# Immunocytochemical staining for lymphatic and blood vessels with frozen sections: using LYVE-1 for lymphatic vessel and von Willebrand factor for blood vessel marker

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ABSTRACT

Almost all human tissues are supplied with lymphatic vessels, yet histological structure and function of lymphatic vessels are still elusive. Most immunocytochemical studies have been performed with the routinely formalin-fixed and paraffin-embedded tissue. This study aimed to investigate lymphatic and blood vessels immunocytochemically with frozen sections to explore the masked lymphatic and blood vessels in the routinely processed normal tissues. Several normal monkey tissues were frozen in liquid propane, and frozen sections were immunostained for lymphatic and blood vessels using LYVE-1 for lymphatic vessels and von Willebrand factor for blood vessels. In the liver, sinusoids were immunostained panlobularlly for LYVE-1 compared to LYVE-1 immunostaining limited in the pericentral lobe with the routinely processed liver. In the jejunal villi, lacteals were surrounded by scattered small venous capillaries. In the large intestines, a few longitudinal lymphatic vessels were detected in lamina propria. Pancreatic islets were surrounded by baskets of rich venous capillaries. Glomerular capillaries were diffusely positive for von Willebrand factor, suggesting leaking capillaries for von Willebrand factor. Prostate had scattered lymphatic and numerous blood vessels in the stroma with nerve bundles in the fibrous capsule. Many lymphatic and blood vessels were immunostained only in frozen sections, which were not immunostained in the routinely processed tissues. Routine processing masks antigen sites of lymphatic and blood vessels. Therefore, immunocytochemical staining with frozen sections is superior to routinely embedded tissues and this immunostaining would further add histopathological information on tumor spread and liver and kidney biopsies.

**Key words:** frozen sections; formalin-fixed and paraffin-embedded; immunocytochemistry; LYVE-1; von Willebrand factor.

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**Ethical approval:** animal care of rhesus monkeys (Macaca mulatta) was provided by the veterinary staff of Animal Resources of the Oregon National Primate Research Center in accordance with National Institutes of Health for care and use of laboratory animals. This study was approved by the Animal Use Committee of the Oregon National Primate Research Center.

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## Introduction

Almost all human organs are supplied by lymphatic vessels except brain, spinal cord, cartilage, bone marrow and eye lenses,[1] yet structure and function of lymphatic vessels in human tissues are still elusive.[2-6] Most immunohistochemical studies had been performed with the routinely formalin-fixed and paraffin-embedded (FFPE) tissues and the precise presence of lymphatic and blood vessels in each organ had not been thoroughly investigated at histological and histopathological levels.[7-10] Immunocytochemical staining for lymphatic and blood vessels is crucial for evaluating cancerous tissues at pathology laboratories but optimal and detailed immunocytochemical staining for the two vessels has not been studied and somehow has been hindered with the FFPE tissues. Formalin fixation, which requires more than 6 h at 25°C results in cross-linking of target proteins and reduces immunoreactivity with specific antibodies.[11] Furthermore, paraffin-embedding through heat exposure in molten wax at 56-65°C for some h will lead to loss of antigenicity.[11-14] We used frozen sections for immunostaining for lymphatic and blood vessels to explore better immunostaining than in FFPE tissues since more epitopes have been detected in frozen sections which have not been revealed with FFPE sections.[15,16] The currently commercially available immunocytochemical markers for the two vessels have been used with the routine FFPE tissues at pathology laboratories.[7-10] The more specific lymphatic markers include LYVE-1 (lymphatic vessel endothelial hyaluronic acid factor receptor-1) and podoplanin, which is a membrane glycoprotein found on lymphatic vessel endothelium and is immunohistochemically stained by the monoclonal antibody, D2-40.[16-20] Immunocytochemical markers for blood vessels include CD31 (platelet endothelial adhesion molecule PECAM-1, found on endothelial cells), CD34 (single-class transmembra-ne silomucin protein, its antibody is used for hematopoietic progenitor cells, positive for blood vessel endothelial cells but not usually lymphatic vessel endothelium), von Willebrand factor (vWF, binds Factor-8, a clotting factor in blood, platelet aggression and adhesion to the cell wall of injured vessels), laminin, collagen IV and VEGRF-1.[21] All blood vessel markers are pan-endothelial markers.[21] We had previously performed immunocytochemical staining for the two vessels with frozen sections of normal monkey tissues[22-24] and are now presenting an immunostaining method with frozen sections.

