5 g NFDM in 50 ml PBS (see Notes 4 and 5).
3. Permeabilization solution: Prepare a 10% v/v stock solution of Triton X-100 in water to be stored at 4°C (this can be kept for ~1 year). Dilute 50 µl of 10% Tx-100 into 10 ml of Block solution (0.05% Tx-100 final) (see Note 6).
4. Primary and secondary antibodies (see Notes 7–9): See Subheading 3.4.2 for details on preparing Antibody Stain Solutions.

3. Methods

The methods outlined below describe (1) the preparation of primary human microvascular endothelial cell monolayers; (2) isolation and culture of primary human effector lymphocytes; (3) dynamic live-cell imaging of lymphocyte TEM; and (4) immunofluorescence staining and high-resolution, fixed-cell confocal imaging of lymphocyte TEM. The experimental system used throughout this protocol is designed to model the process of effector lymphocyte homing to microvascular sites of peripheral tissue inflammation (see Note 10).

3.1. Preparation of Endothelium

The following protocols are designed as a guideline for preparation of confluent primary endothelial monolayers to serve as basic in vitro models of the vasculature. The procedures described herein focus on primary human lung (HLMVECs) and dermal (HDMVECs) microvascular endothelial cells (see Note 11), with emphasis on generating optimal endothelial cell health and recapitulation of physiologic vascular phenotype and function.

3.1.1. Sterilizing Coverslips

For some of the protocols described below, endothelial cells will be grown on glass-coated coverslips placed in cell culture dishes. In a cell culture hood, use forceps to place a single 12-mm circular coverslip into each of the desired number of wells of a 24-well cell culture plate. With the cover off, place each plate in the back of the hood and sterilize by leaving the UV light on for 1–2 h (see Notes 12 and 13).

3.1.2. Fibronectin Coating of Plates

1. Thaw a fresh 100 µl aliquot of fibronectin (FN) stock and dilute to 1× (20 µg/ml) with 5 ml PBS (see Note 14).
2. Add enough 1× FN to culture flasks or dishes to cover surfaces completely (see Note 15). Use the following recommendations:

Culture vessel	Minimum volume (ml)
T75 flask	4
100-mm dish	3
T25 flask	2
6-well plate	1
60-mm dish	1
Bioptechs Delta-T4 dish	0
24-well plate w/coverglass	0.3–0.5

3. Store the dish in culture hood (without UV lamp on) for 1–2 h at room temperature or overnight at 4°C.
4. Aspirate FN immediately before adding medium. It is not necessary to wash plates to remove residual FN.

3.1.3. Starting HLMVECs/HDMVECs

1. Coat a T25 flask with FN, as described in Subheading 3.1.2.
2. Remove FN and add 5 ml complete medium.
3. Pre-incubate in 37°C cell culture incubator.
4. Thaw a vial of frozen HLMVECs or HDMVECs in a 37°C water bath with occasional gentle agitation for ~2–3 min. Proceed until a barely visible piece of ice remains. Immediately transfer cells to T25 flask containing pre-warmed media. Gently swirl and place in incubator at 37°C.
5. Change the media after ~4–6 h. Continue to change media approximately every 48 h (or when media becomes slightly yellow) until the cells reach ~90–95% confluency.

3.1.4. General Splitting and Expansion of Endothelial Cells

1. Grow cells to ~90–95% confluency. This may take 2–5 days. If media becomes yellow, it should be replaced with fresh, pre-warmed complete media as often as necessary until confluency is reached.
2. For splitting, remove media and rinse with HBSS.
3. Remove HBSS and replace with minimum volume of fresh 1× trypsin (0.5 ml for T25 or 1.5 ml for T75). Gently swirl to cover all surfaces with trypsin. Incubate at room temperature for ~3–6 min.
4. Monitor the detachment of the cells from the plate using a low-power light microscope. An occasional gentle tap of the side of the flask can be applied to help determine the extent of detachment (see Note 16).
5. When majority of cells appear rounded or detached, add 1 volume (i.e., equal to the trypsin volume added) of 1× trypsin inhibitor and gently triturate trypsin/trypsin inhibitor solution over the surfaces of the flask to detach all cells.

6. Count endothelial cells with a hemocytometer: At ~90% confluency, T25 flask will yield ~1–1.5 and a T75 flask ~3–4.5 million cells, respectively.
7. Adjust concentration to 0.5 million cells/ml by adding pre-warmed complete EGM-2 MV media.
8. Transfer aliquots of cells to the appropriate FN-coated dishes. For general maintenance/expansion of endothelial cultures, cells should be seeded at ~0.5 million cells (1 ml) for T25 and ~1.5 (3 ml) million cells for T75, and grown in 5 or 10 ml total EGM-2 MV media, respectively (see Note 17).
9. Gently swirl and place the plates in the incubator.
10. Change the media within 6–12 h of plating. Media should be changed approximately every 48 h or as needed, according to media color. Repeat this process approximately every 2–4 days until the cells reach 90–95% confluency (see Note 18).

3.1.5. Preparative Plating of Endothelial Cells for Imaging Experiments

1. FN coat the requisite number of Delta-T dishes (for live-cell imaging as described in Subheading 3.3) or 24 wells containing coverglass (for fixed-cell confocal imaging, as described in Subheading 3.4) as described above (Subheading 3.1.2).
2. Starting with endothelial maintenance flasks that are ~90–95% confluent (and have been passaged less than 6 times) trypsinize, count, and adjust the endothelial cell density to 0.5 million/ml, as in Subheading 3.1.4.
3. To 24 wells, add ~50–100 µl of 0.5 million cells/ml (i.e., 25,000–50,000) to 0.5 ml media. To Bioptechs Delta-T dishes, add ~200–300 µl of 0.5 million cell/ml to 1 ml media.
4. Place in cell culture incubator (see Note 19).
5. Change media within 4–6 h and again at 12–16 h.

3.1.6. Endothelial Cell Transfection

For many experiments (especially live-cell fluorescence imaging), expression of fluorescent protein-tagged probes is extremely useful, if not required. Endothelial cells are highly resistant to transient transfection by cationic lipid-based approaches. Amaxa Nucleofection, using an Amaxa Electroporator and HMVEC-L Nucleofector kit, however, allows for highly efficient (typically, 30–60%) transfection of HLMVECs and HDMVECs with many fluorescent probes (see Note 20). The following is protocol modified from those provided by the manufacturer (Invitrogen/Lonza).

1. Prepare T25 or T75 flasks (as needed) of HLMVECs or HDMVECs to a final density of 90–95% confluency (see Notes 21–23).
2. Prepare Delta-T plates and/or 24-well dishes containing coverglass by FN coating as described above (Subheading 3.1.2).

