

Studies on the Tumor Vasculature and Coagulant Microenvironment

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Abstract

Angiogenesis represents one aspect in the complex process that leads to the generation of the vascular tumor stroma. The related functional constituents include responses of endothelial, mural, bone marrow-derived, and resident inflammatory cells as well as activation of coagulation and fibrinolytic systems in blood. Multiple molecular and cellular effectors participate in these events, often in a tumor-specific manner and with changes enforced through the microenvironment, genetic evolution, and responses to anticancer therapies. To capture various elements of these interactions several surrogate assays have been devised, which can be mechanistically useful and are amenable to quantification, but are individually insufficient to describe the underlying complexity and are best used in a targeted and combinatorial manner. Below, we present a survey of angiogenesis assays and experimental approaches to analyze vascular events in cancer. We also provided specific examples of validated protocols, which are less described, but enable the straightforward analysis of vascular structures and coagulant properties of cancer cells *in vivo* and *in vitro*.

Key words Angiogenesis, Tumor microenvironment, Coagulation, Endothelial cells, Endoglin, Pericytes, Alpha smooth muscle actin, Proliferation, Tissue factor

1 Introduction

The vascular microenvironment defines many essential features of the neoplastic process including metabolic exchange, cellular trafficking, hormonal communication, angiocrine niche effects, metastasis, and paraneoplastic syndromes such as thrombosis and cachexia [1–3]. It is important to consider that vascular responses to tumor growth and dissemination are complex and form a continuum comprising hemostatic, inflammatory, and vessel wall-related mechanisms. While the term “angiogenesis” is often used to describe these events globally, the specific meaning is restricted to vessel formation from pre-existing blood vessels, which is but a fragment of the vascular milieu [3].

The multistep nature of angiogenesis is now understood in considerable molecular detail including the key regulators and cellular events involved [3]. Thus, pre-existing quiescent capillary endothelial

cells (phalanx cells) receive stimulatory signals exemplified by the gradient of vascular endothelial growth factor (VEGF) surrounding hypoxic or transformed cells. To this stimulus, endothelial cells respond by activation of key signaling pathways involving VEGF receptor 2 (VEGFR2), NOTCH/DLL4, ephrins, integrins, and other effectors resulting in the formation of structures known as angiogenic sprouts. These events begin with structural rearrangements in the capillary vessel wall including enlargement of the vascular diameter resulting in the formation of “mother vessels,” followed by dropout of pericytes surrounding the endothelial tube, focal proteolysis of the endothelial basement membrane, and directional invasion of endothelial cell cohorts (sprouts) in the direction of the stimulus [3, 4]. Each of these formations consists of a leading single endothelial tip cell (high expressor of VEGFR2 and DLL4), followed by migrating columns of stalk cells, some of which exhibit mitotic activity. The sprout extension eventually leads to anastomosis with other sprouts/vessels to form a complete loop, a transition mediated by myeloid cell populations, and followed by the establishment of the new lumen, resumption of blood flow, and ultimately an increase in vascular density. Thus endothelial cell division, survival, migration, and morphogenesis involved in different steps of these processes could serve as surrogates of angiogenic activity and frequently represent the basis of the respective assays (Tables 1, 2, and 3) [5–7].

However, several key elements of tumor neovascularisation are not captured by these measurements. For example, tumor cell access to blood vessels may occur through non-angiogenic processes such as vasculogenesis, the recruitment of endothelial progenitor cells to form elements of the endothelial or perivascular milieu. In addition, processes of vessel splitting, vascular cooption, vasculogenic mimicry, endothelial transdifferentiation of cancer cells, and vessel invasion may contribute to the vascular networks in cancer [8]. Components of the circulating blood such as platelets, fibrin, and coagulation factors as well as their receptors such as tissue factor (TF) in perivascular and cancer cells also contribute in various ways to blood vessel formation, and functionality. For example, in human glioblastoma, the high expression of TF coincides with exuberant neovascularisation, intraluminal thrombotic vaso-occlusion, and regions of hypoxia [9–11]. Thus, coagulant properties of cancer and stromal cells may profoundly alter the vascular microenvironment. Moreover, larger vessels, external to the tumor mass, may adapt to and facilitate the demands of the intratumoral vascular networks through processes of arteriogenesis and arterio-venogenesis [4, 12, 13]. Therefore, measurement of angiogenic responses is often insufficient to understand the salient features of the vascular milieu associated with cancer.

