Chapter 2

Immunohistochemical Methods for Measuring Tissue Lymphangiogenesis

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Abstract

The field of lymphatic research has benefited enormously from the discovery of "marker" proteins that permit not only the identification and quantitation of lymphatic vessels in tissue sections for tumor pathology but also the isolation of primary lymphatic endothelial cells for basic research. This chapter focuses on the use of these markers for the immunohistochemical analysis of lymphangiogenesis in both frozen and paraffin-embedded tissue sections and discusses current protocols including newer versions employing biotin tyramide amplification and their associated problems.

Key words Lymphatic, Lymphangiogenesis, LYVE-1, Podoplanin, Immunofluorescence, Peroxidase, Chalkley grid

1 Introduction

The measurement of lymphangiogenesis is of great significance in understanding the role of this process during many pathological conditions. Clearly the most prominent example has been cancer, specifically the metastatic spread of tumors through lymphatic vessels; other examples include lymphedema, wound healing, and inflammation [1, 2]. The measurement of lymphangiogenesis within a tissue is usually approached by assessing the density of lymphatic vessels, although in the case of tumor vessels, it should be borne in mind that isolated measurements of this kind can mask artifactual increases that are caused by tissue compression and fortuitous growth next to preexisting lymphatic networks as well as genuine proliferation [3].

Traditionally the microscopic identification of lymphatic vessels has relied on the skilled analysis of morphology, absence of red blood cells within the lumen, and negative staining for blood vascular markers. However, the ready availability of lymphatic marker proteins has simplified this task [3–5]. Here we describe the use of two of these markers, LYVE-1 [6, 7] and podoplanin [8], to

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identify lymphatic vessels in frozen or paraffin sections of the human tissue. The level of lymphangiogenesis itself is determined by measurement of lymphatic vessel density (LVD) either throughout the tissue specimen or within vessel "hot spots" using the Chalkley point graticule method (*see* refs. 9, 10), combined with estimation based on markers of nuclear division [3]. In addition, we outline the use of a particularly sensitive immunostaining technique (Dako CSA kit) that can be used to detect extremely low levels of these or other novel target antigens expressed by lymphatic vessels [11].

2 Materials

2.1 General

1. Slide staining tray with lightproof lid.
2. Coplin jars.
3. PBS.
4. Hydrophobic pen (Abcam).
5. Normal goat serum.
6. Fetal calf serum.
7. Polyclonal rabbit antihuman LYVE-1 (Abcam, R&D Systems, Reliatech).
8. Mouse monoclonal antihuman podoplanin D2-40 (Signet Laboratories).
9. Mouse monoclonal antihuman Ki-67 (BD Pharmingen).
10. D2-40 (Dako, Abcam, Thermo Scientific).
11. Clear nail varnish.
12. Chalkley eyepiece graticule.

2.2 Frozen Sections

1. Acetone.
2. Paraformaldehyde. (Dissolve 8 g of paraformaldehyde in 90 ml water on a heated stirring block in a fume hood. Add a few drops of 10 M NaOH to help the powder dissolve. Return the solution to neutral pH using 1 M HCl and an indicator paper. Store at −20 °C in small aliquots. To use, melt the solution in a heated water bath then add an equal volume of 2× PBS.)

2.3 Paraffin Sections

1. Microwave-safe dish and slide rack.
2. Citroclear® (HD Supplies).
3. Ethanol.
4. 10 mM sodium citrate pH 6.0/Dako target retrieval solution.

2.4 Immunohistochemistry

1. Bovine serum albumin.
2. EnVision kits (HRP anti-mouse, HRP anti-rabbit, and G|2 doublestain) (Dako).

3. Catalyzed Signal Amplification (CSA) System (Dako K1500).
4. Aquamount (BDH).

2.5 Immunofluorescence

1. Goat anti-rabbit IgG Alexafluor 488 conjugated (Molecular Probes).
2. Goat anti-mouse IgG Alexafluor 568 conjugated (Molecular Probes).
3. Streptavidin, Alexafluor 488/568 conjugated (Molecular probes).
4. Vectashield with Dapi (Vector Laboratories).