## Materials and Methods

Normal tissues from *Macaca mulatta* (rhesus monkey) were procured by necropsy at the research laboratory at Oregon Primate National Research Center, Beaverton, OR, USA. Processing frozen tissues for immunocytochemical staining was initially developed at Dr. Robert Brenner's laboratory at Oregon National Primate Research Center, Beaverton, OR[22-24] and was briefly described before.[21] Fresh tissues included liver, pancreas, GI tract (duodenum, jejunum and large intestine), kidney and prostate. For preparing tissue, fresh pieces of tissues (1 x 1 x 0.5 cm) were cut into blocks in Hank's solution in a Petri dish. After removing the extra Hank's solution by placing the tissue blocks on the filter paper, the tissue blocks were placed on the aluminum foil where tissue blocks were embedded in OCT matrix (Fisher Scientific, Pittsburgh, PA, USA) and were wrapped up with aluminum foil to form a square shape. The date of tissues prepared, organ identification and other information were marked on the aluminum foil with ball-pointed pen on the outer side of aluminum foil. Prior to processing tissues, liquid nitrogen was poured into a container, and a plastic bag was placed in the liquid nitrogen pool, to which propane gas was blown into the plastic bag to produce liquid propane. The tissue blocks were carefully handled with tweezers during the tissue handling, not directly touching them with fingers to avoid damaging tissues. Tissue blocks wrapped up in aluminum foil were frozen in the liquid propane and were stored in a deep freezer at -70°C until several tissue blocks were frozen sectioned in the later days. Frozen sections (5 to 7µm) were prepared on a Hacker/Bright Cryostat (Fairfield, NJ, USA) and were mounted on Super Frost Plus slide (Fisher Scientific) and stored at -70°C at deep freezer. For immunohistochemical staining, tissue sections on glass slides were placed on ice at 4°C in Petri dishes and lightly fixed with 2% paraformaldehyde in phosphate buffer at pH 7.4 for 10 to 15 min at room temperature and immersed twice for 2 min in 85% ethanol at room temperature. After fixation, tissue slides were thoroughly rinsed with PBS. Slides were then treated for 20 min with either normal goat or rabbit serum and then incubated with the LYVE-1, D2-40 or vWF primary antibody solution overnight at 4°C. After rinsing and immersion in blocking serum again, sections were incubated with second antibody (1:200 dilutions) for 30 min at room temperature (Table 1). Final visualization was achieved with the ABC kit (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine tetrachloride (Dojindo Molecular Technology, Rockville, MD, USA) for brown coloration. A batch of stored frozen section slides of about 20 were processed for each immunostaining in our hand. Another set of normal monkey liver and large human intestine and pancreas were fixed in a mixture of 2% formaldehyde and 2% paraformaldehyde and were treated with antigen retrieval procedure with citrate buffer pH 6.2. Then, liver tissue slides were double immunostained for LYVE-1 in brown color and vWF in blue color using Vecstatin and Vector SG (Vector Laboratories). Pancreatic tissue was used for LYVE-1 immunostaining in brown color counterstained by hematoxylin in blue color to increase the orientation of tissue structure.

## Results

### Liver and pancreas

In the frozen sections of the liver, sinusoids were immunostained panlobularlly for LYVE-1 while LYVE-1 immunostaining was only around the pericentral lobe and the peri-portal lobe was negative for LYVE-1 immunostaining in the FFPE tissues (Figure 1 A,B). Veins and arteries in the periportal area were positively immunostained for vWR in both in the frozen section (Figure 1C) and the FFPE tissues (Figure 1D). Double immunostaining for LYVE-1 and vWF with paraffin-embedded tissue showed sinusoidal LYVE-1 immunostaining in the pericentral lobe for blue color while brown vWF immunostaining in veins and arteries in the portal lobe stroma (Figure 1D). In the paraffin-embedded pancreatic tissue, pancreatic islets were diffused immunostained for LYVE-1 in brown color while adjacent insulinoma tissue was negative for LYVE-1 (Figure 1E). Frozen sections of pancreas showed abundant tangled basket of venous capillaries in and around the islets and arterial and venous blood vessels in the stroma were strongly immunostained for vWF (Figure 1F).