Table 1
In vitro angiogenesis assays

Assay	Parameter measured	Principle	Endpoints	References
Endothelial proliferation assay	EC proliferation	Proliferation of ECs in response to angiogenic factors or inhibitors	MTS assay, BrdU or ³ H-thymidine uptake, and PCNA or Ki-67 antigen detection	[5, 30]
Endothelial survival assay	EC survival in GF-depleted medium	The ability of exogenous factors to substitute for paracrine EC survival factors	MTS assay, ³ H-thymidine incorporation, apoptosis assays, and cell count	[7, 31]
Scratch/stencil (“wounding”) assay	EC migration across denuded area	EC motility as element of angiogenesis	Number of cells migrated across the “wound”	[5, 30] [32, 33]
Boyden chamber assay	EC migration across a filter (with or without a matrix coating)	EC motility or invasion reflects elements of angiogenesis	Number of cells migrated across the filter	[5, 30] [34–36]
Tube formation assay	EC morphogenesis on a 2-D or in a 3-D matrix	EC network and tube formation reflect cell functionality	Tube length, number of tubes, and number of branch points	[5, 30] [37–39]
Microfiber co-culture angiogenesis assay	Capillary network formation onto matrix-implanted microfibers	EC network and tube formation resemble angiogenesis	Immunostaining, vessel length, branch points, and volume	[40]
Microfluidic co-culture system	Endothelial cell responses to GF gradients	EC network and tube formation in multicellular context	Immunostaining, visualization of endothelial structures	[5, 41]
Angiogenic sprouting from EC-coated beads	Capillary sprout formation in fibrin gels	EC sprout and tube formation from cell layers exposed to a stimulus	Immunostaining or visualization of endothelial structures	[42]
Embryoid body (EB) assay	Vascular structures of matrix-embedded EBs containing ESCs	Vasculogenesis and angiogenesis are recapitulated by endothelial progenitor cells	Whole-mount immunostaining for EC markers	[7]

Abbreviations: *BrdU* bromodeoxyuridine, *EB* embryoid body, *EC* endothelial cell, *ESCs* embryonic stem cells, *GF* growth factor, *MTS* 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, *PCNA* proliferating cell nuclear antigen

Table 2
In vivo angiogenesis assays

Assay	Parameter measured	Principle	Endpoints	References
Rat mesentery window assay	New blood vessels in the mesentery	Angiogenic tissue implanted into the mesentery triggers angiogenic growth	Vessel count, density, and perfusion	[43]
Chick chorioallantoic membrane (CAM) assay	New blood vessels in CAM	Implantation of angiogenic material (e.g., tumor cells) into CAM triggers chick blood vessel formation	Vessel counts, analysis of structures, and perfusion	[5, 30] [44–46]
Zebrafish model of angiogenesis	Formation of cardinal and connecting vessels	Blood vessels are visualized in transparent fish and through the use of fluorescent tags	Vessel counts, structures, and perfusion	[5, 30] [47, 48]
Corneal micropocket angiogenesis assay	Corneal neovascularization and sprouting	Cornea is avascular and vessel ingrowth is induced by implanting pellets containing angiogenic factors	Vessel counts, length, diameter, density	[5, 49–51]
Matrigel plug angiogenesis assay (matrix invasion assay)	Recruitment of endothelial cells and formation of new blood vessels	Matrigel plugs contain angiogenic growth factors and extracellular matrix that support ingrowth of blood vessels upon subcutaneous implantation into mice	Fixed or frozen sections are stained for EC markers; and plugs can be lysed for hemoglobin content or vascular tracers in the vascular bed, as a measure of the vascular volume	[5, 26, 52, 53]
Angiogenesis chamber assays	New vessel formation between implanted nylon filters	Test substance is sandwiched between nylon mesh layers and vessels are quantified	Vessel counts, diameter, density, and perfusion	[5, 6, 54–62]
Tumor-associated angiogenesis assays	Changes in density and cellular constituents of vascular structures in a growing tumor mass in experimental animals	In situ analysis of the complete neovascularization process is assessed using vascular markers (e.g., ECs, pericytes, basement membrane)	Tumor tissues are immunostained for EC (and other) markers; vascular density, architecture maturation, and perfusion	[5, 30] [13, 63, 64]

(continued)

Table 2
(continued)

Assay	Parameter measured	Principle	Endpoints	References
Tumor-associated EC growth and sprouting assay	Measurement of EC mitogenic activity in the tumor mass	The assay distinguishes between dividing (active) and nondividing ECs	Co-staining for markers of proliferation (PCNA) and ECs (CD31, CD105)	[15]
Endothelial spheroid assay	Sprouting and network formation of EC spheroids grafted into mice	Human EC spheroids are implanted into mice and form vascular structures	Whole-mount immunostaining; or fixed, sectioned, and stained for EC and mural cell markers	[65]
Hollow fiber assay	Vascular network formation to hollow fibers	Hollow fibers containing cancer cells are used as standardized space to attract and quantify the ingrowth of new blood vessels	Microscopic imaging of tumor-associated blood vessels; testing viability of cancer cells	[66]

Abbreviations: *CAM* chorioallantoic membrane assay, *EC* endothelial cell, *PCNA* proliferating cell nuclear antigen

Table 3
Ex vivo angiogenesis assays

Assay	Parameter measured	Principle	Endpoints	References
Aortic ring assay	Vessel outgrowth from aortic explants	Outgrowth of new sprouts from ECs present in the aortic lumen and wall	Microscopic analysis of new sprouts, number of branch points, enhanced by whole-mount immunostaining	[5, 15, 67–71]
Explant angiogenesis assays	Vessel outgrowth from explants of carotid artery, saphenous vein, vena cava, thoracic aorta, fetal or adult metatarsals, placental blood vessels	Assays measure vessel outgrowth from the pre-existing ECs in the vasculature of explanted tissues	Vessel counts, number of branch points, and whole-mount immunostaining	[5, 72–79]
Retina angiogenesis assay	Vessel growth in retinal explants	Vessel growth in mouse retina is visualized microscopically	Sprout formation, vascular structures and architecture, vessel density	[80, 81]

