

Review

Visualisation and stereological assessment of blood and lymphatic vessels

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Summary. The physiological processes involved in tissue development and regeneration also include the parallel formation of blood and lymphatic vessel circulations which involves their growth, maturation and remodelling. Both vascular systems are also frequently involved in the development and progression of pathological conditions in tissues and organs. The blood vascular system circulates oxygenated blood and nutrients at appropriate physiological levels for tissue survival, and efficiently removes all waste products including carbon dioxide. This continuous network consists of the heart, aorta, arteries, arterioles, capillaries, post-capillary venules, venules, veins and vena cava. This system exists in an interstitial environment together with the lymphatic vascular system, including lymph nodes, which aids maintenance of body fluid balance and immune surveillance.

To understand the process of vascular development, vascular network stability, remodelling and/or regression in any research model under any experimental conditions, it is necessary to clearly and unequivocally identify and quantify all elements of the vascular network. By utilising stereological methods in combination with cellular markers for different vascular cell components, it is possible to estimate parameters such as surface density and surface area of blood vessels, length density and length of blood vessels as well as absolute vascular volume. This review examines the current strategies used to visualise blood vessels and lymphatic vessels in two- and three-dimensions and the basic principles of vascular stereology used to quantify vascular network parameters.

Key words: Visualisation of vascular and lymphatic vessels, Endothelial cell, Mural cell and basement membrane component identification, Three dimensional vascular casts, Two dimensional sectioning, Stereology

Introduction

The circulatory system is comprised of cardiovascular and lymphatic vascular systems. The cardiovascular system supplies tissues with oxygen, nutrients and hormones and removes cell metabolic waste products for excretion. The effective function of this system relies on conduction of nutrient-rich and oxygenated blood from the heart, by the musculo-elastic aorta which branches into muscular arteries and arterioles which conduct the blood to capillaries. Capillaries, whose thin walls are built of a single layer of endothelial cells surrounded abuminally by the basement membrane encasing pericytes (when present), are the site where the exchange of nutrients, oxygen and other compounds required for adequate parenchymal cell function and survival takes place. Capillaries are also a site where cell metabolic waste products, including carbon dioxide, are returned back into the circulation. The capillary network coalesces into the thin-walled venous system that carries the deoxygenated blood to the lung for oxygen enrichment. The vascular system also supplies the liver and kidney. Both organs have multiple roles which involve purification of the circulating blood. The capacity of the vascular system to perform these functions is in part dependent on blood pressure which is required to continuously distribute blood to peripheral tissues (Hall, 2010).

Blood pressure also causes plasma (containing both proteins and an occasional blood cell) to leak through the capillary walls into the interstitial space leading to loss of blood plasma volume and an increase in

interstitial fluid. Most of this fluid is reabsorbed by tissue cells and venules. However, a small percentage of the interstitial fluid from the interstitial space, which contains high molecular weight compounds, including protein, is collected by the initial lymphatic vessels (lymphatic capillaries) and forms a clear fluid, called lymph (Alitalo et al., 2005; Witte et al., 2006). This drainage function is possible because of the distinctive structure of lymphatic capillaries having a discontinuous basement membrane (Pflücke and Sixt, 2009) and button-like endothelial cell junctions (Baluk et al., 2007), which together with lymph backflow, control the opening and closing of endothelial flap valves. These endothelial cell flaps are formed by positioning of the adjacent endothelial cells whereby at the junction of the adjacent endothelial cells, the edge of one cell overlaps the edge of the neighbouring cells (Alitalo et al., 2005; Witte et al., 2006; Baluk et al., 2007). This flapping valve opens only to the interior of the capillary. The subsequent movement of the lymph along the lymphatic vessels is due to semilunar lymphatic valves projecting from the lymphatic wall into the lymphatic vessel lumen which prevent backflow and intrinsic contractility of the lymphatic vessels (Witte et al., 2006).

Lymphatic capillaries are absent from the central nervous system, bone marrow and avascular tissues such as cartilage, cornea and epidermis (Alitalo et al., 2005). In addition to blind-ending initial lymphatic capillaries and draining collecting lymphatic vessels, the lymph passes through lymph nodes, where it is filtered and screened for foreign antigens. The lymph collected from the peripheral tissue coalesces in the thoracic duct and it is emptied into the venous system at the juncture of the left internal jugular vein and the subclavian vein (Alitalo et al., 2005). Alterations in the lymphatic system whereby the conduction of lymph from the peripheral tissue to the vascular circulation is prevented results in a build up of interstitial fluid evident as swelling or oedema (Alitalo et al., 2005; Witte et al., 2006; Jurisic and Detmar, 2009) and is termed lymphoedema.