3 Methods

3.1 Pretreatment of Frozen Sections

3.1.1 Fixing

1. Allow slides of sections of approximately 10 μm thickness to equilibrate to room temperature.
2. Label slides with a pencil noting specimen and primary antibody(s) to be used.
3. (a) If the podoplanin antibody D2-40 is to be used, then cover section with 4 % paraformaldehyde for 10 min. Rinse slides carefully over a sink using a wash bottle of PBS (*see* **Note 1**).
   (b) If D2-40 is not being used, then place slides in a coplin jar containing 100 % acetone for 2 min to fix and then remove and air-dry.
4. Place slides in a jar of PBS for approximately 5 min to allow embedding compound around the sections to dissolve.

3.1.2 Blocking

1. Carefully dry the slides using tissue or paper towel and draw around each specimen with a hydrophobic pen to retain the small antibody volumes on the section.
2. Place slides in a staining tray and block nonspecific antibody-binding sites by applying approximately 200 μl of 5 % v/v goat serum in PBS to each section and incubating for 20 min (*see* **Note 2**).
3. Rinse slides carefully over a sink using a wash bottle of PBS (*see* **Note 1**) and place in a jar of PBS for 5 min.
4. Proceed to the appropriate section for either immunohistochemistry or immunofluorescence.

3.2 Pretreatment of Paraffin Sections

3.2.1 Dewaxing

1. Label slides with a pencil noting the specimen and primary antibody(s) to be used.
2. Place slides in a coplin jar containing Citroclear® for approximately 5 min. Remove and drain.

3. Place slides in a second coplin jar containing Citroclear® for approximately 5 min. Remove and drain (*see* **Note 3**).
4. Place slides in a coplin jar containing 100 % ethanol for approximately 5 min. Remove and drain.
5. Place slides in a second coplin jar containing 100 % ethanol for approximately 5 min. Remove and drain.
6. Place slides in a coplin jar containing 50 % v/v ethanol for approximately 5 min. Remove and drain.
7. Place slides in a coplin jar containing water for approximately 5 min. Remove and drain.

3.2.2 Antigen Retrieval

1. Preheat a covered microwave-safe dish containing enough 10 mM citrate buffer (pH 6.0)/Dako antigen retrieval buffer to cover a rack of slides to 100 °C in a microwave.
2. Place the rack of slides into the heated buffer, re-cover, and simmer for 10 min.
3. Place sections in a jar of PBS for approximately 5 min to cool.

3.2.3 Blocking

1. Carefully dry the slides using tissue or paper towel and draw around each specimen with a hydrophobic pen to retain the small antibody volumes on the section.
2. Place slides in a staining tray and block nonspecific antibody-binding sites by applying approximately 200 µl of 5 % v/v goat serum in PBS to each section and incubating for 20 min (*see* **Note 2**).
3. Rinse slides carefully over a sink using a wash bottle of PBS [1] and place in a jar of PBS for 5 min.
4. Proceed to the appropriate section for either immunohistochemistry or immunofluorescence.

3.3 Immunohistochemistry

3.3.1 Dako EnVision Staining

Primary Antibody

1. Carefully dry slides and place in the staining tray.
2. Apply peroxidase block from the Dako EnVision kit (blocks endogenous peroxidase activity that is present in certain tissues) to cover each section. Incubate for approximately 5 min.
3. Rinse slides carefully over a sink using a wash bottle of PBS and place in a jar of PBS for 5 min.
4. Carefully dry slides and place in the staining tray.
5. Apply primary antibody (anti-LYVE-1 or D2-40) at 5 µg/ml in 1 % w/v BSA in PBS. Use approximately 200 µl per section. Incubate for 30 min [4].
6. Rinse slides carefully over a sink using a wash bottle of PBS and place in a jar of PBS for 5 min.

Secondary Antibody and Mounting

1. Carefully dry slides and place in the staining tray.
2. Apply enough of the appropriate Dako EnVision secondary HRP conjugate to cover each section (anti-rabbit for LYVE-1, anti-mouse for D2-40). Incubate for 30 min.
3. Rinse slides carefully over a sink using a wash bottle of PBS and place in a jar of PBS for 5 min.
4. Mix the EnVision peroxidase substrate as per the instructions and apply to each section. Incubate for 5–10 min.
5. Wash slides using distilled water from a wash bottle.
6. Place slides in a jar of hematoxylin solution for approximately 2 min.
7. Wash slides with normal tap water.
8. Carefully dry slides and place in the staining tray.
9. Apply a few drops of Aquamount medium to each section and place a coverslip on top.
10. Invert each slide and press down firmly on a flat pile of paper towels to evenly spread the mounting medium.
11. Seal each slide by painting around the edge of the coverslip with clear nail varnish.