Table 4
Assays measuring non-angiogenic vascular activities in cancer

Assay	Parameter measured	Principle	Endpoints	References
Tumor arteriogenesis and arterio-venogenesis assays	Formation of extra-tumoral “feeding” vessel in mice	Macroscopic blood vessels outside of the tumor mass undergo enlargement to meet the demands of the tumor microcirculation	Diameter of the feeding vessel, blood flow measured by ultrasound, and influx of BM cells in the vessel wall	[4, 13]
Assays for circulating ECs and EPCs	Potential for vasculogenesis	CECs and CEPs and their viability reflect the systemic regulation of tumor neovascularization and antiangiogenic therapies	FACS detection of EPCs and CECs in blood (VEGFR2 ⁺ /CD45 ⁻ cells)	[82–85]
Tissue factor-dependent coagulation (TF PCA)	Cellular clotting potential	Tumor cell coagulant activity mediated by TF contributes to thrombosis and angiogenesis in cancer	Generation of activated clotting factor X (FXa)	[22, 24]

Abbreviations: *BM* bone marrow, *CEC* circulating endothelial cell, *CEP* circulating endothelial progenitor, *EC* endothelial cell, *EPC* endothelial progenitor cell, *FACS* fluorescence-activated cell sorting, *TF PCA* tissue factor pro-coagulant activity

While providing detailed experimental protocols for diverse processes involved in tumor neovascularization exceeds the scope of this chapter, we include the synopsis of the relevant literature and methods in Tables 1, 2, 3, and 4, for reference. In this regard, several assays have been generated for in vitro (Table 1), in vivo (Table 2), and ex vivo (Table 3) studies of angiogenesis and of related vascular processes (Table 4), in response to test substances and conditions (e.g., anti-angiogenic and pro-angiogenic factors, or tumor cells). The simplicity of these assays must, however, be balanced with their combinatorial use and careful interpretation, as they capture only small fragments of the underlying biology, and they often do not reflect the distinctive and heterogeneous properties of angiogenic circuits and regulators in different molecular tumor subtypes [14]. The protocols included in this chapter describe immuno-localization of endothelial cells (Subheading 3.1), mural cells/pericytes (Subheading 3.1), and coagulant/tissue factor-expressing cells in tissue sections in situ, as well as quantitative characterization of tissue factor activity of cultured cells.

Immuno-localization of endothelial cells in paraffin sections of tumor tissue can be accomplished using several lineage markers, such as CD31, CD34, vWF, lectin binding, and tumor endothelial

markers (TEMs) [15, 16]. Here, we describe the use of anti-CD105 (endoglin) staining of mouse tumor tissue sections, as a robust and effective strategy to highlight tumor blood vessels [17]. In this protocol, CD105 is combined with staining for Ki-67 to highlight proliferating endothelial cells specifically [13, 15, 18].

The dynamics of the tumor neovascularization process and responses to antiangiogenic therapies can be inferred from staining for markers of endothelial cells (above), basement membrane, and mural cells such as pericytes and smooth muscle cells [19, 20]. In particular, the tight pericyte coverage of endothelial tubes along with structural changes in the vascular pattern often signifies vessel maturation or normalization [3]. Here, we describe the combined use of anti-CD105 (endoglin) staining of endothelial cells and anti-alpha smooth muscle actin (aSMA) staining in mouse tumor tissue sections to reveal the changes in vessel maturation [17].

Tissue factor (TF) is a transmembrane receptor for the coagulation factor VII/VIIa, which is expressed on perivascular cells and is often upregulated by cancer cells, activated endothelium, inflammatory cells, or stroma [21]. TF renders cancer cells pro-coagulant and mediates their angiogenic and metastatic responses through fibrin deposition, or intracellular signaling transduced by protease-activated receptors and integrins [22, 23]. The expression of TF by cancer cells is often reflective of the degree and pathway of malignant transformation as documented in glioblastoma, medulloblastoma, and other malignancies [10, 14, 24]. Here, we describe an effective protocol to stain tumor sections for TF antigen.

The expression of TF by cancer cells is often indicative of their malignant transformation [22] and their ability to respond to stimulation upon exposure to TF ligands such as coagulation factor VII/VIIa. These interactions play a role in the regulation of gene expression [25], as well as pro-angiogenic, pro-inflammatory, invasive, and growth responses of cancer cells upon exposure to effectors of the coagulation system [14, 26]. Detection of tissue factor pro-coagulant activity (TF-PCA) represents a sensitive method to measure the level of biologically active TF on the surface of cancer cells. In this assay, TF is allowed to bind to activated VIIa and convert inactive coagulation factor X to an active form (Xa), which can be measured by conversion of the S-2765 chromogenic substrate. Here, we describe a simple and effective protocol to measure TF PCA using cultured cancer cells.