Traditionally, vascular and lymphatic networks have been identified and quantified by a number of techniques such as visualisation of three-dimensional vascular networks following infusion of materials (resin, or India ink in gelatine) to form solid or semi-solid vascular casts, which are then identified by eroding or 'clearing' the surrounding tissue. The entire three-dimensional branching network can be observed by light microscopy or scanning electron microscopy, recorded and measured. Alternative three-dimensional labelling techniques involve infusion of biotinylated or fluorescently-labelled endothelial cell markers which when perfused into a vascular bed can label the entire vascular network, which is visualised in three-dimensional whole mount tissues. Measurements of the vascular network can then be undertaken. Perhaps the most commonly employed technique to measure the parameters of vascular networks is to embed tissues in paraffin blocks, cut very thin sections (5-7 μm) and

immune-label vessels with specific blood vascular or lymphatic endothelial cell markers. Vascular and lymphatic measurements are then possible using stereology. Stereology is "a technique that enables one to obtain data on the number of identifiable objects in a three-dimensional structure by sampling in two dimensions" (Howard and Reed 2005, pg. xvii) and is extensively used both in materials science and in biology.

For animal and human tissues stereology permits estimation of the total number of structures of interest, length, surface area, volume or three-dimensional spatial distribution or orientation of the structure of interest, such as blood vessels in a three-dimensional organ by sampling the organ in two dimensions (Weibel and Gomez, 1962; Weibel, 1972, 1975, 1989; Cruz-Orive and Weibel, 1990; Howard and Reed, 2005; Dockery and Fraher, 2007). In biological sciences, such estimations are performed with the aid of light microscopy (McDonald and Choyke, 2003), electron microscopy (Nyengaard and Gundersen, 2006; Witgen et al., 2006), confocal microscopy (Czymbek et al., 2001; Wagner et al., 2006) and the application of suitable geometric probes (Cruz-Orive and Weibel, 1990; Howard and Reed, 2005; Nyengaard and Gundersen, 2006; Dockery and Fraher, 2007).

A third broad area which provides visualisation and measurement of vascular systems is high resolution medical imaging. The improvement in resolution capacity of modern medical imaging technologies such as longitudinal micro computed tomography (Abruzzo et al., 2008), positron emission tomography and magnetic resonance imaging now enables *in vivo* non-invasive visualisation of functional vascular beds (McDonald and Choyke, 2003; Ocak et al., 2007; Arkudas et al., 2010). In addition, new technologies such as intra-vital video microscopy for longitudinal temporal studies (MacDonald and Chambers, 2008) and the use of synchrotron radiation to visualise blood vessels (Eppel et al., 2009) have now been reported. In the future, these techniques are likely to be combined with stereological methods to measure the vascular and lymphatic parameters within any *in vivo* research model. Medical imaging technology has not been included in this review. Instead, the following review will examine current approaches in visualising blood vessels in two- and three-dimensions and stereological methods used to evaluate vessel parameters such as vessel number, volume, size and length.

Visualization of blood vessels

To examine the vascular parameters following changes in homeostatic physiological tissue conditions or the effect of a pathological condition on the existing vasculature, the development of new blood vessels during embryogenesis or wound healing, or to assess the development of a vascular network in a three-dimensional tissue engineered construct (extracellular

matrix plus cells implanted *in vivo*) it is necessary to clearly identify and delineate the vessel structures from the surrounding tissue. Arteries, veins and capillaries are easily identified in tissue sections by their specific morphology. The basic blood vessel wall structure includes tunica intima, composed of endothelium and abluminal basement membrane, tunica media composed of pericytes in the capillaries and vascular smooth muscle cells in arteries and veins, also embedded in the basement membrane, and tunica adventitia, composed of connective tissue containing small nerves and small blood vessels (*vasa vasorum*) supplying the blood vessel itself. Blood vessels can be visualised in two dimensions (i.e. tissue sections) or three dimensions (i.e. vascular casts). However, distinguishing between some vessel types, for example, capillaries and post-capillary venules, is much harder as no clear morphological distinction criteria or specific cell markers exist that would clearly distinguish the two vessel types.