3. Add 1 or 0.5 ml of complete EGM-2 MV culture media to Delta-T or 24 wells, respectively, and pre-incubate/equilibrate plates in a humidified 37°C/5% CO_2 incubator.
4. Harvest and count endothelial cells, as described in Subheading 3.1.4.
5. Centrifuge the required number of cells (0.5 millions cells per sample) at 200 × *g* for 5 min at room temperature.
6. Resuspend the cell pellet carefully in 100 µl room temperature Nucleofector solution per sample.
7. Combine 100 µl of cell suspension with 1–5 µg of DNA. Transfer the cell/DNA suspension into certified cuvette; the sample must cover the bottom of the cuvette without air bubbles.
8. Close the cuvette with the cap. Insert the cuvette with cell/DNA suspension into the Nucleofector cuvette holder of the Amaxa electroporator and apply electroporation program S-005.
9. Take the cuvette out of the holder once the program is finished.
10. Add ~500 µl of the pre-equilibrated culture media to the cuvette and gently remove the cell suspension from the cuvette using the plastic transfer pipettes provided in the Nucleofection kit.
11. For experiments using Delta-T dishes, partition the cell suspension from one reaction equally between two dishes containing pre-warmed media. For experiments using 24 wells, partition one reaction equally into four separate wells (see Note 24).
12. Incubate the cells in a humidified 37°C/5% CO_2 incubator and change media 4–6 h, and again at 12–16 h post-transfection.

3.2. Preparation of Effector Lymphocytes

The following protocols are designed as a guideline for isolation and culture of primary human Th1 effector-like lymphocytes (see Notes 25 and 26) for investigation of the cell biological mechanisms of TEM, and are optimized for maximal retention of viability and physiologic characteristics.

3.2.1. Acquiring Blood Sample

1. Place a fresh sheet of bench paper on the benchtop. Gather an elastic tourniquet, 21-gauge butterfly needle/tubing, Band-Aids, and ethanol wipes on bench paper.
2. Prepare blood donor form and complete informed consent process (see Note 27).
3. Sterilely transfer 10 ml of dextran and 7 ml of citrate solution to a sterile 10-cm tissue culture dish in the hood. Use the dish lid to prop the dish at a slight angle.

4. Uncap a fresh sterile 60-ml syringe and draw up the dextran/citrate mixture. Invert the syringe, expel excess air, and recap the syringe until blood drawing.
5. A trained phlebotomist should attach a butterfly needle/tubing to the dextran/citrate-containing syringe and commence blood draw via venipuncture (see Note 28). Continue drawing until the syringe is filled.
6. Remove the needle from the subject and *using extreme caution*, replace cap on the needle. Then gently invert the syringe 3–4 times to mix the blood with the dextran/citrate.
7. Affix the inverted syringe to the surface of the hood with tape and let stand for 30 min (Fig. 2). The red blood cells (RBCs) will sediment into a dark red lower phase, and the serum,

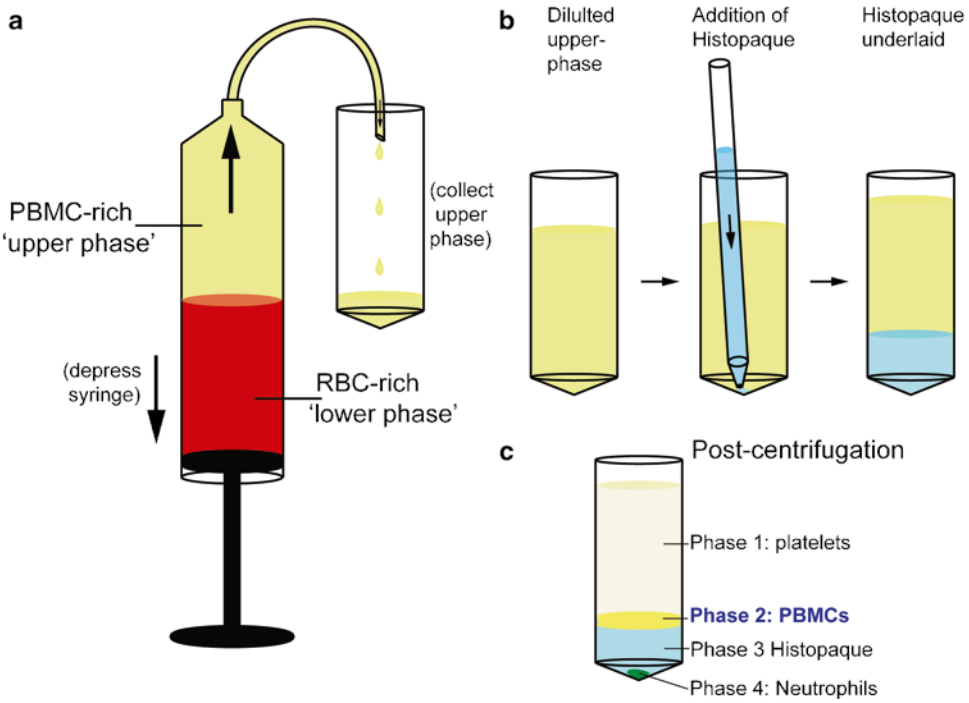

Fig. 2. Extracting the PBMC Phase of Whole Blood. (**a**) Schematic of a syringe containing whole blood mixed with dextran and citrate affixed to a surface inverted. Following a 30–60-min incubation at room temperature, the sample will spontaneously separate into an upper PBMC-rich phase and a lower RBC-rich phase. Collection of the upper phase is accomplished by cutting the tubing connecting the syringe to the butterfly needle and placing the free end of the tube into a 50-ml collection tube, followed by slow depression of the syringe. (**b**) After the "upper phase" is diluted with PBS, Histopaque is underlaid by slowly pipetting into the bottom of the tube (see *center panel*). After addition, a sharp interface between the "upper phase" and the Histopaque should be evident (see *right panel*). (**c**) Following centrifugation, the sample will have separated into four phases: (1) a large (25–30 ml) milky upper phase, composed largely of blood platelets; (2) a dense white cloud of PBMCs termed the "buffy coat" concentrated at the original blood sample–Histopaque interface; (3) an underlying ~7-ml phase of relatively clear Histopaque; and (4) a pellet containing neutrophils and residual RBCs. Collection of PBMC is accomplished by careful aspiration of phase 1 with a pipette, followed by collection of phase 2 without perturbation of phase 4.

white blood cells (lymphocytes, monocytes, and neutrophils), and platelets will form a pale yellow upper phase.