2 Materials

Reagents may be obtained from a number of suppliers with the exception of specific validated antibodies or products, as indicated. The compatibility with formalin-fixed tissues may differ between different antibodies which may result in high background and low

specificity. High-quality deionized water should be used to prepare all the indicated buffers and solutions. The procedures require access to general wet lab space, basic histology equipment (tissue processing and embedding stations, slides) cell culture and microscopy facilities.

1. *Slide dewaxing reagents*: 100 % Xylene and 99 % ethanol should be diluted as described for the intended use in immunostaining protocols.
2. *Buffers*: Dulbecco's phosphate-buffered saline (DPBS, used as supplied) and Tris-buffered saline (TBS, pH 7.6) should contain the final concentrations of 20 mM Tris and 150 mM NaCl. For Tris-buffered saline (TBS, pH 7.4), prepare the solution containing 50 mM Tris, 120 mM NaCl, 2.7 mM KCl, and 3 mg/mL BSA.
3. *Tissue permeabilization, antigen unmasking, and blocking reagents*: For permeabilization 0.3% Triton X-100 is prepared in DPBS. Vector Antigen Unmasking Solution (Vector Labs Burlington, ON) containing 1% citrate is prepared by mixing 2.5 mL of the supplied stock with 247.5 mL of distilled water. Hydrogen peroxide (H₂O₂) should be diluted to 1.5% final concentration in TBS. Bovine serum albumin (BSA) for tissue blocking is prepared at 1% concentration by dissolving 0.1 g of BSA powder in a total final volume of 10 mL DPBS. Blocking rabbit serum is prepared at 10% concentration in DPBS. For blocking tissue normal donkey serum (NDS) is used at the final concentration of 5%, prepared by diluting 200 µL of NDS stock in 4 mL of DPBS.
4. *Primary antibodies*: Immunostaining procedures described herein are sensitive to the antibodies used and we have used the following products: Anti-mouse Endoglin/CD-105 Affinity Purified Goat IgG (AF1320; R&D Systems, Minneapolis, MN, USA; used at 1:100 dilution); Anti-Ki-67 rabbit polyclonal antibody (RM-9106, ThermoFisher, Ottawa, ON, Canada; used at 1:200 dilution); Rabbit Anti-Alpha Smooth Muscle Actin Antibody (ab5694; ABCAM, Toronto, ON, Canada; used at 1:100 dilution); Sheep Anti-human TF Antibody (SATF-IG; Affinity Biologicals, Ancaster, ON, Canada; used at 1:100 to 1:10,000 dilution).
5. *Secondary antibodies*: Chicken Anti-goat, Alexa Fluor 488, IgG, H+L (A21467; Life Technologies Inc., Burlington, ON, Canada; dilution 1:200) and Donkey Anti-rabbit Alexa Fluor 594 IgG, H+L (A21207; Life Technologies, Eugene, OR, USA; dilution 1:200).
6. *Immunohistochemistry reagents, and slide-mounting media*: Vectastain Elite kit (PK-4006) and ImPACT DAB (SK-4105), VectaMount Mounting Medium (H-5000) from Vector Labs, Burlington, ON, Canada.

7. *TF-PCA reagents and supplies*: The assay requires cell culture supplies (cell culture media, cell culture-grade trypsin/ethylene-diaminetetraacetic acid (EDTA) solution, plasticware including 24-well culture plates, pipettes, biosafety cabinet), and a timer. The key assay reagents include Factor VIIa (FVIIa; ADG407act), Factor X (FX; 527), both from Sekisui Diagnostics (Lexington, MA, USA), 2 mM solution of Chromogenic Substrate (S-2765; Chromogenix, Mississauga, ON, Canada), and Rabbit Brain Thromboplastin (RBT) TF activity standard (Sigma, Oakville, ON, Canada; 2 mM/mL solution). In addition, the procedure requires preparation of the anhydrous CaCl₂ (100 mM), acetic acid (50%), and protein lysis buffer solutions (0.7 mL basic lysis buffer: 2 mL 10% Triton-X-100; 400 μL 0.5 M EDTA, pH 8.0; 200 μL 1 M Tris-HCl, pH 7.0; 0.175 g NaCl; 6 mL glycerol; 11.4 mL H₂O, supplemented with 170 μL of 7× Mini protease inhibitor, 10 μL of 100 mM phenylmethanesulfonyl fluoride (PMSF), 100 μL of 0.2 M sodium orthovanadate, and 50 μL 1 M sodium fluoride) as well as protein quantification reagents.