3.3.2 Dako Catalyzed Signal Amplification (CSA) Staining

The Dako CSA System offers a more sensitive immunohistochemical (IHC) staining procedure for the detection of extremely low levels of marker antigen. The method involves the peroxidase-catalyzed oxidation and deposition of biotinylated tyramide, followed by a secondary reaction with streptavidin peroxidase.

1. Carefully dry slides and place in staining tray.
2. Apply peroxidase block from Dako CSA kit (blocks endogenous peroxidase activity that is present in certain tissues). Incubate for approximately 5 min.
3. Place in a fresh buffer (0.05 M Tris–HCl pH 7.6 containing 0.3 M NaCl and 0.1 % w/v Tween 20).
4. Apply protein block solution from Dako CSA kit. Incubate for 5 min, but do not rinse off protein block solution.
5. Tap off excess protein block solution and apply primary antibody. Use approximately 200 µl per section and incubate for 15 min.
6. Rinse gently with buffer and place in up to three fresh buffer baths for 3–5 min.
7. Apply Link antibody (anti-mouse or anti-rabbit) from Dako CSA kit. Incubate for 15 min.
8. Repeat *step 6*.
9. Apply streptavidin-biotin complex (prepared 30 min in advance as per CSA instructions) and incubate for 15 min.

10. Repeat *step 6*.
11. Apply amplification reagent from Dako CSA kit and incubate for 15 min.
12. Repeat *step 6*.
13. Apply streptavidin peroxidase from Dako CSA kit and incubate for 15 min.
14. Repeat *step 6*.
15. Apply substrate-chromogen (prepared in advance as per CSA instructions) and incubate for 5 min. Rinse gently for 5 min in distilled water.
16. Place slides in a jar of hematoxylin solution for approximately 2 min.
17. Wash slides with normal tap water.
18. Carefully dry slides and place in the staining tray.
19. Apply a few drops of Aquamount medium to each section and place a coverslip on top.
20. Invert each slide and press down firmly on a flat pile of paper towels to evenly spread the mounting medium.
21. Seal each slide by painting around the edge of the coverslip with clear nail varnish.

3.4 Immunofluorescence

3.4.1 Standard Immunofluorescence Staining

Primary Antibodies

1. Carefully dry slides and place in the staining tray.
2. Apply primary antibodies (anti-LYVE-1 and D2-40) at 10 μg/ml each in 5 % v/v FCS/PBS. Use approximately 200 μl per section. Incubate for 30 min (*see* **Note 4**).
3. Rinse slides carefully over a sink using a wash bottle of PBS and place in a jar of PBS for 5 min.

Secondary Antibodies and Mounting

1. Carefully dry slides and place in the staining tray.
2. Dilute both fluorescently labeled secondary antibodies together 1 in 500 in 5 % v/v FCS/PBS and apply approximately 200 μl to each section. Incubate for 30 min, ensuring that the light-proof lid is in place for this step.
3. Rinse slides carefully over a sink using a wash bottle of PBS and place in a jar of PBS for 5 min.
4. Carefully dry slides and place in the staining tray.
5. Apply a few drops of Vectashield to each section and place a coverslip on top.
6. Invert each slide and press down firmly on a flat pile of paper towels to evenly spread the mounting medium.
7. Seal each slide by painting around the edge of the coverslip with nail varnish.
8. Store slides at 4 °C in a lightproof box.

3.4.2 Dako CSA Kit Immunofluorescence Staining

1. Perform *steps 1–12* as for Dako CSA kit immunohistochemistry.
2. Add fluorescently labeled streptavidin 1 in 200 in 5 % v/v FCS/PBS and apply approximately 200 μl to each section. Incubate for 30 min, ensuring that the lightproof lid is in place for this step.
3. Rinse slides carefully over a sink using a wash bottle of PBS and place in a jar of PBS for 5 min.
4. Carefully dry slides and place in the staining tray.
5. Apply a few drops of Vectashield to each section and place a coverslip on top.
6. Invert each slide and press down firmly on a flat pile of paper towels to evenly spread the mounting medium.
7. Seal each slide by painting around the edge of the coverslip with nail varnish.
8. Store slides at 4 °C in a lightproof box.