While arteries, veins and capillaries differ from each other by their anatomical structure, the capillary structure further differs in each tissue due to functional specialisation of the capillary bed as required by that particular organ (Lokmic and Mitchell, 2008). For example, capillaries in the renal glomeruli where blood filtration takes place are fenestrated and have a thick basement membrane, while those in skeletal muscle lack fenestrae and have a much thinner basement membrane. Therefore, it is necessary to clearly identify capillaries from the surrounding tissue by using endothelial cell-specific markers to aid capillary visualisation and the subsequent quantification. Lymphatic capillaries can also be difficult to morphologically distinguish from blood capillaries and post-capillary venules and are therefore mostly examined by labelling the lymphatic vessels by specific lymphatic endothelial cell markers.

Visualisation of blood vessels in two-dimensions

Visualisation of blood vessels in tissue sections relies on the specific identification of vascular and lymphatic endothelium (Fig. 1). This identification is achieved by antibody-mediated detection of endothelial cell specific products (such as von Willebrand factor for blood vascular endothelium), endothelial cell receptors (i.e. VEGFR-3 for the detection of the lymphatic endothelium), glycoproteins (such as CD31 for blood vascular and lymphatic endothelium, and CD34 for blood vascular endothelium) and use of labelled proteins (i.e. lectins for blood vascular endothelium) that specifically bind to sugars expressed on the endothelial cell surface. However, it remains to be determined which endothelial cell markers can be used to distinguish between the vessels that are angiogenic (growing capillaries) from those that are not (stable capillary networks) within the same tissue compartment. The following section will briefly discuss the most commonly used markers to detect the blood vascular and lymphatic endothelium.

Identification of blood vessels by labelling endothelial cells

a) von Willebrand factor (vWF)

vWF is a large multimeric glycoprotein stored in Weibel-Palade bodies in endothelial cells, subendothelial connective tissue and in α -granules in platelets. Release of vWF mediates platelet adhesion to injured blood vessel walls and exposed subendothelial connective tissue and enables platelet aggregation to form a platelet plug (Sadler, 1998). Although vWF is an intrinsic secretory component of blood vascular endothelial cells and therefore quite reliable as an EC marker, vWF should be used with caution since it has been reported that the alveolar wall capillaries of the lung are mostly negative for vWF (Muller et al., 2002). The staining intensity of vWF increases gradually with increasing vessel diameter (i.e. greater expression in arterial and venular endothelial cells than in capillary endothelial cells) (Pusztaszeri et al., 2006; Baluk and McDonald, 2008). In addition, the interpretation of vessel staining in heavily haemorrhagic and traumatised tissues may be compromised due to heavy secretion of vWF into injured tissue during haemorrhage and trauma (Miettinen et al., 1994).

b) CD31

Cluster of differentiation (CD) molecule 31 (CD31), also known as platelet cell adhesion molecule 1 (PECAM-1) is a 130 kDa glycoprotein that mediates platelet cell adhesion to endothelial cells and plays a role in many physiological events including angiogenesis (DeLisser et al., 1997), interaction of endothelium and leukocytes during inflammation (Woodfin et al., 2007), maintenance of adherens junctions and permeability, organisation of intermediate filament cytoskeleton and control of endothelial apoptotic events (Ilan and Madri, 2003). CD31 expression on endothelium (Fig. 1A,B) is detected on endothelial cell-cell junctions that can easily be seen by light microscopy and therefore distinguished from CD31 staining of platelets, monocytes, macrophages and neutrophils (Newman and Albelda, 1992). However, the limitation of using CD31 is that it is expressed by both the blood vascular and lymphatic endothelial cells (Baluk and McDonald, 2008; Clasper and Jackson, 2009) thus preventing accurate estimates of either the lymphatic or blood vessel parameters, if used alone.

c) CD34

CD34, a member of the sialomucin family of surface molecules, is a glycosylated type I transmembrane glycoprotein (Krause et al., 1996). Systematic examination of seven monoclonal CD34 antibodies developed to detect CD34 expression in human tissues showed that CD34 is expressed on capillaries of most