3.2.2. Isolation of Peripheral Blood Mononuclear Cells

1. Sterilely transfer 20 ml of Histopaque-1077 to a 50-ml test tube, let stand at room temperature, and set aside.
2. In the hood, drench the tubing connecting the butterfly needle and syringe, as well as a pair of scissors (open and close scissors during this process to ensure all cutting surfaces are sterilized).
3. Carefully cut the tubing about 3 in. from the tip of the syringe and place the newly cut end of the tubing in a sterile 50-ml conical test tube (Fig. 2a). Transfer the free needle to an appropriate "sharps disposal" waste container immediately.
4. Collect the yellow upper phase of the blood sample by slowly depressing the syringe causing this phase to be expelled into the 50-ml conical test tube (Fig. 2a). Proceed at a modest pace; it should take ~1–2 min to collect the majority of the upper phase (30–35 ml). Stop collection when ~1–2 ml of this phase remains.
5. Briefly set aside the collected upper phase.
6. Detach the syringe from the surface of the hood and expel the RBCs into a 200-ml beaker containing 50–100 ml bleach. Let stand for 5 min. Dispose in sink. Rinse excessively with running water.
7. Split the volume of the upper blood phase equally into two sterile 50-ml conical tubes (usually ~15–17 ml each).
8. Add sterile PBS to each tube to bring the volume to ~38–40 ml. Cap each and invert several times.
9. Using a 10-ml pipette gently underlay 10 ml of room temperature Histopaque-1077 in each of the two test tubes (Fig. 2b). A sharp interface should form between the clear Histopaque on the bottom and the yellow upper phase of the blood on top.
10. Transfer to a benchtop swinging bucket centrifuge. Spin at $600 \times g$ for 30 min at room temperature with no brake applied during deceleration.
11. Collect the tubes and place in a cell culture hood. The samples should now contain four phases: (1) a large (25–30 ml) milky upper phase, composed largely of blood platelets; (2) a dense white cloud of peripheral blood mononuclear cells (PBMCs) termed the "buffy coat" concentrated at the original blood sample–Histopaque interface; (3) an underlying ~7-ml phase of relatively clear Histopaque; and (4) a pellet containing neutrophils and residual RBCs (Fig. 2c).

12. Sterilely remove and discard the upper phase into a waste beaker containing bleach.
13. Next, without touching the underlying pellet, remove the PBMCs/buffy coats (in ~10 ml volume each for each tube) and transfer each one to a separate sterile 50-ml tube.
14. Add bleach to the remaining material and discard.
15. To the extracted buffy coat, add ~40 ml of sterile PBS. Cap and invert several times.
16. Centrifuge at 280 × *g* in a swinging bucket table-top centrifuge for 10 min at room temperature with brake on low.
17. Discard the platelet-rich supernatants and resuspend the PBMCs-rich pellets in 5 ml sterile PBS via trituration.
18. Add 45 ml PBS to each tube and centrifuge at 180 × *g* for 10 min with brake on low.
19. Discard the supernatant and resuspend PBMC pellets from both tubes in a total of 12 ml of PBS containing 1 mM calcium and 1 mM magnesium.

3.2.3. Preparation of Effector Lymphocytes

1. To each of three T75 flasks, aliquot ~10–15 ml of sterile gelatin (Subheading 2.2, step 5), swirl to coat bottom surface, and incubate at 37°C for 30 min.
2. Remove gelatin and rinse each flask once with 10 ml sterile PBS.
3. Aliquot 4 ml of PBMCs (Subheading 3.2.2) to each of the three flasks. Incubate at 37°C for 30–60 min to allow adhesion of monocytes to the gelatin-coated surface.
4. Pre-warm 15 ml of RPMI-PHA media in a separate T75 flask.
5. Upright the gelatin-coated, PBMC-containing T75 flasks and gently remove the monocyte-depleted lymphocytes contained in the liquid at the bottom.
6. Pool the lymphocyte from all three flasks in a 15-ml conical test tube and centrifuge at 230 × *g* for 3 min.
7. Resuspend pellet in 15 ml of pre-warmed RPMI-PHA media, transfer to a T75 flask, and incubate at 37°C overnight.
8. After 24 h of growth in RPMI-PHA, add 15 ml of additional pre-warmed RPMI-PHA media and transfer lymphocytes to T150 flask.
9. After 72 h of growth in PHA, centrifuge lymphocytes (230 × *g*, 3 min) in two 15-ml test tubes.
10. Remove as much supernatant as possible without perturbing the cell pellet.

11. Resuspend each pellet in 15 ml of pre-warmed RPMI-IL-2 media (see Note 29), transfer to T75 flasks, and place in cell culture incubator.
12. Expand cells by adding 15 ml of fresh RPMI-IL-2 media within ~48 h.
13. Continue to expand/split cells in RPMI-IL-2 every 24–48 h as needed (based on media color) thereafter (see Note 30). The resulting effector lymphocytes can be used for TEM experiments after 3 days in IL-2 and can be continuously cultured and used for further experiments for an additional ~5 days thereafter.

3.3. Live-Sample Dynamic Imaging of TEM

The following protocols are designed as a guideline for setting up and performing live-cell imaging studies of leukocyte diapedesis that will provide a high degree of topological detail. Specifically, these methods are designed for high-resolution, live-cell dynamic imaging using an inverted wide-field light microscope. In this case, the protocol is written for an AxiovertS200 microscope outfitted with a 63×, 1.4 NA PlanApo objective coupled to a CCD camera, automated fluorescence filter cube turret, and Axiovision time-lapse image acquisition software (see Note 31). Selected fluorescent probes (to be introduced into endothelium) will include actin-GFP and membrane-RFP (mRFP), which will be used in concert with differential interference contrast (DIC) bright-field imaging (see Notes 32–34).

3.3.1. Preparing Endothelium

1. Co-transfect primary HLMVECs with actin-GFP and membrane-RFP via Amaxa Nucleofection and plate onto Delta-T live-cell imaging culture plates, as described in Subheading 3.1.6 (see Notes 32–34).
2. Change media within 4 h and again between 12 and 16 h post-transfection (see Note 35). Imaging experiments should be performed 48–72 h post-transfection.
3. At 4–12 h prior to the imaging, replace media with fresh media containing 50 ng/ml TNF-α-transfected endothelium (see Notes 36 and 37). Once TNF-α is added, endothelial cell proliferation largely ceases and, as endothelial cells become activated, stress fibers and contractility develop that appose endothelial monolayer integrity. One must be sure that at the time of initiating imaging experiments that the monolayers are intact with well-formed intercellular junctions free of gaps (see Note 38).

3.3.2. Basic Setup

1. To initiate an imaging experiment, prepare Buffer A and fixative solution (Subheading 2.3) and turn on/set up the imaging system.

2. Thoroughly clean the imaging objective with ethanol and objective lens paper.
3. Only after all buffers are prepared and imaging equipment/acquisition software setup are confirmed, take a sample of cultured lymphocytes and determine the density by counting with a hemocytometer (see Note 39).
4. Remove a Delta-T plate containing transfected and activated HLMVECs from cell culture incubator. Using a disposable transfer pipette, rapidly remove media and rinse once by adding ~1 ml of pre-warmed Buffer A. Aspirate Buffer A and then add 0.5 ml of Buffer A.
5. Prepare the underside of the Delta-T dish by successive cleaning with water and then with ethanol-soaked Kimwipes (Fisher).
6. Add fresh objective oil and mount Delta-T dish on the heating stage adaptor and immediately turn on to equilibrate to 37°C (will take ~2–3 min) (Fig. 3a).