3 Methods

3.1 *Fluorescent Multicolor Tissue Immunostaining (IF)*

Multicolor immunofluorescent staining protocols can be challenging; yet they are increasingly used to reveal complex features of cells in their natural milieu. One practical approach to this question is to employ a three-color procedure, including staining for two different antigens (red and green fluorescence, respectively) and a nuclear counterstain (DAPI-blue). Examples of such protocols compiled in this section are relatively straightforward and amenable to modifications and to imaging by standard fluorescent microscopy (*see Note 1*). For practical reasons, a double immunostaining is optimized for a 3-day cycle:

1. (*Day 1*) *Dewaxing and rehydration of tissues*: Processing tissue samples, embedding, and sectioning are described in other sources easily accessible in the literature [27]. Once the sections are cut and mounted on slides the staining begins with the following steps: Place the 5 μm thick paraffin-embedded tissue sections in 100% xylene (histological xylene) in the fume hood for 10 min at room temperature followed by two xylene washes for 5 min each. Remove slides and place in three consecutive washes of 99% ethanol for 5 min each. Sequentially wash the slides in dilutions of ethanol of 95%, 80%, 70%, and 50% and then running tap water to remove any trace of ethanol. For anti-TF staining, the slides are deparaffinized and rehydrated according to a protocol provided by Abcam with slight modification, including the following sequential washes: xylene (3×7 min), xylene 1:1 with 100% ethanol (3 min),

- 100% ethanol (2 × 3 min), 95% ethanol (3 min), 70% ethanol (3 min), and 50% ethanol (3 min). Rinse under cold tap water.
2. (*Day 1*) *Microwave antigen retrieval*: Place roughly 200 mL of the 1% vector antigen unmasking solution in a glass Coplin jar (250 mL) and heat the solution in a microwave to 95 °C using a thermometer or temperature probe. At 95 °C, place the rack of slides into the antigen retrieval solution. Replace the Coplin jar into the microwave and ensure that the temperature is maintained at 95 °C to 100 °C by periodic heating of the microwave for 15 min. At the end of the 15 min, and using a glove to protect against the hot glass surface, remove the Coplin jar and place on the bench at RT for another 20 min. After 20 min, transfer the slide rack to a clean Coplin jar containing DPBS. Perform three DPBS washes of 5 min each of the slides. For anti-TF staining, antigen retrieval is carried out using antigen unmasking solution for 20 min at 98 °C.
 3. (*Day 1*) *Permeabilization*: Prepare a solution of 3% Triton-X in DPBS. Place slides in Triton-X solution for 10 min at RT. At the completion of the permeabilization step, rinse slides in three washes of DPBS for 5 min each. For anti-TF staining, the slides are allowed to cool for 30 min after antigen retrieval and are subsequently washed with running tap water (10 min), TBS (2 × 5 min), and TBS containing 0.025% Triton X-100 (2 × 5 min).
 4. (*Day 1*) *Blocking unspecific signal*: Prepare a solution of 1% bovine serum albumin. Mix thoroughly and then filter using a 0.22 μm syringe filter. Remove each slide from DPBS, wipe away excess solution, and place 1% BSA on the entire tissue. Place the slide with 1% BSA in a humidified chamber and incubate for 30 min. For anti-TF staining the slides are blocked for 2 h with 10% rabbit serum in TBS containing 1% BSA.
 5. (*Day 1*) *Primary antibody binding—first staining*: While incubating in BSA, make the working solution of the primary antibody (*see Note 5*). For Endoglin staining a 1:100 dilution of goat anti-mouse Endoglin/CD105 (AF1320, mouse Endoglin Affinity Purified Goat IgG, R&D Systems, Minneapolis, MN, USA) in DPBS gives strong and specific signal. Maintain the antibody working solution on wet ice (4 °C) until ready for use. When the blocking step is complete, remove a slide from the humidified chamber, remove excess blocking buffer, and wipe the edge of the tissue with blotting paper. Without letting the tissue dry, add enough primary antibody to cover the tissue completely, and place it in the humidified chamber overnight at 4 °C. Complete the same procedure with the other slides. For anti-TF staining, the slides are incubated at 4 °C overnight in a humidified chamber with the TF primary antibody (sheep

anti-human TF, SATF-IG, Affinity Biologicals) at 1:10,000 dilution in TBS containing 1% BSA. While anti-TF staining can be combined with other multicolor protocols, it can also be reduced to a faster immunohistochemical cycle aiming at visualization of TF expression in tumor tissue. In such a case, the slides exposed to the primary antibody are processed through the following steps: the slides are washed (TBS containing 0.025% Triton X-100: 2 × 5 min) and then blocked for endogenous peroxidase activity for 1 h at 4 °C in a H₂O₂ solution; the Vectastain Elite kit for sheep IgG antibody detection is used according to the manufacturer's instructions for incubation with the biotinylated secondary antibody and ABC reagent; and ImPACT DAB (3,3'-diaminobenzidine) peroxidase substrate was used to detect biotinylation prior to counterstaining with hematoxylin or methyl green.