### Small and large intestines

In the duodenum, small linear lymphatic vessels were revealed in the lamina propria and smooth muscle layer, and large circumferential lymphatic vessels were diffusely distributed between

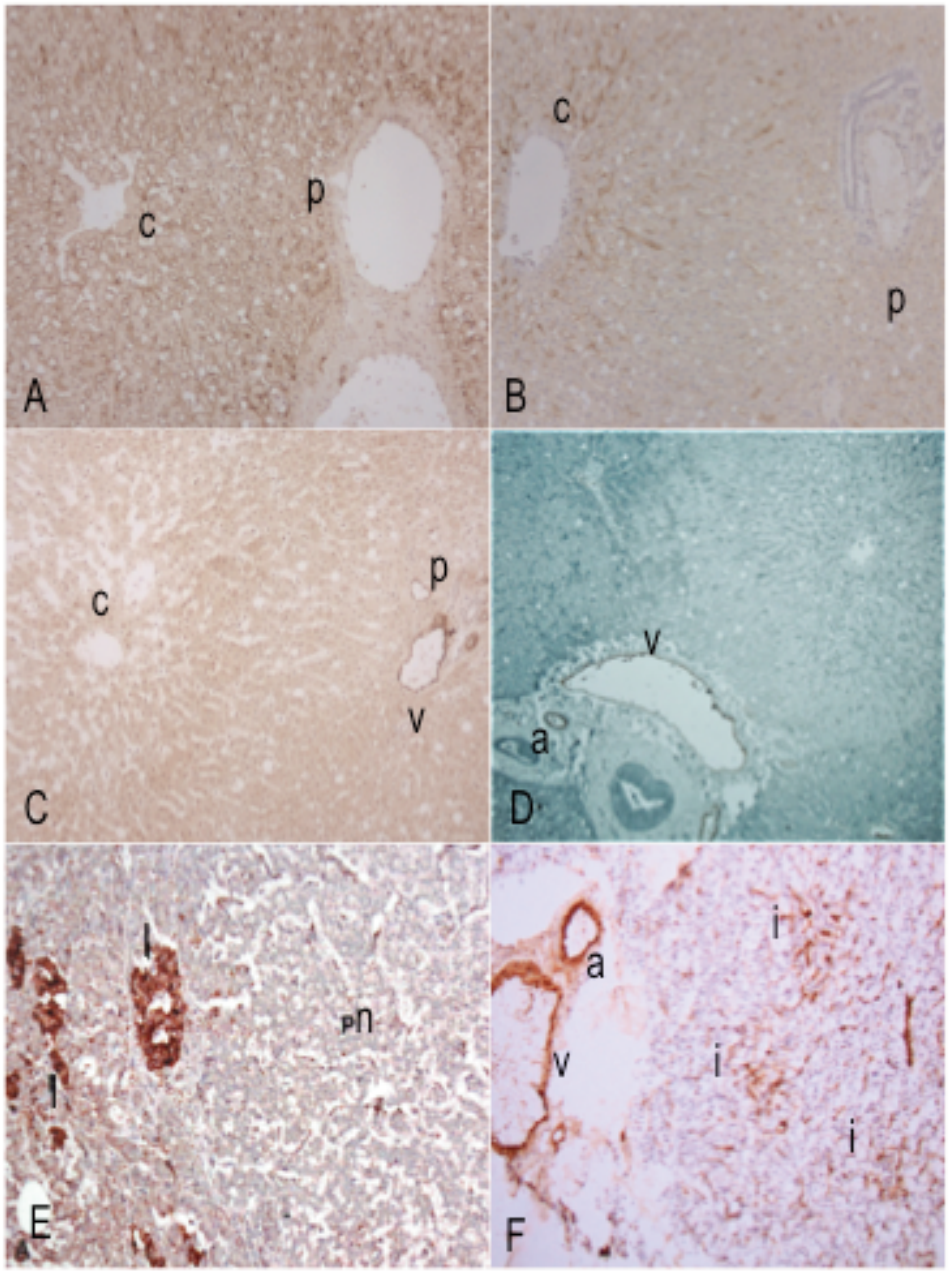


**Figure 1.** Liver and pancreas. **A,B,E**) LYVE-1. **B,F**) vWF. **D**) LYVE-1 and vWF double immunostained. **A-C,F**) Frozen sections. **D,E**) FFPE sections. Hepatic sinusoids were panlobularly positive to LYVE-1 in frozen section (**A**) while positive LYVE-1 immunostaining was limited to pericentral lobe in the paraffin-embedded sections (**B,E**). Blood vessels in portal area were positive for vWF in brown color both in frozen and paraffin-embedded sections; sinusoids were negative to vWF immunostaining with no brown color (**C,D**). Pancreatic islets were densely positive for LYVE-1 in brown color but insulinoma, a pancreatic neuroendocrine tumor was negative to LYVE-1 with no brown color (**E**). Frozen section of pancreas showed abundant tangled basket of venous capillaries in and around the islets and big arterial and venous vessels were strongly positive for vWF in brown color (**F**). a, artery; c, central vein; i, islet; p, portal area; v, vein; **A-F**) 200 x.

**Table 1.** Antibody staining conditions.

| Antibody | Manufacturer | Frozen sections dilution | Paraffin-sections dilution |
|---|---|---|---|
| Goat anti-human LYVE-1 | R and System, Minneapolis, MN, USA | 1: 1,200 | 1:100 |
| Mouse monoclonal D2-40 | Signet Laboratories, Dedham, MA, USA | 1:100 | 1:100 |
| Rabbit human vWF | Dako System, Carpenteria, CA, USA | 1:800 | 1:100 |