3.3.3. Live-Cell Imaging

1. Bring the objective into contact and use bright-field imaging to find the focal plane.
2. Switch to fluorescence imaging and identify a field of interest using the ocular lenses (see Note 40).
3. Identify fields in which brightly fluorescent-positive transfected endothelial cells are present that appear healthy with well-formed intercellular junctions.
4. Switch imaging to CCD camera and adjust acquisition parameters (e.g., exposure time, detector gain, and binning). Be sure that mean fluorescence signal intensity in each channel falls between 25 and 75% of the dynamic range of the detector (see Note 41).
5. Remove a volume of lymphocytes from culture equivalent to 0.5–1 million cells. Transfer to a 15-ml conical tube and centrifuge at 200 × *g* for 3 min. Aspirate media and gently but thoroughly resuspend lymphocytes in 20–40 ml of Buffer A.
6. Begin live-cell imaging of endothelial cells by successive capture of sets of DIC, green fluorescence, and red fluorescence images with an acquisition interval of ~5–30 s per set (see Note 42).
7. Acquire 30–60 s of baseline imaging of endothelium.
8. During acquisition, apply ~5 ml of concentrated lymphocytes to the center of Delta-T imaging field by inserting the tip of a small volume (P-5 or P-20) pipette into the media close to the center of the objective and ejecting slowly.
9. Image for ~30 s to allow for cells to settle into the imaging field. For an average imaging system using a 63× objective, ~10–20

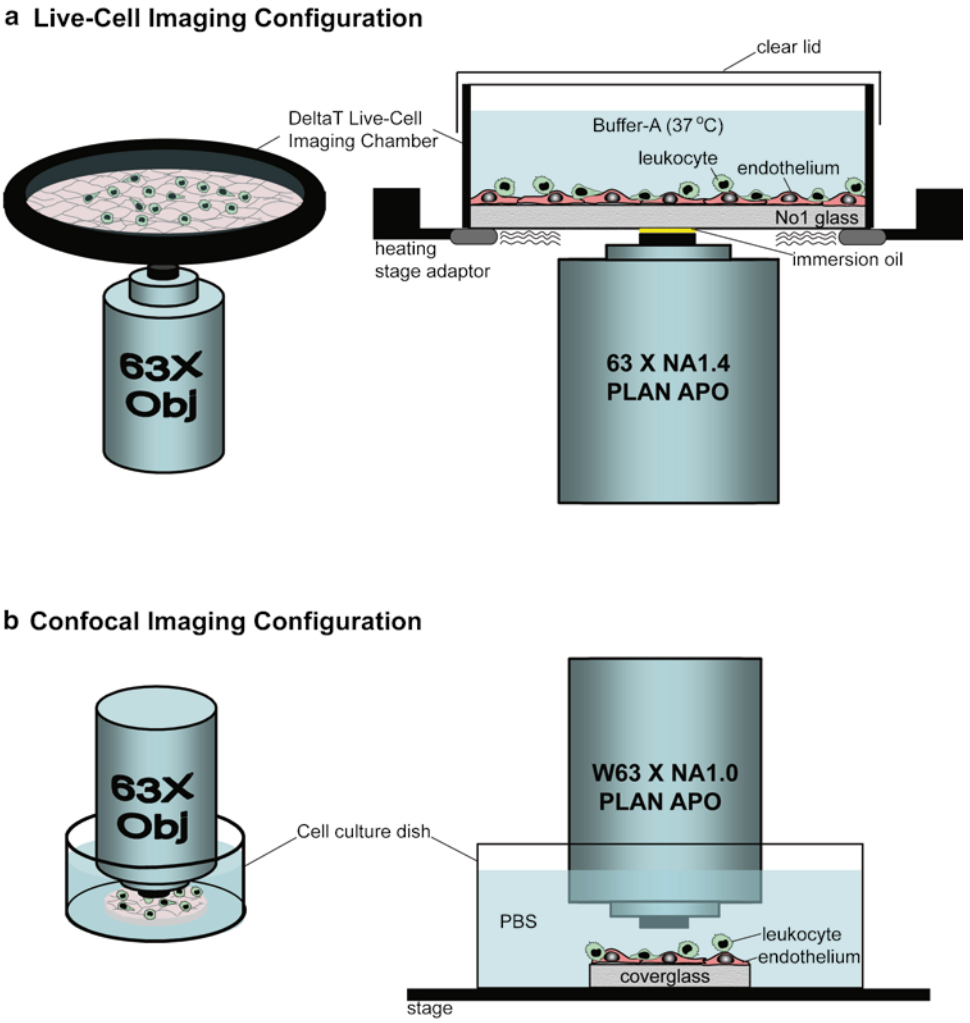

Fig. 3. Live-Cell and Confocal Imaging Configurations. (**a**) Live-Cell Imaging. A monolayer of endothelial cells (*pink*) grown on a Delta-T live-cell imaging chamber and immersed in Buffer A is mounted on a heating stage adaptor and equilibrated to 37°C. Lymphocytes (*green*) are added to the top of the chamber and lymphocyte-endothelial interactions are imaged live via time-lapse epifluorescence/wide-field microscopy. (**b**) Confocal Imaging. Endothelial cells grown on 12-mm circular cover glass in 24 wells are incubated with overlaid lymphocytes in Buffer A for various times, followed by fixation and immunofluorescence staining. The cover glass is then transferred to 6-well or 60-mm culture dishes containing PBS and imaged by confocal microscopy using an upright microscope coupled to a water-dipping objective.

cells per field is optimal (see Note 43). If the lymphocyte number is too low, repeat steps of lymphocyte addition and analysis until an appropriate density is reached. Continue imaging over the course of ~20–60 min (see Notes 44–47).

10. Analyze results. Potential analysis of resulting videos is almost limitless. The design of these imaging experiments will yield high spatial and temporal resolution dynamic of lymphocyte and endothelial morphological changes and behavior during TEM. Diverse parameters such as rate/efficiency of TEM,

spreading and polarity of lymphocytes, speed and quality of lateral migration, route of diapedesis, and formation dynamics of transmigratory cups and podosome represent just some of the potential information to be extracted from such studies. Methods for performing actual measurements of these parameters are equally diverse (26, 32, 33, 44) and beyond the scope of this chapter. However, the majority of required analyses can generally be achieved with a few basic morphometric tools available in most image acquisition or offline analysis software packages (including free programs, such as NIH Image) (see Note 48).

3.4. Immunofluorescence Staining and Confocal Imaging of TEM

The following protocol describes a fixed time point time course for examining lymphocyte–endothelial interactions by confocal imaging (see Note 47).