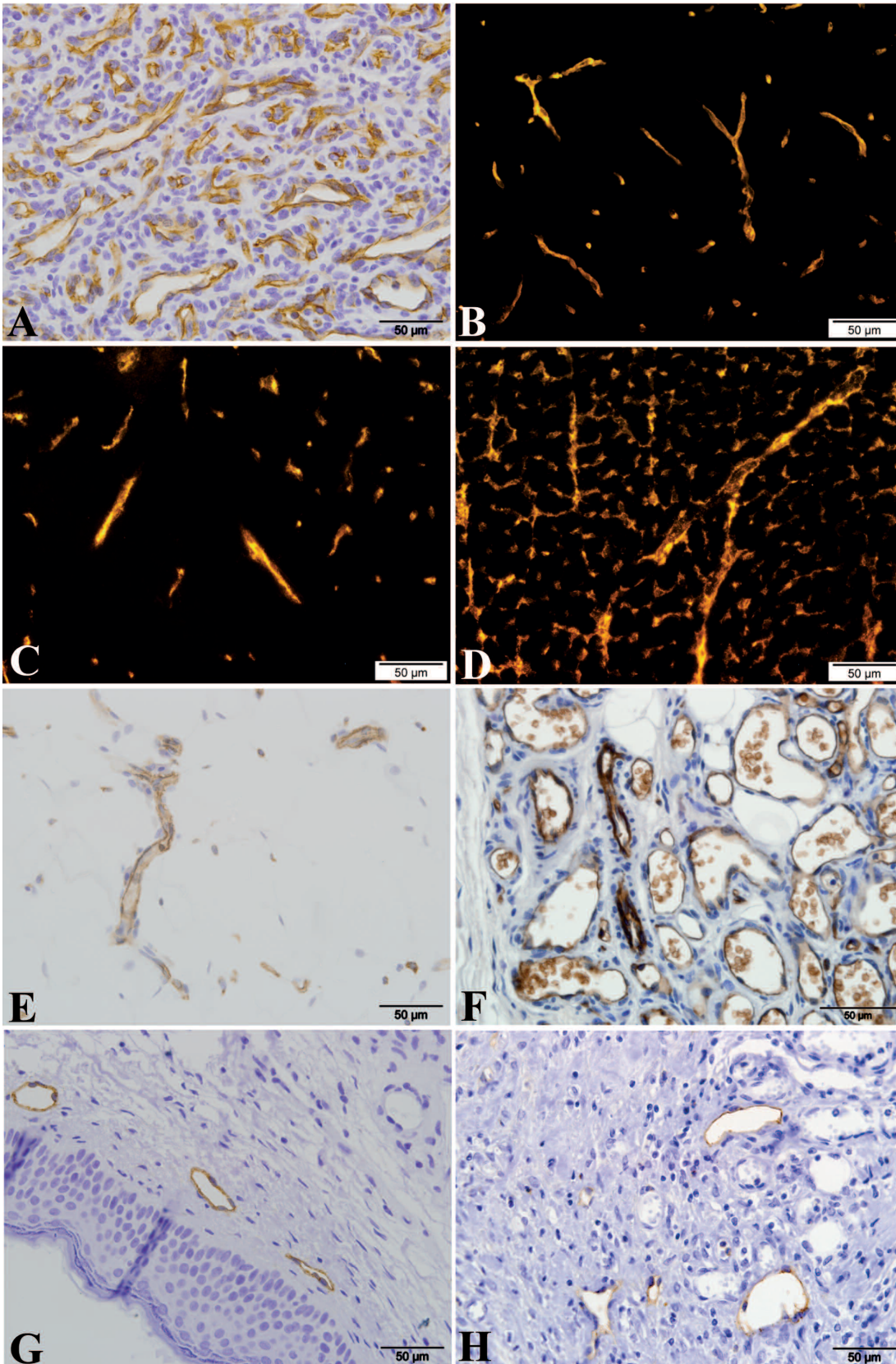


Fig. 1. Immunolabelling of vascular and lymphatic endothelium. Human vascular endothelium can be clearly distinguished with CD31 (clone JC70A, tissue: infantile haemangioma) (A), while CD31 clone MEC13.3 is used in labelling vascular endothelium in mouse (B, brain tissue). CD34 (clone RAM 34) is commonly used to identify mouse endothelium as seen in brain (C) and heart tissue (D). Lectins such as *B. Simplicifolia* can distinguish vascular endothelium in rat cutaneous tissue (E) while *U. Europaeus* labels human vascular endothelium (F, tissue: infantile haemangioma). Human lymphatic vessels are commonly identified by monoclonal antibody D2-40 directed against human podoplanin (G, human skin tissue), whereas anti-podoplanin antibody clone HG19 identifies podoplanin on rat lymphatic endothelium (I, 7-day rat wound tissue).

tissues, with the exception of fetal liver and adult central nervous system where only a small percentage of capillaries were labelled (Fina et al., 1990). It has been suggested that CD34 expressed by lymph node endothelium mediates lymphocyte adhesion to high endothelial venules via L-selectin expressed on lymphocytes (Nielsen and McNagny, 2008).