Note: For double immunofluorescent labeling of slides using the Dako CSA kit, a second compatible antibody of a different species that does not require significant amplification can be added after *step 3*. The protocol outlined in Subheading 3.4 for conventional immunofluorescence staining should then be followed.

3.5 Identification of Lymphatic Vessels

3.5.1 Detection of Lymphatic Vessels

Lymphatic vessels should be clearly visible as stained structures within the tissue. However there may be a wide variation in size and morphology. Initial lymphatics may appear as small structures (diameter range 10–50 μm), while larger capillaries may appear as elongated structures, sometimes with a collapsed lumen (diameter range 100–200 μm). Tumor-associated lymphatic vessels may appear as small basketlike structures within the tumor mass or as a continuous endothelium surrounding a tumor embolus [12]. Figure 1 shows typical lymphatic morphologies in normal and tumor tissue detected by immunohistochemistry with antiserum against LYVE-1. Additionally the contrast in morphology between a lymphatic and a blood vessel can be seen.

3.5.2 Confirmation of Lymphatic Identity

Positive staining of a structure for a single lymphatic marker protein should not be regarded as definitive proof of lymphatic identity. For sections stained with antibodies to LYVE-1 and podoplanin by immunofluorescence, it is a simple matter to check the co-expression of marker proteins. For specimens stained by immunohistochemistry, we recommend the staining of consecutive serial sections with each antibody to confirm identification. Two-color staining is also possible using immunohistochemistry, for example, by combining peroxidase and alkaline phosphatase-conjugated antibodies. However, it is this author's opinion that the procedure is more satisfactory for mutually exclusive staining of distinct cell types or for cases where the two markers localize to different