3.4.1. Setting Up Fixed Time Point TEM

1. Prepare IL-2-cultured effector lymphocytes as in Subheading 3.2.3.
2. Prepare 24-well plates containing at least four wells (duplicates of four time points: 5, 10, 20, and 40 min; see Note 49) with coverslips and confluent HLMVECs, as described in Subheading 3.1.5.
3. At 4–12 h prior to the imaging, replace media on endothelial cell with fresh media containing 50 ng/ml TNF-α (see Notes 36–38).
4. On the day of experiment, prepare fresh Buffer A and fixative (Subheading 2.3).
5. Take a sample of cultured lymphocytes and determine the density by counting with a hemocytometer.
6. Transfer a lymphocyte culture volume equivalent to at least 3×10^5 lymphocytes/sample (e.g., 2.4×10^6 for the eight HLMVECs well prepared above) to a 15-ml conical tube and centrifuge at $200 \times g$ for 3 min. Remove the supernatant and resuspend the lymphocyte pellet in Buffer A at a concentration of 6×10^5 lymphocytes/ml.
7. To begin the TEM experiment, remove the endothelial cells from the cell culture incubator, (proceeding one sample at a time) successively aspirate media, and replace with 0.5 ml of Buffer A/lymphocyte suspension (3×10^5 lymphocytes/sample) and replace in 37°C incubator (see Note 50).
8. After appropriate incubation times, noted above, aspirate Buffer A and add sufficient fixative solution to cover the sample completely (e.g., in 24-well plate, ~300–500 µl; in a 6-well plate, ~1–1.5 ml; and in a Delta-T dish, ~0.5–1.0 ml). Incubate at room temperature for 5–10 min (see Note 51).
9. Rinse the sample 3× with PBS (see Note 52).

3.4.2. Sample Staining

The following protocols are designed as a guideline for immunofluorescence labeling of isolated adherent cells for the purposes of confocal fluorescence microscopy (see Note 53). In this procedure, staining will be for endothelial ICAM-1, lymphocyte LFA-1, and total sample F-actin, a combination previously established to be highly informative for understanding the topology of leukocyte–endothelial interactions (26, 32, 33) (see Note 54). See Notes 55–58 for general tips on handling procedures for sample staining.

1. Aspirate PBS. Add 0.5 ml permeabilization solution per well (see Note 59).
2. Incubate at room temperature for 5–10 min.
3. Rinse the sample 1× with PBS. Aspirate PBS.
4. Add 0.5 ml Block Solution per well and incubate at room temperature for 10–15 min (see Note 60).
5. To prepare stain solution, aliquot a volume of Block Solution equivalent to 0.3 ml × the total number of samples to be stained and then add directly conjugated antibodies (see Notes 61 and 62) to human ICAM-1 (IC1/11-488) and LFA-1 (TS2/4-Cy3) at 10 μg/ml (see Note 63) and phalloidin-647 stock solution at 10 μl/ml, as recommended by the manufacturer (Invitrogen).
6. Centrifuge stain solution (at ~10,000 × *g* in a microfuge for 2 min) to pellet any antibody/stain aggregates. Recover the supernatant (see Note 64).
7. Aspirate the block solution from samples and replace with aliquots of stain solution. Incubate at room temperature for 30 min in the dark (see Note 65).
8. Remove the stain solution from samples and collect into a clean Eppendorf or 15-ml conical tube (see Note 66).
9. Wash the samples three times with PBS (allowing ~30–60-s incubations for each) (see Note 67). Aspirate the PBS from samples.
10. Add fixation solution and incubate at room temperature for 3–5 min (see Note 68).
11. Rinse three times with PBS.

3.4.3. Confocal Imaging

1. This protocol is designed for use with an upright Zeiss LSM510 laser-scanning confocal microscope used with a 63× water-dipping objective (see Note 69). To accommodate the width of a water-dipping objective, transfer samples on 13-mm glass coverslips from 24-well plates to 6-well plates or 60-mm dishes (containing ~4 ml of PBS) using fine tweezers and a "hook-tipped" syringe needle (see Notes 70–72). The samples can be either stored at 4°C for imaging at a later time

(see Note 73) or transferred to the microscope to initiate imaging immediately. If the samples are stored at 4°C, allow to warm to room temperature before initiating imaging (see Note 74).

2. To begin imaging, place the 6-well plate containing samples on the microscope stage. Be sure that the sample-containing cover glasses are lying in the center of each well and then lower the 63× dipping objective into one well (Fig. 3b).
3. Use the bright-field imaging mode and ocular lenses to locate the focal plane.
4. Switch to epifluorescence and inspect the sample via the ocular lenses (see Note 40).
5. Select a field of interest.
6. Switch to laser-scanning mode.
7. Using the fast-scan mode and working in the widest (i.e., non-zoomed or -cropped, see Note 75) field possible, individually adjust laser power and gain of each channel to optimize signal such that, ideally, the specific signal intensity reaches at least ~25% and not more than 75% of the dynamic range of the detectors.
8. Using manual focus controls, quickly scan through the *Z*-axis and identify the upper and lower limits of sectioning (typically all of the information should be contained within a thickness of ~15 mm).
9. Select the *Z*-axis section thickness in the range of ~0.2–1.0 μm (see Note 76).
10. Finally, zoom/crop the imaging field to the specific region of interest (see Note 75) and conduct scan (see Note 77). In addition to having very bright and specific fluorescence signal in the sample, acquiring high-resolution 3D imaging requires iterations of scanning and making adjustments to acquisition parameters (see Notes 76 and 77). The objective is to obtain maximal *x*–*y*- and *z*-axis resolution, without appreciable photobleaching.
11. Analyze images. The resulting optical sections can be subjected to extensive analysis (e.g., Pearson's co-localization analysis), orthogonal viewing, and digital 3D rendering to extract information from the sample, and ultimately better understand the mechanisms for TEM (see Note 78). The precise protocols to be employed will depend on the experimental questions (see Note 78).

4. Notes

1. Store at 4°C and use for no more than ~3 months.
2. Though vendors can make a difference in some cases, generally, similar cell-culture pretreated polystyrene culture plates from diverse vendors are acceptable.
3. Phenol-free RPMI can be used instead of HBSS.
4. Always prepare this solution fresh, i.e., same day of use.
5. Total volumes will change based on sample needs.
6. While 0.5% Tx-100 works well for many applications, it may be necessary to vary this value, typically in the range of ~0.01–0.1%, to produce optimal results (i.e., that balance sufficient permeabilization with minimal sample disruption).
7. Antibody staining can use two main strategies: (1) Primary antibodies that are directly conjugated to fluorochrome or (2) unconjugated primary antibody followed by secondary antibody that is fluorochrome conjugated.
8. Non-antibody fluorescent dyes (e.g., Phalloidin-488 (actin stain), ToPro-3 (nuclear stain), and Lyso-tracker-Red (lysosomal stain)) may also be used in combination with antibody staining. Protocol details will vary according to the manufacturer's instructions.
9. Special notes on fluorophores: (a) For imaging, new generation fluorophores (i.e., Alexa and Cy dyes) are vastly superior to more traditional fluorescein (e.g., FITC) and rhodamine (e.g., TRITC, texas red) derivatives. (b) UV wavelength dyes (e.g., DAPI) are fine for epifluorescence microscopy, but may not be compatible with laser-scanning confocal microscopy, as most systems are not equipped with the appropriate UV lasers for exciting such fluorophores. Thus, alternatives that fall in the green, red, or far-red categories (e.g., TopPro3, Invitrogen) must be used. (c) Far-red dyes (e.g., Cy5 and Alexa 647) are not visible to the naked eye and should only be used when a third color is needed. (d) Generally speaking, ideal fluorophores for imaging are as follows: Green channel – Alexa 488, Cy2, eGFP, and eYFP; red channel – Alexa 546, Cy3, and mCherry; and far-red channel – Cy5, Alexa 647, and mPlum(FP).
10. Though focused on one specific context, in general these protocols offer a widely applicable platform of approaches relevant for modeling of broad leukocyte–endothelial interactions.