6. (*Day 2*) *Secondary antibody—first staining*: Continue with the double-staining protocol, remove the slides from the humidified chamber, and wash off the primary antibody by placing slides in a Coplin jar of DPBS for 5 min. Repeat the washes three times for 5 min each. Make up a solution of 1:200 dilution of the respective secondary antibodies. This includes the chicken anti-goat Alexa 488 antibody (for anti-CD105 staining). Remove the excess of DPBS by wiping the edge of the tissue and then apply enough of secondary antibody to completely cover the surface of the tissue section. Incubate in the humidified chamber for 45 min. Immediately thereafter, tip the slides to remove the secondary antibody and wash in DPBS in a Coplin jar for a total of five times in DPBS for 5 min each (also *see* **Note 6**). While the slides are still wet, verify the quality of staining under the fluorescent microscope (CD105-positive endothelial cells located around blood vessel lumens should exhibit bright and specific green fluorescence).
7. (*Day 2*) *Blocking tissues for the second staining*: Second stain is intended to reveal the functional states of endothelial cells such as proliferation (Ki67) or association with pericytes/smooth muscle cells (α SMA), the latter a feature of mature blood vessels. For anti-Ki-67 and anti- α SMA antibodies, the blocking buffer consists of 5% normal donkey serum (NDS). Make the solution of 5% NDS (by diluting 200 μ L of NDS in 4 mL of DPBS). Wipe excess solution from the edge with a tissue and place enough 5% NDS to cover the tissue completely (*see* also **Notes 2–4**). Place the slide in a humidified chamber for 30 min.
8. (*Day 2*) *Primary antibody binding—second staining*: Make up the working dilutions of the respective primary antibodies for the second staining. For the Ki-67 immunostaining, the primary antibody should be diluted at 1:200 in 0.3% Triton X-100 in DPBS. For the α SMA staining make a 1:100 dilution

using the blocking buffer as diluent. Before applying the antibody solutions, remove the blocking buffer by blotting the excess of liquid and add enough primary antibody to completely cover the tissue section (never wash the slides after the blocking step as the blocking buffer must remain on the tissue to prevent unspecific binding of the antibody). Place the slides in a humidified chamber and place in the refrigerator at 4 °C overnight.

9. (*Day 3 Secondary antibody—second staining*): Prepare the solutions of the appropriate secondary antibodies. For staining with anti-Ki-67 and anti- α SMA primary rabbit antibodies, prepare the anti-rabbit secondary antibody (Alexa Fluor donkey anti-rabbit 594 IgG (H+L)) at a dilution of 1:200 in 0.3% Triton X-100 in DPBS. Leave the solution on ice. Remove the slides from the refrigerator and place in 0.3% Triton X-100/DPBS for five washes of 3 min each at room temperature (RT). Add the prepared secondary antibody solution to completely cover the tissue and place the slides in a humidified chamber at RT. Incubate with the secondary antibody for 45 min. When the incubation is complete, remove the slides and place them in 0.3% Triton X-100/DPBS for five washes of 3 min each. Using the fluorescent microscope (while the tissues are still wet), quickly examine whether proper staining has been achieved; for example Ki-67 will appear as bright red nuclear staining in dividing cells, and other patterns of staining such as diffuse or nonnuclear stain may signify lack of specificity.
10. (*Day 3 Mounting slides for storage for analysis*): Mount slides by placing the cover slip over the tissue using Vectashield Hard Set Mounting Media with DAPI. This will highlight nuclei with blue fluorescence while protecting the tissue. Maintain slides in the dark and in a cold space (refrigerator, cold room) until used for analysis.
11. (*Slide viewing, interpretation, and image analysis*): Stained tissues may be examined visually and micro photographed at different times after completion of the procedure. Image acquisition can be performed using appropriate fluorescent or confocal microscope while ensuring that any unnecessary and protracted exposure to microscope or laser light is avoided, as this may lead to photo-bleaching of the signal. Fluorescent double staining is particularly useful in seeking information as to vascular geometry (e.g., density) and functional state of cells within and around blood vessels. For example, the vascular caliber and density could be combined with the analysis of numbers, percentages, and distribution of proliferating endothelial cells (CD105⁺/Ki67⁺) versus their quiescent counterparts (CD105⁺/Ki67⁻). These features may exhibit regional differences within tumor tissue as shown in Fig. 1. In this