inner and outer muscle layer (Figure 2A). There were venous capillaries at the tip of villi, and many blood vessels were present in submucosa (Figure 2 A,B). In the jejunum, large longitudinal lymphatic vessels, lacteals, were in the villi surrounded by small blood vessels (Figure 2 B,C). In the large intestines, there were some longitudinal lymphatic vessels in lamina propria and numerous longitudinal lymphatic vessels in muscle bundles (Figure 2D). There were small venous capillaries in lamina propria (Figure 2F). In the paraffin-embedded human large intestine, there were a few longitudinal lymphatic vessels into the lamina propria spreading from the submucosa counterstained in blue color by hematoxylin (Figure 2G).

### Kidney and prostate

There were a few linear lymphatic vessels around the arteries in the cortex (Figure 3A) while there were many small lymphatic vessels perivascularly in the medulla (Figure 3B). D2-40 immunostaining was positive for Bowman's capsule while LYVE-1 did not immunostain Bowmann's capsule (Figure 3 A-C). Glomerular capillaries were positive for vWF in full thickness of epithelial basement membrane and both thin-walled veins and thick-walled artery were positive for vWF immunostaining (Figure 3D).

In the prostate, there were scattered lymphatic and numerous venous vessels in the stroma with nerve bundles in the fibrous capsule (Figure 3 E,F).

## Discussion

The presence of lymphatic vessels in many organs has been overshadowed and delayed than that of blood vessels due to a lack of specific markers of lymphatic vessels.[7-10]

We would like to discuss the different immunostaining patterns of several normal tissues by comparing the currently presented results using frozen sections with the previously reported results