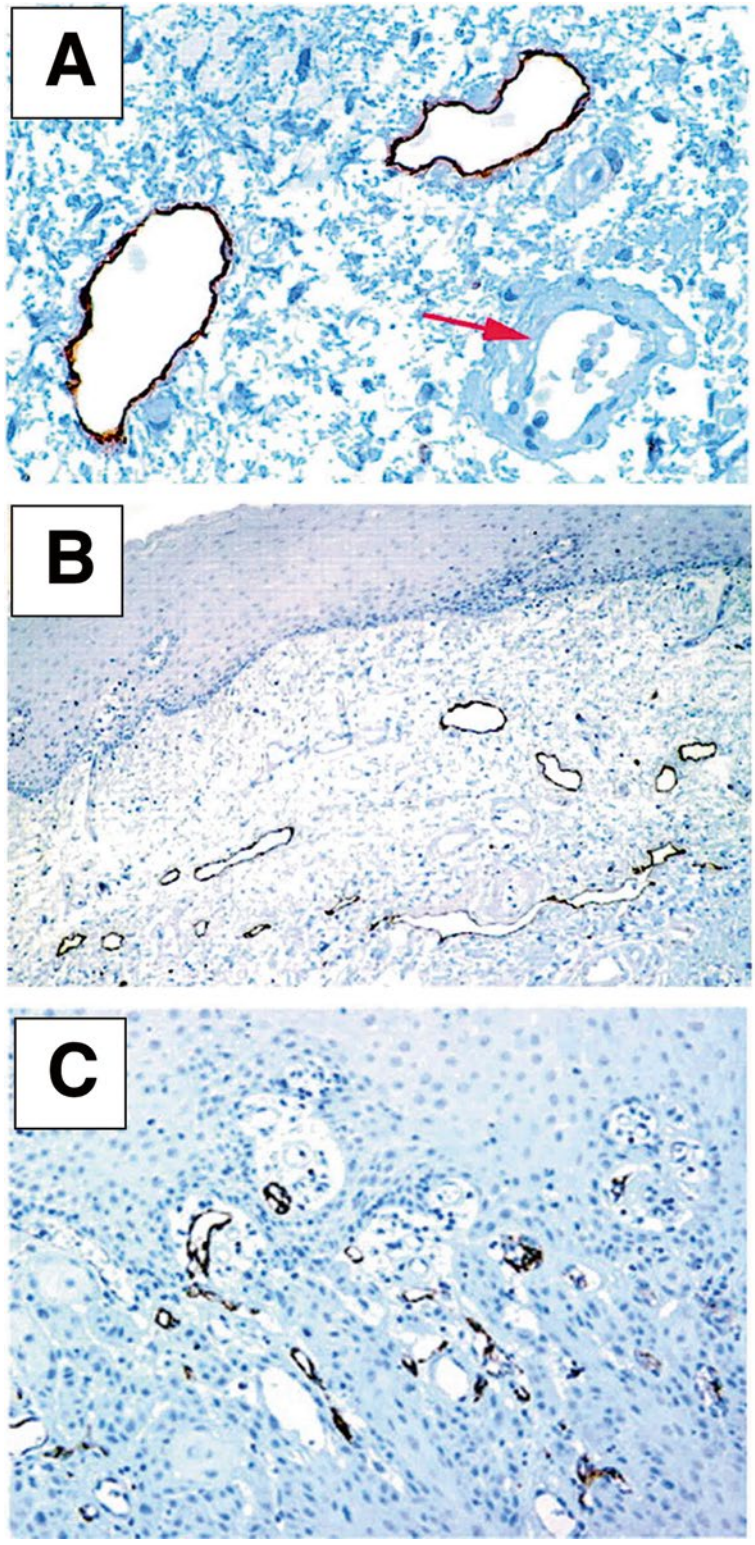

Fig. 1 Immunohistochemical staining of lymphatic vessels in soft tissue. Patent lymphatic vessels of the normal human tongue show strong staining of LYVE-1, while the erythrocyte-containing blood vessel (*red arrow*) shows no staining and a typical thickened vessel wall (×32 objective) (**a**). Abundant LYVE-1 positive lymphatic vessels can be seen beneath the epithelium (×10 objective) (**b**). LYVE-1 positive intratumoral lymphatic vessels within squamous carcinoma of the tongue (×20 objective) (**c**). Adapted from Beasley et al. [12]

regions of the same cell as described later. Regardless of whether immunohistochemistry or immunofluorescence staining is chosen, the results should still be interpreted with caution (*see* **Note 5**). Discrimination between lymphatic vessels and blood vessels is rarely a problem when using these markers together (*see* **Note 6**).

3.5.3 Vessel Hot Spots

In normal tissue, e.g., the dermis, lymphatic vessels may be evenly distributed. However, in tumors these vessels may be concentrated within discrete areas termed "hot spots" induced by agents enriched in the local microenvironment such as lymphangiogenic growth factors (VEGF-C, VEGF-D, PDGF, etc.). Hot spots rather than randomly chosen areas are frequently targeted for tumor vessel counts in the assessment of lymphangiogenesis, although the validity of this practice has been disputed (*see*, e.g., ref. 13).

3.6 Measurement of Mean Lymphatic Vessel Density

This method relies upon the ability of the observer to distinguish discrete lymphatic vessels and count them within a known area, thus giving an actual measurement of lymphatic vessel density within the plane of the section.

3.6.1 Calculation of Area of View

Consult the microscope manufacturer's handbook to obtain the field of view distance in mm for each objective lens to be used. Use this number to calculate the area of view with the formula:

$$\text{Area}(\text{mm}^2) = \pi(\text{field of view} \,/\, 2)^2$$

3.6.2 Counting of Lymphatic Vessels

1. Using a low power objective, a field of view containing lymphatic vessels (e.g., hot spot) should be identified, and a suitable objective lens should be chosen to magnify this area. This size of objective should be constant throughout all of the samples.
2. Each discrete individual stained lymphatic structure (see above), irrelevant of size, is counted as a vessel, and the total within the area of view is recorded.
3. A different region within the same section is then chosen and the vessel number again recorded. This is repeated at least three times and the mean vessel number is calculated.
4. The process is repeated for each section, making sure to keep the objective lens constant.

3.6.3 Calculation of Lymphatic Vessel Density

1. The vessel density is calculated for each section:

$$\text{Lymphatic vessel density}\,(\text{mm}^{-2}) = \text{mean vessel number} \,/\, \text{area of view}\,(\text{mm}^2)$$

2. The application of a suitable statistical method can be used to compare mean vessel densities between tissues and hence levels of lymphangiogenesis (*see* **Note 7**).