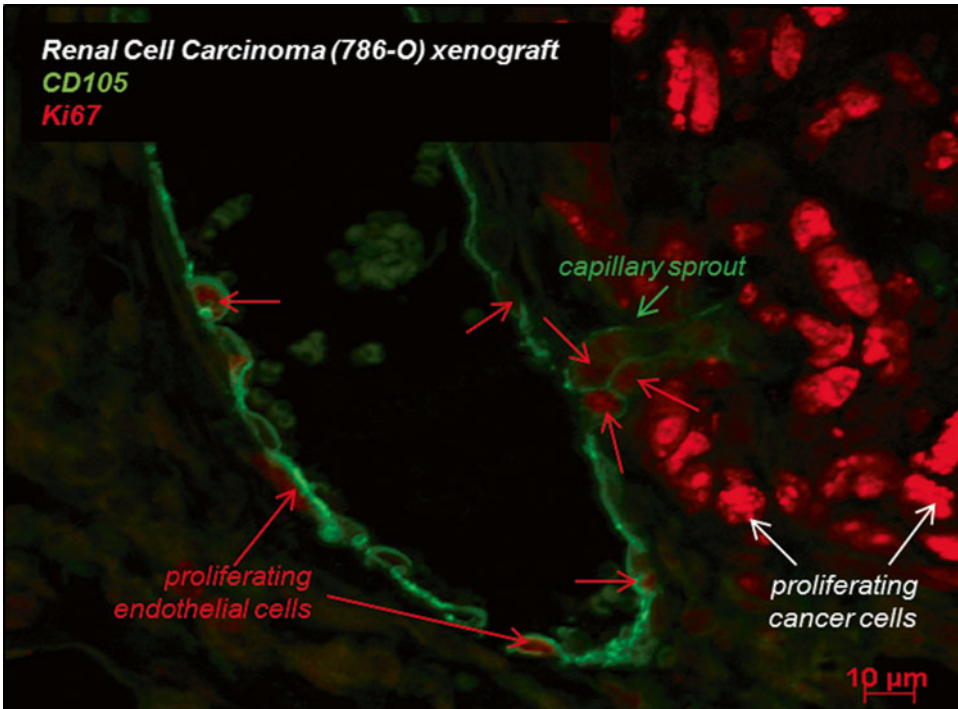


Fig 1 Immunofluorescent staining of tumors for proliferating endothelium. Since normal endothelial cells remain largely quiescent, proliferation of these cells is a hallmark of vascular growth (angiogenesis and arteriogenesis). Human clear cell renal cell carcinoma (ccRCC) cells, 786-O, were injected subcutaneously into immune-deficient SCID mice and the resulting tumors were collected, fixed, sectioned, and co-stained for the endothelial cell marker (CD105, *green*) and proliferation marker (Ki-67, *red*). Nuclei of proliferating endothelial cells within a larger vessels (arteriogenesis) and capillary sprout (angiogenesis) are clearly positive for Ki-67, which also stains CD105-negative tumor cells. Image adapted with permission from [13]

image, proliferating endothelial cells can be found mostly in vascular sprouts, or in tumor regions undergoing vascular remodeling, such as segments within the wall of larger feeding vessels. Of note is the fact that dividing cancer cells surrounding blood vessels exhibit CD105⁻/Ki67⁺ staining pattern. In Fig. 2, double staining for CD105 and α SMA reveals blood vessels containing pericyte coverage (mature blood vessel). Layers of pericytes or mural cells (smooth muscle cells in larger vessels) are visualized as structures with the CD105⁻/ α SMA⁺ staining pattern located in the outer aspect of the vessel wall. Immunostaining also offers the opportunity to perform some quantification of the features of interest. Using the protocol outlined above, dividing endothelial cells or mature blood vessels may be enumerated under high magnification (400 \times) and expressed as numbers per high-power field or per number of blood vessels. Such assessment is often performed to compare vascular features under different treatment conditions, in

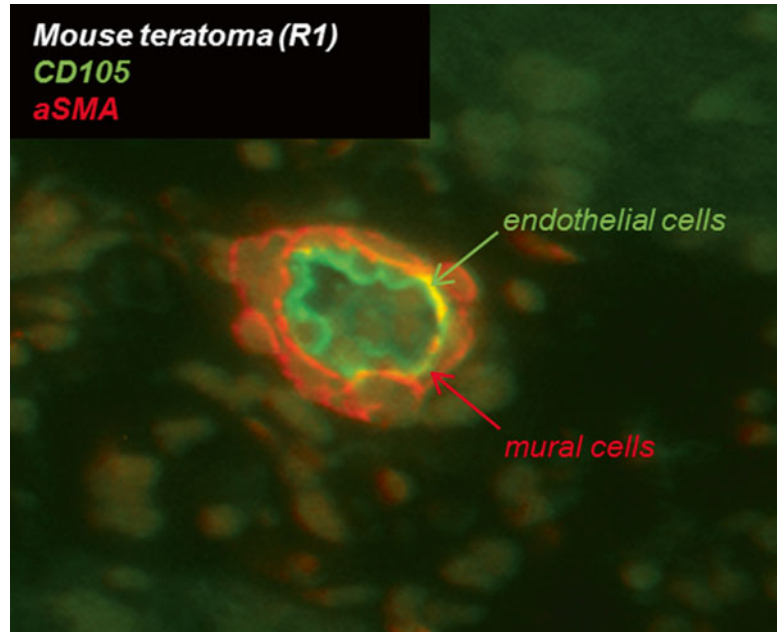


Fig. 2 Immunofluorescent staining of tumors for endothelial and mural cells. Murine embryonic stem cells (R1) were injected into immune-deficient SCID mice to form aggressive teratoma. The tumor tissue was collected, fixed, and stained for markers of endothelial cells (CD105, *green*) and mural cells, such as pericytes and smooth muscle cells (α SMA, *red*). The image depicts the close association of mural and endothelial cells in a small arteriole. Image adapted from [29]

distinct tumor types, or between micro-regions within the same tumor (e.g., center and periphery, or in hypoxic regions). Another aspect of the vascular milieu is revealed by immunohistochemical staining for TF expression. In the protocol, described TF-positive cells are distinguished by brown coloration, often in regions of hypoxia, inflammation, or malignant growth. This signal can be quantified as percent area stained for TF multiplied by average staining intensity, as evaluated by independent blinded observers. The percent area is measured using the ImageJ software, and the staining intensity is assessed on an arbitrary scale of 1–4. However, the specific nature of tissue analysis depends on the underlying research question as described earlier [13, 15, 18, 28].