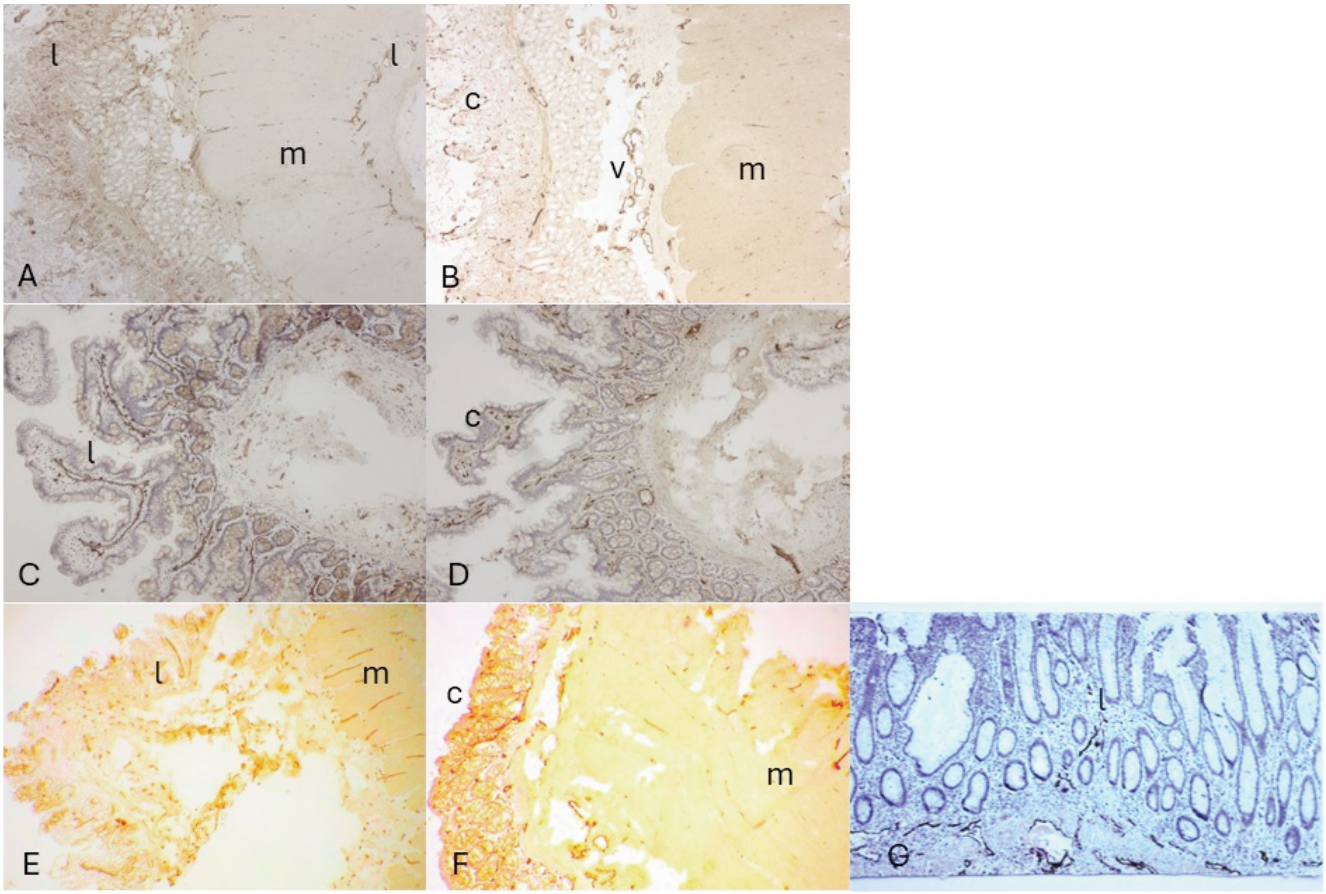


**Figure 2.** Small and large intestines. **A,C,E,G**) LYVE-1. **D,F**) vWF immunostained. **A-**F) Frozen sections. **G**) FFPE section. In the duodenum, there were several vertical lymphatic vessels in lamina propria and smooth muscle layers with abundant circumferential lymphatic vessels between the two smooth muscle layers (**A**). There were venous capillaries at the tip of villi, and many veins were present in submucosa (**B**). In the jejunum, dilated lacteals were surrounded by small venous capillaries (**C,D**). In the large intestines, several vertical lymphatic vessels were detected in lamina propria, and numerous longitudinal lymphatic vessels were in smooth muscle layers (**E**). There were small venous capillaries in lamina propria and several longitudinal venous vessels in smooth muscle layers (**F**). In the paraffin-embedded large human intestine, there were a few longitudinal lymphatic vessels in brown color in the deep lamina propria spreading from the submucosa counterstained in blue by hematoxylin (**G**). c, venous capillary; l, lymphatic vessel; m, smooth muscle bundle; **A-F**) 185x; **G**) FFPE tissue, 270 x.