11. These protocols are also generally applicable to diverse human (e.g., umbilical vein, coronary artery, heart microvascular, and bladder microvascular) and mouse (e.g., heart, lung, and skin microvascular) endothelium, though with various cell-specific tweaks.
12. Alternatively, glasses can be flame sterilized, by dipping each one briefly in ethanol followed by flaming with a Bunsen burner inside the cell culture hood and then placing each in cell culture plate wells. This requires more manipulation initially, but ultimately can save some time. Anecdotal experience suggests that some endothelia may grow preferentially on flamed glass.
13. Mouse primary endothelium is relatively more challenging to grow on glass compared to human.
14. HBSS can be substituted.
15. When using 24-well plates with circular coverglass, attention must be paid to the complete coverage of the glass. Initially, the glass is more hydrophobic than the plastic and will repel the FN solution or even cause the cover glass to float on top. Agitation of the plates will help to begin the coating process, making the glass increasingly hydrophilic.
16. Do not excessively swirl or knock the plate during this stage; while it may help to remove cells, it will also cause cell damage and aggregation.
17. Seeding endothelial cells at significantly lower densities may lead to low viability and extremely slow growth.
18. It is not recommended to use endothelial cells beyond ~6 passages, as phenotypic drift may seriously compromise the quality/physiologic relevance of the endothelial monolayers formed.
19. When preparing monolayers to be used in experiments, seeding density should be sufficiently high that following initial attachment and spreading (i.e., with ~4–6 h of plating), confluency will be ~80%. This will allow ~100% confluency to be reached by ~12–16 h. Cells should subsequently be cultured further for a minimum of 24 h (ideally 48–72 h) before any experimental manipulations. This regime allows intercellular junctions to form and at least a minimal amount of differentiation toward an integrated vascular monolayer to occur.
20. Even with the Amaxa approach, some constructs may be expressed poorly due to issues of stability, toxicity, or aspects of construct design (e.g., promotor type used).
21. Transfection results are highly dependent on health of the endothelial cells. Ideally, cells should be passaged 3–4 days prior to transfection and be at relatively early total passage number (i.e., p2–p4).

22. At 90% confluency, T25 and T75 flasks typically yield ~1.5 million and ~4 million cells, respectively.
23. A single Nucleofection reaction required 0.5 million endothelial cells.
24. Viability will vary depending on type and health of the endothelium and the plasmids transfected.
25. From the same starting point of isolated primary human blood, one can readily also isolate/culture naïve lymphocytes, memory lymphocytes, neutrophils, monocytes, and NK cells for similar use in in vitro TEM studies.
26. Generally, similar protocols can be used for derivation of mouse Th1 and memory lymphocytes, neutrophils, and monocytes from spleen with some variations.
27. An alternative strategy to drawing blood specifically for experiments is to purchase "blood leukopacks" from nearby hospitals, blood banks, or commercial vendors (e.g., Cambrex). Leukopacks are bags of fresh human blood cells collected from normal peripheral blood by automated apheresis in anticoagulant followed by density gradient centrifugation. Each leukopack contains a mixture of monocytes, lymphocytes, platelets, plasma, and red cells. This not only offers convenience over setting up IRB-approved human subject protocols for drawing blood, but also tends to be an expensive option. Protocols to work with leukopacks and quality of resulting cell preparations are generally similar to those of blood drawn freshly in house.
28. Details of appropriate blood drawing techniques and safety protocols are beyond the scope of the current chapter. Those interested in learning more on this topic should consult the appropriate human subjects research administrators within their host institutions.
29. RPMI-IL15 media (RPMI base media supplemented with 10 μg/ml of IL-15; R & D Systems) can be used as an alternate here to produce a relatively more memory, rather than effector, T cell phenotype.
30. Based on FACs analysis, resulting cells are typically ~97% CD3+, ~50% CD4+, ~50% CD8, ~95% CD45RO+, and CD56- and thus represent a mixture of CD4 and CD8 effector lymphocytes. One can readily further purify subpopulations using immuno-magnetic bead sorting (e.g., MACS, Miltenyi Biotec, Germany) if desired.
31. Generally, similar types of image acquisition can be achieved using inverted scopes/CCD camera setup from diverse vendors.

32. Membrane-tagged fluorescent proteins (e.g., mYFP and mRFP) are expressed with high efficiency (~50–70%) and at high levels in endothelial cells and provide a crisp delineation of individual cells and an extremely sensitive readout for endothelial topological changes during interactions with leukocytes (26). Actin-GFP is expressed somewhat less efficiently in endothelium and provides important additional topological information and more general insights into endothelial cytoskeletal responses to interactions with leukocytes (26). DIC imaging provides exquisite detail of all cell surface dynamics and complements the fluorescence information.
33. Co-transfection of diverse combinations of markers can be employed to address distinct questions. Particularly interesting markers for TEM studies include fluorescent protein fusion contructs of ICAM-1, VCAM-1, PECAM-1, VE-cadherin, tubulin, caveolin, and Rho family GTPases. Further advanced imaging can be obtained by combining topological markers (e.g., mRFP) with ratiometric- or FRET-based biosensors that readout localized signaling activities.
34. The protocols described here do not include any specific fluorescent markers for the lymphocytes, as primary blood leukocytes remain extremely poorly transfectable, by virtually all known approaches. The combination of DIC and endothelial mRFP which can serve as an indirect readout for leukocyte topological dynamics (26) can provide a great deal of information on leukocyte morphology. However, when desired, fluorescence can be introduced to leukocytes by staining with fluorescent cytoplasmic (e.g., BCECF or SNARF) or membrane (e.g., DiI) dyes or with fluorescent-conjugated antibody fAb fragments that target leukocyte cell surface markers (e.g., LFA-1), although these strategies are not optimal.
35. Amaxa transfection is extremely cytotoxic. Only a fraction of the input cells will survive and effectively adhere to the culture plates. To ensure that these grow as healthy monolayers, it is important to remove the dead cells and debris by gentle swirling and exchanging the media.
36. The current protocols model inflammatory leukocyte–endothelial interactions. As such, the endothelium must be activated to upregulate the expression of adhesion and chemoattractant molecules appropriately, which drive leukocyte adhesion and TEM. TNF-α arguably represents the most potent and broadly relevant endothelium-activating cytokine. However, alternative activation by other cytokines (such as IL-1β or interferon-γ) or by distinct stimuli (such as hypoxia, thrombin, or lipopolysacharide) may be substituted when modeling distinct inflammatory settings.