CD34 is widely used as a marker of vascular endothelial cells (Fina et al., 1990; Baumheter et al., 1993, 1994; Young et al., 1995; Nielsen and McNagny, 2008), with the strongest expression of CD34 reported in capillaries (Fig. 1C, D), followed by arteries, veins, arterioles, and venules (Muller et al., 2002, 2002a; Pusztaszeri et al., 2006). However, the use of CD34 for vascular morphometry in human tissues is limited due to its variable expression on immature and poorly formed vessels as observed in granulation tissue (Suster and Wong, 1994), presence on fibrocytes (Barth et al., 2002) and in stromal cells of some tumours (Kutzner, 1993; van de Rijn, et al., 1994a,b; Barth et al., 2002). In order to delineate between lymphatic and blood vessels in tissues, a combination of CD34 and CD31 are frequently used.

d) Lectins

Direct application of biotinylated or fluorescently-conjugated lectins enables identification of blood vessels on tissue sections. Commonly used lectins for visualisation of blood vessels include *Lycopersicon esculentum* lectin, *Griffonia (Bandeiraea) simplicifolia* lectin and *Ulex europaeus I (UEA-I)* lectin. Lectins are non-enzymatic and non-immune sugar binding proteins which may covalently bind, with high specificity, to a soluble carbohydrate or to a carbohydrate moiety which is a part of a glycoprotein or glycolipid (Sharon and Lis, 2001). *Lycopersicon esculentum* lectin, purified from tomatoes, binds to trimers and tetramers of N-acetylglucosamine oligomers and glycoproteins such as glycophorin and Tamm-Horsfall glycoprotein (Nachbar et al., 1980). *Griffonia (Bandeiraea) simplicifolia* lectin I, isolectin B4, which is derived from *Griffonia simplicifolia*, an evergreen woody climbing shrub native to West and Central Africa binds to sugar galactose present on mammalian endothelial cells (Alroy et al., 1987; Greene et al., 1990) (Fig. 1E). Both the *Griffonia (Bandeiraea) simplicifolia* and the tomato lectin are used to label endothelial cells in rodent vasculature. *Griffonia (Bandeiraea) simplicifolia* lectin has been used to estimate the vascular volume in developing rat arteriovenous (AVL) chamber constructs (Lokmic et al., 2007) and following the in vivo inhibition of the fibrin matrix polymerisation in the AVL construct (Lokmic et al., 2008a). The tomato lectin has been effectively used to visualise and examine rodent tumour vascularisation (Bergers et al., 2003). The UEA-I, isolated from furze gorse, an evergreen shrub (*Ulex europaeus*) seeds selectively binds to glycoproteins and glycolipids containing α -linked fucose residues, such as ABO blood

group glycoconjugates. UEA-I lectin is used to label human blood vessels (Fig. 1F) (Holthofer et al., 1982). While relatively inexpensive, limitations to the use of lectins in clearly visualising blood vessels in tissue sections include their extensive binding to microglial cells (Streit and Kreutzberg, 1987) and subsets of leukocytes (Lee et al., 1987; Matsumoto et al., 2007). In addition, UEA-I lectin binds to normal epithelium and a wide range of epithelial tumour cells (Ko et al., 1996).

e) Other endothelial cell-specific markers

Less frequently employed endothelial cell markers for visualisation of blood vessels include endoglin (CD105), an accessory protein of the transforming growth factor beta (TGF- β) receptor system (Dallas et al., 2008), antibody MECA-32, a monoclonal antibody specific for the mouse embryonic and adult vascular endothelium (Hallmann et al., 1995) and monoclonal antibody Pathologie anatomie Leiden-endothelium (PAL-E). The use of endoglin as an endothelial-specific marker is limited as it is also expressed by pericytes (Crisan et al., 2008), vascular smooth muscle cells, bone marrow stromal fibroblasts, progenitor B cells, interstitial macrophages as well as vessels in gastrointestinal, breast, prostate and head and neck malignancies (Dallas et al., 2008).

MECA-32, an antibody which reacts with mouse plasmalemmal vesicle protein which is present on the surface of most endothelial cells, can detect small arterioles and venules, but not capillaries in the skeletal and cardiac muscle and in the case of brain blood vessels, it is present only until embryonic day 16 of mouse gestation (Hallmann et al., 1995). In addition, MECA-32 expression in cardiac muscle and the central nervous system blood vessels can be induced by inflammation (Engelhardt et al., 1994).