using FFPE sections as follows. In the liver, hepatic sinusoids are known to be positive to lymphatic markers in the FFPE tissue, but not diffusely in the entire hepatic zones where oxygen-rich zone 1 in the peri-portal zone is negative but oxygen-poor zones 2 and 3, pericentral venous zone are positive for LYVE-1.[25-27] There are two types of sinusoidal epithelia in liver in this context. Type 1 hepatocytes are: LYVE-1$^{-}$, CD34$^{hl}$CD14 in the oxygen-rich zone 1 while type 2 sinusoidal epithelia are LYVE-1$^{+}$, CD32$^{hl}$, CD36$^{mid\text{-}lo}$ in oxygen-poor zones 2 and 3.[25-28] It was considered oxygenation was stronger in zones 2 and 3 which were venous capillaries with more LYVE-1 attached in the epithelia while type 2 epithelia are arterial capillaries with less LYVE-1 attached. Hepatic sinusoids are large capillaries being positive in zones 2 and 3 in FFPE sections but only patchy immunostained for vWF in the literature.[25-28] Hepatic sinusoids were negative for vWF in both frozen section and FFPE sections in this study (Figure 1 C,D). Since all epithelia of undamaged veins and arteries were positively immunostained for vWF in the frozen sections, vWF is attached to the undamaged arterial epithelia to be immunostained for vWF. In FFPE sections, zone 1 was positive and zone 2 was reportedly negative for CD31 while CD 34 stained stronger for arteries than venules, which supports that zone 1 is arterial capillaries.[27-30] While this LYVE-1 positive