3.2 Tissue Factor Procoagulant Activity Assay (TF-PCA)

Measurement of TF procoagulant activity of cancer cells is useful and complementary to the aforementioned staining of tissues and cells for the TF antigen. The presence of TF staining does not always correspond to the cellular ability to activate the coagulation cascade as intracellular, encrypted TF, or alternatively spliced TF are inactive in this respect. The simple and effective quantitative TF-PCA can be performed on cultured cancer cells:

1. *Establishing adherent cultures of cancer cells.* Plate each cell line or condition in quadruplicates in a 24-well plate, and grow cells to 70–80% confluence. The cells should be seeded at densities that would not require prolonged culture to achieve these conditions (ideally cells should be grown for 1–3 days) or lead to uneven distribution of cells in wells, altered viability, or other unspecific differentials (*see Note 7*).
2. *Preparation of the TF standard curve and stock solutions.* Once cell cultures are at optimal density, the 1-day TF-PCA begins with preparation of the TF standard curve. To accomplish this, serial dilutions of the Rabbit Brain Thromboplastin (RBT; Sigma 44213) standard, a source of TF/thromboplastin activity, are prepared in TBS, pH 7.4, according to Table 5, and 200 μL aliquots of each dilution are added into empty wells of a 24-well plate. Prepare the stock solutions of all reagents in quantities sufficient for all wells containing cells or standard. From these ingredients, prepare the reagent mix containing 281 μL TBS, pH 7.4, 2 μL 5 nM FVIIa, and 2 μL 150 nM FXa per well.
3. *Setting up the TF-PCA reaction:* Wash the cells three times with pre-warmed TBS, pH 7.4. Add 285 μL of reagent mix solution to each well. At 10-s intervals, add 15 μL of 100 mM CaCl_2 to each well and incubate the cells with solutions for 30 min at 37 °C (this is important as TF activity reaction is time sensitive).
4. *Developing color reaction.* While the cells are incubating, add 20 μL of chromogenic substrate to wells of 96-well plate. At 10-s intervals in the order as in the previous step, add 200 μL of solution from each well of the 24-well plate to the 20 μL substrate in the 96-well plate. This will mix the activated factor X (FXa) from wells containing cancer cells (or standard) with the chromogenic substrate, the cleavage of which results in a color reaction. This reaction is indicative of the FXa activity, which is quantitatively generated by the TF/VIIa complex on the surface of cancer cells. Incubate for 3–5 min at 37 °C. At 10-s intervals, add 20 μL of 50% acetic acid to stop the reaction.

Table 5
Dilution table

Component	Dilution			
	1	0.5	0.25	0.125
RBT standard (μL)	3	450	450	450
TBS, pH 7.4 (μL)	897	450	450	450
Total volume (μL)	900	900	900	900

5. *Reading the TF-PCA reaction.* Place the 96-well plate containing samples and RBT standards in a plate reader and record the absorbance at 405 nm.
6. *Normalization.* Wash the 24-well plate twice with PBS, and lyse the cells for protein quantification. Interpolate the units of TF activity from the RBT standard curve, and normalize to total protein (μg). Additional modification, use of TF-neutralizing antibodies, and related methods to assess the interplay between the coagulation system and the tumor vasculature are reviewed elsewhere [1–3].

4 Notes

1. It is essential to use positive and negative controls with every immunostaining (usually tissue samples known to exhibit the expression of the respective markers). It is also essential to pay attention to subcellular localization of the staining, e.g., membrane/cytoplasmic for CD105 or nuclear for Ki67.
2. Do not place the slides in direct contact with running water but rather allow water to flush over the slides.
3. When applying the primary antibody completely cover the surface of tissue sections and never wash the slides after the blocking step, as the blocking buffer must remain on the tissue to prevent unspecific binding.
4. When removing the blocking buffer, wipe the edge of the tissue with blotting paper and never let tissue dry.
5. For double staining, carefully select the antibodies. For example, the secondary antibody of the second stain must be devoid of any cross-reactivity against all the primary and secondary antibodies used in the first stain. It must also be matched to the species and class of the primary immunoglobulin in the second stain, and carry a fluorophore that has non-overlapping emission spectra with the first staining cycle. It is also practical to avoid antibodies reactive with host tissue immunoglobulins (e.g., anti-mouse antibodies to stain mouse tumors), as blocking off these signals often complicates the protocol.
6. After incubation with the secondary antibody and washing, and while slides are still wet, check one slide to verify, under fluorescence, if the staining was successful (vessels will appear bright green).
7. For TF-PCA, having a proper cell culture and control cells and especially timing the reaction using 10-s intervals, as indicated, are essential as FXa generation and substrate cleavage are dynamic processes.

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