3.7 Chalkley Counting

This method does not rely on the observer's assessment of individual vessels, but instead effectively measures the area covered by vessels [9]. A Chalkley eyepiece graticule is required to fit the microscope. This is a rotatable graticule marked with 25 randomly placed spots. Due to the requirement to be able to see the graticule markings against the background of the illuminated slide, this method is only suitable for sections stained by immunohistochemistry, not immunofluorescence.

3.7.1 Determining the Chalkley Count

1. Using a low power objective, a field of view containing lymphatic vessels (i.e., hot spot) should be identified, and a suitable objective lens should be chosen to magnify this area. This size of objective should be constant throughout all of the samples.
2. The graticule is carefully rotated until the maximum number of dots overlaps lymphatic vessels (note that these do not need to be separate vessels), and the number of these dots (maximum score 25) is recorded. *See* Fig. 2 for an example of this.
3. The procedure is repeated a minimum of three times using different regions within the same section.
4. The mean number of dots overlapping lymphatic vessels on a section is the Chalkley count.
5. The application of a suitable statistical method can be used to compare Chalkley counts between tissues (applying cutoffs where appropriate) and hence levels of lymphangiogenesis (*see* **Note 7**).

3.8 Lymphatic Endothelial Cell Proliferation

The basis of this method is that by co-staining with both a lymphatic marker and a proliferation marker, it allows identification of actively dividing lymphatic endothelial cells and hence an accurate measurement of ongoing lymphangiogenesis at the time the tissue was taken. As this method requires two-color staining, it is perhaps most suitable for immunofluorescence; however two-color immunohistochemistry may be used as the lymphatic marker, and proliferation marker proteins are located on the cell surface and nucleus, respectively. Extreme care must be taken in the identification of lymphatic vessels as only a single lymphatic marker antibody is used.

3.8.1 For Frozen Sections

1. Frozen sections must be fixed and permeabilized to allow access to the nuclear compartment. Follow the instructions described in Subheading 3.1.1 for fixing with paraformaldehyde.
2. Before blocking, rinse the section and incubate for 5 min with 0.5 % (w/v) Triton X-100 in PBS.
3. Rinse with PBS and proceed to blocking.