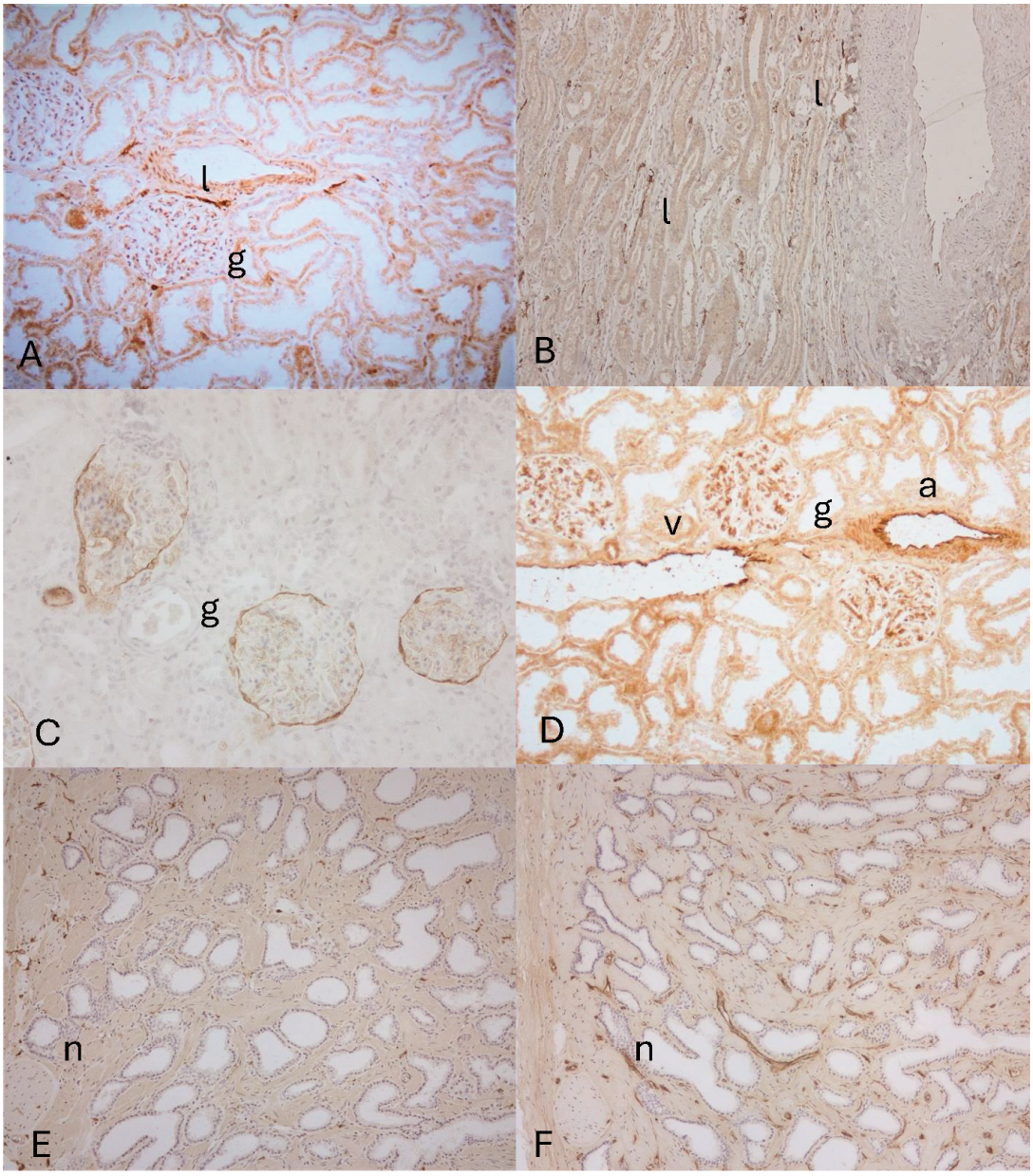


**Figure 3.** Kidney and prostate. **A,B,E**) LYVE-1. **C**) D2-40. **D,F**) vWF immunostained. **A-G**) Frozen sections. There were a few periarterial linear lymphatic vessels in the renal cortex (**A**), while there were more perivenous lymphatic vessels in the medulla (**B**). Bowmann's capsule was positive to D2-40 (**C**). Glomerular capillaries were positive for vWF in full thickness of epithelial basement membrane as well as artery and vein (**D**). In the prostate, there were scattered small lymphatic and numerous venous vessels in the stroma with nerve bundles in the fibrous capsule (**E,F**). a, artery; g, glomerulus; l, lymphatic vessel; v, vein; **A-C**) 185 x; **E,F**) 370 x.

immunostaining in the entire hepatic sinusoids in the frozen sections may imply a phenotypic feature of sinusoidal endothelial component of lymphatic vessel.[27,28] In FFPE embedded and frozen sections of normal human liver, sinusoidal epithelium was positive for LYVE-1 but was negative for D2-40 and vWF.[29] LYVE-1 and Prox-1 were positive in the FFPE embedded human cirrhotic liver but was restrictive for the tumor margins and surrounding normal liver tissue while it was negative to hepatocellular tumors.[30]

Abundant venous capillaries in and around islets were detected in the only frozen sections (Figure 1F), not in the paraffin-embedded sections (*not shown*). Presence of these abundant venous capillaries in and around islets are supported by 5 to 20 times more blood circulation in islets than acinar tissue despite that islets account for 1 to 2% of pancreatic tissue.[31] In FFPE pancreatic tissues, islets were positive for LYVE-1 while an insulinoma tissue, a pancreatic neuroendocrine tumor, was negative to LYVE-1 immunostaining (Figure 1E).