37. It is also important to note that a key challenge to studying leukocyte TEM in vitro is the formation and maintenance of an intact endothelial monolayer. In the absence of this, any apparent endothelial barrier crossing activities by leukocytes (i.e., TEM) is meaningless. Thus, it is important to start with conditions that allow for confluent monolayers to form and begin to differentiate within 12–16 h of transfection/plating.
38. The process of creating transfected and activated endothelial monolayers with well-formed intact junctions is an empirical one. If monolayers are not well formed, modifications (such as increasing the input cell density, culturing for longer periods before addition of TNF-α, or reduction in either the concentration or treatment time (e.g., 4 h instead of 12 h) of TNF-α application) should be implemented systematically until optimal monolayer conditions are identified.
39. It is critical to minimize the time interval between removal of cells from culture incubators and initiation of imaging. Thus, it is important to first fully prepare all aspects of the experiment and image acquisition. All live-cell time-lapse image systems are equipped with software that drive multidimensional image acquisition. Confirm parameter setup before starting experiment.
40. Effort should be made to work quickly and minimize the exposure of a sample to cytotoxic fluorescence excitation sources. Apply neutral density filters to attenuate excitation source to the minimal usable levels and turn excitation sources off (i.e., close relevant shutters) immediately whenever a pause in imaging activities is required.
41. The basic principle to be applied is to use the minimal amount of excitation energy required to provide good quality brightness and contrast. Generally speaking, exposure times should be set in the range of 200–800 ms. If high-quality images are possible at less than 100 ms, it is likely that the intensity of excitation light source being applied is greater than necessary and should be attenuated (e.g., using a neutral density filter). Alternatively, when required exposure times are longer than 1 s, aberrations due to the relative speed of cell dynamics may appear. Thus, in such settings, it may be necessary to increase the detector gain and/or binning.
42. As with exposure intensity/duration (see Note 41), acquisition rate/frequency must be selected carefully. This selection should be based on the minimum required temporal resolution and duration of imaging, as defined by the speed and duration of the specific behaviors being studied, respectively. Each exposure of the sample to excitation light sources induces some degree of phototoxic damage that hinders

cell viability and photobleaching, which reduces quality of fluorescence signals. The total amount of exposure (intensity and duration of each exposure multiplied by the total number of exposures, which in turn is defined by the frequency of acquisition multiplied by the total duration of imaging) that a given sample can tolerate before cell viability and/or fluorescence signal falls below acceptable levels is finite. *Thus, one should bear in mind that every imaging experiment works on a limited "photon budget."* High-quality, reliable results can be obtained only when all parameters are managed such that the experiment remains well within these limits by "spending" the minimal amount of photons necessary to obtain the required results.

43. Successful and robust imaging analysis requires a carefully balanced number of input lymphocytes in the field of interest. Too few cells may mean that a time-consuming live-cell imaging experiment may only yield one or several leukocyte–endothelial cell interactions to evaluate. Too many cells will make it extremely difficult to discriminate individual interactions, thus precluding meaningful analysis. Moreover, excess amounts of lymphocytes may also overwhelm and, thereby, compromise the health of the endothelium.
44. For high-quality imaging, it is absolutely critical to pay careful attention to the focal plane throughout the course of the experiment.
45. The current model system using effector lymphocytes and activated endothelium should produce completion of TEM by the majority of lymphocytes within ~30–45 min. If for any reason experimental objective requires imaging for much longer durations, careful consideration should be given to issues of evaporation and CO_2/pH. Clear plastic covers for Delta-T plates (Bioptechs) that do not interfere with imaging can offer some protection from evaporation. However, for truly long-term experiments, humidified, CO_2-equilibrated, 37°C environmental control boxes that fit around the entire microscope stage are highly recommended.
46. In many cases, it may be desirable to end the imaging experiment by fixation via removal of Buffer A followed by addition of fixative solution and incubation at room temperature for 5 min, followed by rinsing three times with PBS. This allows for the possibility of performing additional staining of samples and high-resolution, 3D confocal, fixed-cell end-point image analysis (Subheading 3.4).
47. It is important to note that this approach does not include physiological fluid shear forces as normally experienced in blood circulation. One can readily modify these experiments using parallel wall flow chambers (e.g., Biotechs FCS2 chamber)

to include shear force application and determine whether this parameter is important to the phenomena being measured (26, 32, 33). However, since these techniques are invariably more technically challenging and time consuming, it is recommended to develop results first with a static system before progressing to shear systems. Additionally, it must be noted that acute application of fluid shear forces to an in vitro model system will not necessarily make it more physiologic as a whole. While these forces may recapitulate those felt suddenly by leukocytes as they transition from free flow to firm adhesion on endothelium, the acute onset of shear felt by the endothelium itself is entirely non-physiologic (under normal condition, endothelium exhibits a high degree of long-term shear adaptation) and induces aberrant calcium flux, stress fiber formation, and intercellular junction weakening.

48. It must be stressed that the strength of the imaging approach described throughout this protocol is in delineation of fine morphological dynamics and attempting to correlate them with overall TEM behavior. In this way, cell biological mechanisms governing TEM may be uncovered and characterized. Thus, it is up to the investigator's power of observation to first identify and then rigorously characterize/quantify specific morphological dynamics, or changes in such dynamics in response to experimental conditions. This approach provides potential as both an exploratory tool and an assay system. However, the realization of this potential is a highly empirical process, in which measurement protocols often need to be custom developed.
49. These time points are selected to provide representative snap shots of behaviors at major phases of TEM. Extensive experience with this model shows that a small fraction of lymphocytes begin diapedesis within 5 min, whereas the majority complete the process by 30–40 min (26, 32, 33). Time points can/should be freely adjusted to suite experimental questions or distinct model systems.
50. It is recommended that the addition of cells for each time point be staggered (i.e., adding lymphocytes to the longest time point samples first and the shortest ones last) to allow for fixation of all samples together in one step to avoid issues noted below (see Note 51). Alternatively, one can plan a priori to plate each time point in separate 24-well plates.
51. It is possible to over-fix a sample, which may result in damaging of antibody epitopes or fluorescent proteins. This fixative should never be used at 37°C or for more than 30 min.
52. Samples can be transferred to 4°C and stored at this point if desired. Staining performed within several weeks often shows no appreciable difference from immediate staining.

However, for first time analysis, it is highly recommended that samples be stained and imaged immediately.