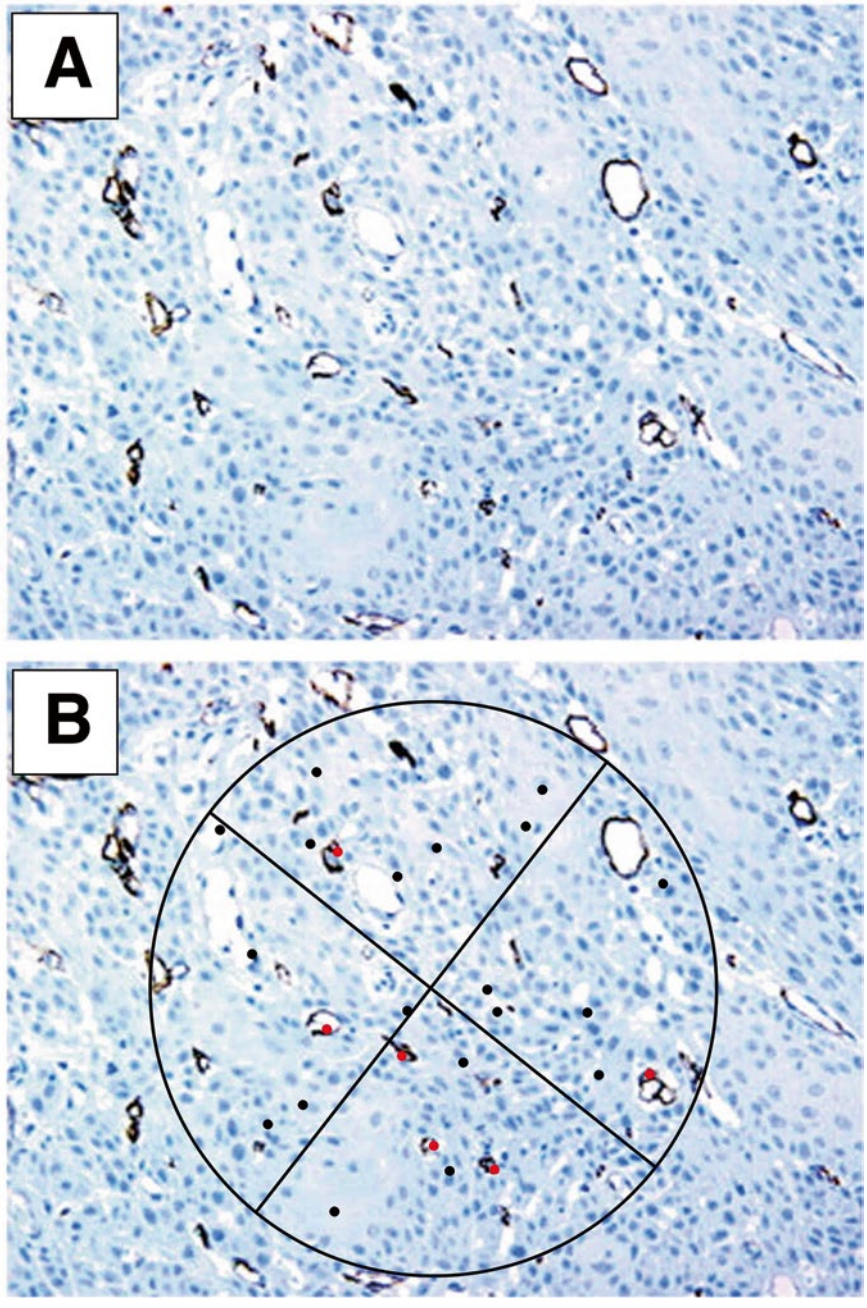

Fig. 2 The Chalkley counting method for estimating lymphatic vessel density. Intratumoral lymphatic vessels of tongue squamous carcinoma stained for expression of LYVE-1 (**a**) are overlayed with a representation of a Chalkley grid (**b**). Dots which overlap lymphatic vessels are highlighted in *red* (×20 objective). Adapted from Beasley et al. [12]

3.8.2 Immunofluorescence

The protocol for frozen or paraffin sections should be followed with the exchange of the podoplanin D2-40 antibody with a mouse monoclonal raised against the proliferation marker Ki-67.

3.8.3 Immunohistochemistry

The Dako EnVision G|2 Doublestain system should be used with anti LYVE-1 and anti Ki-67 according to the manufacturer's instructions.

3.8.4 Evaluation of Staining

1. Proliferating lymphatic vessels will contain cells which are stained positively for both LYVE-1 and Ki-67 expression (*see* Fig. 3). The observer should be aware that tumor tissue and areas of inflammation or wound healing are likely to contain an abundance of proliferating non-lymphatic cells.
2. Mean proliferating lymphatic vessel density may be calculated in a way analogous to the calculation of mean lymphatic density as described in Subheading 3.6 when only vessels containing proliferating cells are scored.
3. Immunohistochemistry may be combined with a Chalkley graticule to give a Chalkley count for proliferating vessels.