Frozen sections of the duodenum showed small thin linear lymphatic vessels and small round venous vessels in villi while vertical lymphatic and venous vessels were present in the smooth muscle layer with bundles of circumferential lymphatic vessels between the inner and outer muscle layers (Figure 2 A,B).[32,33] The jejunum revealed large elongated lymphatic vessels, lacteals and small capillaries in jejunal villi (Figure 2 C,D).[32,33] The large intestines showed a few vertical linear lymphatic vessels and abundant longitudinal lymphatic vessels in smooth muscle layer (Figure 2E) while there were round small venous capillaries in mucosa and vertical venous vessels in smooth muscle layer (Figure 2F). In the well-fixed FFPE sections of normal large human intestine, a few linear lymphatic capillaries spread into lamina propria from submucosa (Figure 3G). Kennedy *et al*. reported absent lymphatic vessels in normal colonic mucosa but some lymphatic vessels in cases with inflammation and neoplasia in FFPE sections.[34] Lymphangiogenesis was observed in colonic polyps and at the margin of colonic carcinoma by LYVE-1 and vWF immunostaining in the FFPE sections[35] as well as in cases of chronic inflammation, would healing, tumorigenesis and tumor invasion.[35-37] Capillaries of angiosarcoma were immunopositive for both podoplanin and vWF.[17,18]

Kidney revealed a few peri-arterial lymphatic vessels in the cortex while there were abundant perivascular lymphatic vessels in the medulla (Figure 3 A,B). D2-40 immunostained Bowman's capsule (Figure 3C). Glomerular capillaries, which are fenestrated capillaries, were diffusely immunostained for vWF in the full thickness of basement membrane, supporting that glomerular capillaries were permeated by vWF (Figure 3D). Glomerular capillaries were reportedly positive for CD31 and CD34 but negative for vWF in the FFPE sections.[38,39] The full thickness of glomerular epithelial membrane was immunostained for vWF in frozen sections in this study while Pusztaszeri *et al.* with the FFPE sections reported partly positive for vWF and positive for CD31 and CD34 immunostaining in glomerular capillaries.[21] This is a major difference in immunostaining between frozen sections and FFPE sections. There have been no reports on LYVE-1 and vWF immunocytochemical staining for nephritic and nephrotic kidney diseases. In FFPE embedded renal cell carcinoma, blood vessel density and lymphatic density were studied using CD34 and lYVE-1, respectively, which showed a better prognostic outcome in cases with high blood density while worse outcome was recorded in cases with high lymphatic density cases.[40]

In the human prostate, rich lymphatic and venous vessels in the fibrous capsule may implicate access of cancer invasion through the fibrous capsule.[41,42]

The main chemical reaction by FF is the cross-links, primarily methyl ridges (-$CH^2$-) between amino acids of proteins, which are beneficial for preserving tissue structure but detrimental to immunohistochemical staining by physically hiding antigens.[11-13] The main advantage of immunocytochemical staining with frozen sections is the superior preservation of antigens, especially heat-sensitive antigens.[14,15] Frozen sections maintain proteins and other biomedical materials in natural states while FFPE tissues undergo harsh processing that damage and mask antigen epitopes.[15] Thus, antigens are not cross-linked or masked in frozen sections and do not require heat-induced antigen retrieval, which is necessary for FFPE tissues for many antigen epitopes.[14] Therefore, frozen sections required less antibodies of LYVE-1 at 1:1,200 dilutions and vWF at 1:800 dilutions compared to 1:100 dilutions of LYVE-1 and vWf for FFPE tissue sections. Liquid propane embedding induces neither ice crystals nor damage protein or rupture of cells,[22-24] which may occur in the routine tissue freezing procedure. The disadvantage of using frozen sections is that the procedure is labor-intensive and can process relatively small tissue blocks about 1x1x0.5cm for animal tissues and 1.5x1x 0.5cm for human tissues and tissue blocks may crack during freezing for larger tissue blocks.

Immunocytochemical staining with frozen sections provided more precise presence of lymphatic and blood vessels in several tissues compared to the routine FFPE sections. Thus, immunocytochemical staining with frozen sections would add more precise histopathological presence of lymphatic and hematogenous spread of malignant tumors[37,40-42] and histopathological significance of lymphatic and blood vessels in liver diseases such as chronic hepatitis, cirrhosis and hepatic carcinoma[29,30,43-45] and kidney diseases including nephrotic and nephritic diseases and renal cell tumors.[38-40,46] Currently, there are no available truly specific immunocytochemical markers for lymphatic and blood vessels, and future availability of such specific immunocytochemical markers will further provide the precise presence of lymphatic and blood vessels in many normal and pathological tissues as well.

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