53. While general principles and procedures are similar, staining of suspension cells, whole-mount tissues, or frozen sections requires modifications. Moreover, immunofluorescence staining for flow cytometry also has distinct procedures and requirements.
54. This basic protocol is also generally applicable to broad combinations of stains (e.g., antibodies to other cell surface or intracellular proteins) and stains for other markers (e.g., lysotracker and mitotracker to mark the lysosome and mitochondrial compartments, respectively).
55. This protocol covers basic procedures common to most immunofluorescence staining needs. However, aspects of this protocol are written specifically for use with an upright microscope coupled to a water-dipping objective. For this, it is recommended that adherent cells be grown in 24-well plates containing 13-mm circular cover glasses.
56. Never let the samples dry. When aspirating and replacing, solutions always move quickly to minimize the amount of time that the sample is exposed to air. If working with a large number of samples, do not perform steps on all samples at once. That is, do not aspirate media or buffer off of 20 samples and then replace buffer in all 20. Rather, quickly aspirate buffer from one (or a few) samples and replace buffer, then move to the next sample (or a small group of samples).
57. Balance speed and efficiency of buffer changes with gentleness. Harsh pipetting will lead to damage or even loss of sample. Use of disposable transfer pipettes (which have relatively large diameter opening compared with pipette tips) allows for rapid delivery of buffer with minimal shear force. Additionally, add buffers to the side of the sample well rather than directly onto the center of the sample.
58. Once fluorophores are introduced (which may mean *a priori* if samples have been transfected to express a fluorescent protein), care should be taken to minimize sample exposure to light (especially direct sunlight). Perform incubation steps inside of a laboratory bench drawer or under a sheet of aluminum foil.
59. Permeabilize samples only when necessary (i.e., intracellular staining is being conducted). If staining only includes extracellular/cell surface markers, it is best not to perform a permeabilization step, as this almost always increases the background signal.
60. BSA, HSA, or FCS may be preferable to NFDM in some cases.

61. Direct antibody conjugation of primary antibodies can readily, quickly, and cost effectively be accomplished with as little as ~100 μg of protein using commercially available Alexa (Invitrogen) and Cy (Amersham) dye micro-labeling kits. Direct conjugation offers greatly increased flexibility (i.e., freedom from issues of selecting primary antibodies derived from distinct species in order to be able to use non-cross-reacting secondary antibodies), convenience (one antibody stain step instead of two, see Note 62), and generally cleaner, more specific staining results.
62. Traditional methods using nonconjugated primary antibodies in conjunction with fluorescent-conjugated secondary antibodies can similarly be used in this protocol by preparing primary antibodies as indicated, but without phalloidin-647, followed by washing, using a second antibody stain (i.e., containing secondary, for example, goat anti-mouse-488 or rat anti-rabbit-Cy3, antibodies; Phalloidin-647 or other non-antibody stains, such as ToPro3, should also be included in this step), and a wash step.
63. It is best to determine optimal concentrations empirically, but 10 μg/ml works well for most antibodies.
64. It is normal to see a significant amount of NFDM pelleted.
65. Longer incubations may sometimes be used/necessary to increase the level of signal. However, this may also increase the nonspecific background staining of the sample as well.
66. Stored at 4°C in the dark, stains can often be reused several times over a period of weeks or months without appreciable loss in stain quality, thereby offering substantial conservation of precious reagents and convenience. However, the shelf-life of a given premade stain solution will vary greatly and must be determined empirically.
67. In some cases, additional washes and/or extended incubations may be useful in reducing nonspecific background staining.
68. This step covalently links stains to the sample and dramatically increases the stability of the staining, allowing appropriately stored samples to be repeatedly imaged over the course of weeks or months. However, some dyes (e.g., Phalloidin-488 and ToPro3) do not seem to be efficiently "fixed" into the sample and may leach out over time. These, therefore, may require a fresh restain immediately before viewing samples that have been stored.
69. Use of an upright microscope and water-dipping objective offers several advantages over oil immersion objectives. The sample preparation technique used for a water-dipping objective is substantially easier than that used for traditional

coverslip mounting (see Notes 70 and 72) and also offers dramatically more stable samples (see Note 72). Additionally, the ability to image the sample directly through an aqueous medium (e.g., PBS) reduces *Z*-axis aberrations, which are strongly promoted by imaging through media of mixed refractive index (e.g., oil, glass, and aqueous sample).

70. First, carefully press the pointed edge of the needle against a hard benchtop at ~15° angle and slowly increase the angle to ~70° while applying pressure. This will result in a "hooked" appearance at the end of the needle that can be used to lift one edge of the coverslip and then raise it until the tweezers can be used to grab and transfer it. Be careful not to allow the sample to be exposed to air for more a couple of seconds and to maintain the coverslip with the samples surface up. It is highly recommended that this approach be practiced with blank coverslips before transferring "real" samples.
71. Samples can also be grown directly on 6-well or Delta-T plates. However, this will require significantly greater numbers of cells and large amounts of (usually precious) staining reagents.
72. If a water-dipping objective is not available, coverslips can also be mounted using traditional techniques to be used with an oil immersion objective mounted on either upright or inverted confocal systems. To do so, place a drop of mounting medium (e.g., Slow Fade, Invitrogen) on a cover glass, then flip the cover glass (sample-side down) on to the medium, and then use nail polish to seal the edges. Be extremely cautious to ensure that the nail polish is completely dry before attempting to image.
73. To store samples for subsequent imaging sessions, make sure that 3–4 ml of PBS remains in each well. Cover the plate with adhesive sealing strips (Fisher) and then with aluminum foil and store at 4°C. Samples stored in 6 wells covered by PBS and protected from light often retain high-quality signal for weeks or months, whereas mounted coverslips may retain reliable, good quality signal typically for only several days.
74. If samples are in the process of warming up during image acquisition, significant drift in focal plane can occur during scanning. This will be particularly problematic if 3D serial sectioning is being attempted.
75. The more zoomed or cropped the field is, the more intense the total laser energy being absorbed per unit area. Thus, the continuous scanning required to adjust parameters will have a higher tendency to photobleach the sample when it is highly zoomed. It is, therefore, recommended to set parameters first using as wide a field as possible and then crop/zoom to smaller regions of interest as the last step before collecting the final scan.

76. Ideally, all scans would be conducted in the lower range to provide optimal 3D information. However, as noted for live-cell imaging (see Note 42), we always need to be aware of the limited "photon budget" when working with fluorescent samples. The smaller the section thickness, the more scans will be required to complete the Z-sectioning. When using a laser-scanning confocal system, it is very easy to photobleach the sample as image acquisition is taking place, such that signal intensity in the sections obtained toward the end of the series may be substantially diminished (or lost all together) compared to those at the beginning. Thus, section thickness and/or laser intensity will often need to be compromised to avoid photobleaching issues. Ultimately, this is an empirical process. After several rounds of trail and error, optimal laser power and section thickness for a given sample can be accomplished.
77. An important tool/parameter for high-quality confocal imaging is the multiple-line scan feature or Kalman filter. In short, this feature causes the laser to raster repeatedly over the same line multiple times, as set by the user. Basic algorithms provide a type of recursive filtration that can dramatically enhance signal to noise. However, this comes with an increase in laser energy input to the sample and thus, increased photobleaching. Again, ultimate imaging quality requires balancing the number of line scans applied, the laser intensity, and the *Z*-axis section thickness.
78. No set analytical procedure exists. As with the dynamic live-cell imaging described in Subheading 3.3 (see Note 48), the analysis to be conducted with confocal serial Z-sections is highly dependent on the questions being asked in any given setting and experiment. Wide-ranging offline image-processing software packages exist (including many free packages) with flexible capabilities.

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