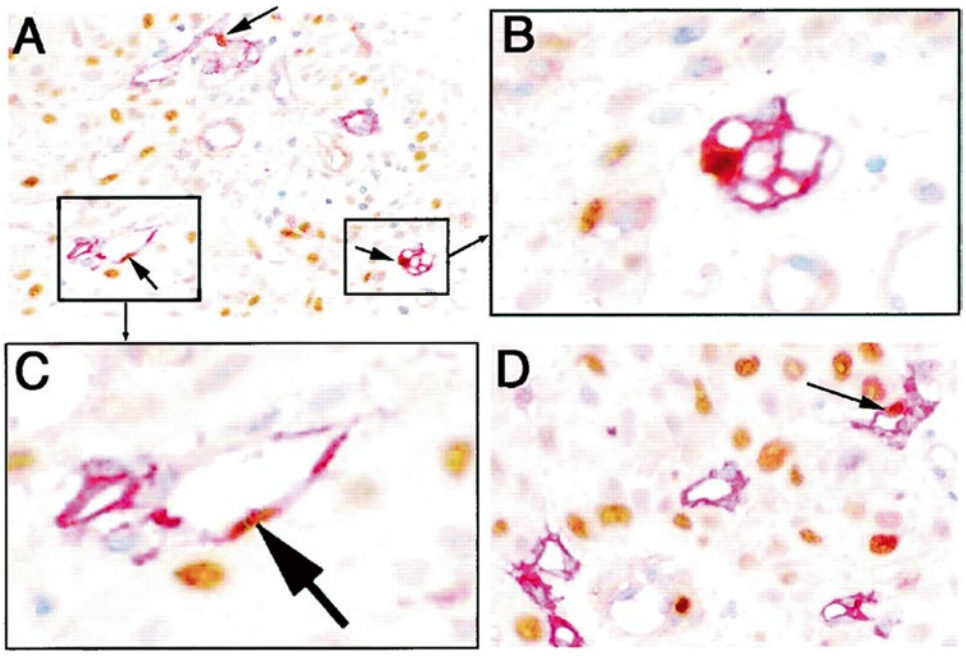

Fig. 3 Immunohistochemical measurement of lymphatic vessel proliferation. Newly dividing intratumoral lymphatic vessels are detected by double staining for the Ki-67 proliferation antigen (*brown* nuclear staining) and LYVE-1 (*pink* membrane staining). Panels (**a**–**d**) show examples of dividing LYVE-1/Ki-67 double-positive small lymph vessels (*black arrows*) surrounded by large numbers of LYVE-1-negative/Ki-67-positive squamous carcinoma cells. Panel (**a**) ×20 objective, panels (**b**) and (**c**) ×100 objective, and panel (**d**) ×40 objective. Adapted from Beasley et al. [12]

4. Using either method, a measurement of the ratio of proliferating to nonproliferating vessels gives an insight into the degree of lymphangiogenesis in the tissue.

3.9 Measuring Lymphangiogenesis in Mouse Tissues

While the analysis of lymphangiogenesis within human tissue samples is clearly important in a diagnostic and prognostic role, it is appreciated that experimental models are widely used both during the study of adult disease and embryonic development. The techniques above are equally valid using mouse tissues providing the correct antibodies are used (*see* **Note 8**). However, the lack of a Dako EnVision kit for rat antibodies limits the immunohistochemistry that can be performed using the particular protocols described above. As an alternative, we suggest use of the Vector Laboratories ImmPRESS™ HRP Anti-Rat IgG, Mouse adsorbed (Peroxidase) Polymer detection kit (Catalogue no. MP-7444).

4 Notes

1. Use a wash bottle with a wide bore spout. Hold the slide almost vertically and aim the jet at a point above the section, allowing the PBS to gently flow down over the section.
2. It is beneficial from this stage onward to ensure that the sections do not dry out. Therefore the slides should be dried in small batches before adding the next solution.
3. When processing large numbers of slides, it may be easier to place the dewaxing solutions in individual small glass tanks and transfer the slides in a rack from tank to tank. Dewaxing solutions may be stored and used for several cycles before replacement with fresh solutions.
4. The use of a negative control antibody is necessary to confirm the validity of the staining. This should either be an isotype-matched antibody or a preimmune serum from the relevant species.
5. LYVE-1, while being a widely used marker protein for lymphatic endothelium, is expressed by other cell types including certain macrophages, liver sinusoidal endothelium, and certain lung alveolar cells. In light of this, single cells staining positive for LYVE-1 should be identified with care, while the use of LYVE-1 as a marker for hepatic lymphatic endothelium is not recommended. It is also worth noting that the expression of LYVE-1 on lymphatic endothelium is downregulated during inflammation. This may clearly lead to an underestimation of lymphatic vessel density during inflammatory conditions. Similarly, podoplanin is expressed by several different cell types (e.g., epithelia and fibroblasts) in addition to lymphatic endothelia and is present in several tumor types, particularly at the

invasive front. Care is therefore required in the interpretation of tumor lymphangiogenesis using this marker. Should problems be encountered with the specificity of LYVE-1 and podoplanin expression in the chosen tissue, antibodies to other marker proteins may be tried. These include the nuclear transcription factor Prox1 and VEGFR3, although again it should be borne in mind that these proteins are also not exclusively expressed by lymphatic endothelium, the former being expressed by hepatocytes and the latter by macrophages and blood vessels associated with tumors and wound healing.

6. Identification of blood vessels in human tissues can be confirmed by positive staining with the antibody PAL-E.
7. It is recommended that all slides are evaluated either single- or double-blindedly by two independent observers to prevent bias.
8. Polyclonal antisera to mouse LYVE-1 are commercially available (R&D Systems, Reliatech, etc.) as is a hamster monoclonal antibody to podoplanin. Most rabbit antisera to human Prox1 appear to cross-react with the mouse protein. The rat antibody MECA32 works well as a mouse blood endothelial-specific marker.

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