PAL-E antibody also reacts with human plasmalemmal vesicle 1 (PV-1) protein (Schlingemann et al., 1985; Niemela et al., 2005) and is thought to play a role in leukocyte transendothelial migration (Keuschnigg et al., 2009). PAL-E is completely absent from the human lymphatic endothelium and could therefore be considered to specifically interact with human blood vascular endothelium, thus allowing a discrimination between blood vessels and lymphatic vessels (Niemela et al., 2005; Baluk et al., 2007). However, the use of PAL-E is also limited as it is not always expressed on arteriolar and arterial endothelium (Schlingemann et al., 1985; Clarijs et al., 2002). Furthermore, like MECA-32, PAL-E is present in the adult brain blood vessels during inflammation only (Leenstra et al., 1990).

Identification of blood vessels by labelling pericytes and vascular smooth muscle cells

In addition to identifying endothelial cells, the vascular structures can also be identified by using

markers specific for vascular smooth muscle cells and pericytes (Fig. 2) that reside abluminally to the endothelium in the part of the vessel called the tunica media. To date, it remains difficult to immunophenotypically distinguish between pericytes and the vascular smooth muscle cells, particularly in the smaller vessels such as post-capillary venules.

The literature suggests that pericytes act as progenitor cells with the potential of developing into osteogenic, chondrogenic and adipose cells (Díaz-Flores et al., 2009) and can regenerate skeletal myofibers in dystrophic immunodeficient mice (Dellavalle et al., 2007). In addition to expressing α -smooth muscle actin (α -SMA) (Fig. 2A) and desmin (Fig. 2B), platelet-derived growth factor β receptor (PDGFR- β), NG-2 proteoglycan (Fig. 2C) and the regulator G-protein signalling RGS5 (Lamagna and Bergers, 2006), pericytes also express mesenchymal stem cell markers CD44, CD73, CD90 and CD105 (Crisan et al., 2008). Like pericytes, most vascular smooth muscle cells are also positive for α -SMA and desmin, plus calponin and caldesmon but are negative for NG-2 (Hughes and Chan-Ling, 2004). However, for the overall visualisation of the microvascular network for stereology it is sufficient that the cells of the tunica media are identified either by the expression of α -SMA or desmin.

Identification of blood vessels by labelling the basement membrane components

The components of the vascular basement membrane, a highly interconnected glycoprotein network, are synthesised by the endothelial cells, the pericytes and the vascular smooth muscle cells (Hallmann et al., 2005). While the precise molecular composition of the basement membrane differs between arteries, veins and capillaries, the major building block is composed of collagen type IV scaffold interconnected to a laminin network (composed of laminin type 411 and 511) via heparin sulphate proteoglycans, perlecan and the nidogens (Lokmic et al., 2008b). The final end-product is a thin sheet-like structure that separates the

endothelial cells from the supporting pericytes in capillaries and vascular smooth muscle cells in other vessel types. In the literature, the most frequently employed antibodies used to visualise vascular basement membranes are antibodies to collagen IV and pan-laminin (Fig. 3A,B), an antibody which detects most laminin forms. However, since these molecules are present in other basement membranes, their correct interpretation and use is tied to the examiner's ability to recognise the histological appearance of different blood vessels and to clearly distinguish them from other tissue components. Often immunohistochemical/immunofluorescent co-localisation of collagen IV or laminin in combination with endothelial, pericyte and/or smooth muscle cell-specific markers is used (Fig. 3C-E).

Visualisation of blood vessels in three dimensions

Improvements in confocal microscopy and development of fluorochrome filters and software that allows the z-stack acquisition, has enabled the user to create true three-dimensional images of the vascularisation of a tissue sample. It is routine to visualise the three-dimensional vascular network either through the *in vivo* injection of antibodies to specific endothelial cell markers (see previous section) or by perfusing the vasculature with space-occupying compounds such as low viscosity resins (Fig. 4), India ink, dextrane and cell-binding lipophilic dyes such as 1,1'-diocadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, commonly known as DiI. The following section will briefly discuss currently used strategies to visualise three-dimensional vascular networks.

Vascular perfusion with space-occupying compounds

a) India ink

Perfusion of India ink/gelatine solution under the animal's own haemostatic pressure (90-100 mmHg) via the vena cava, and subsequent tissue dehydration

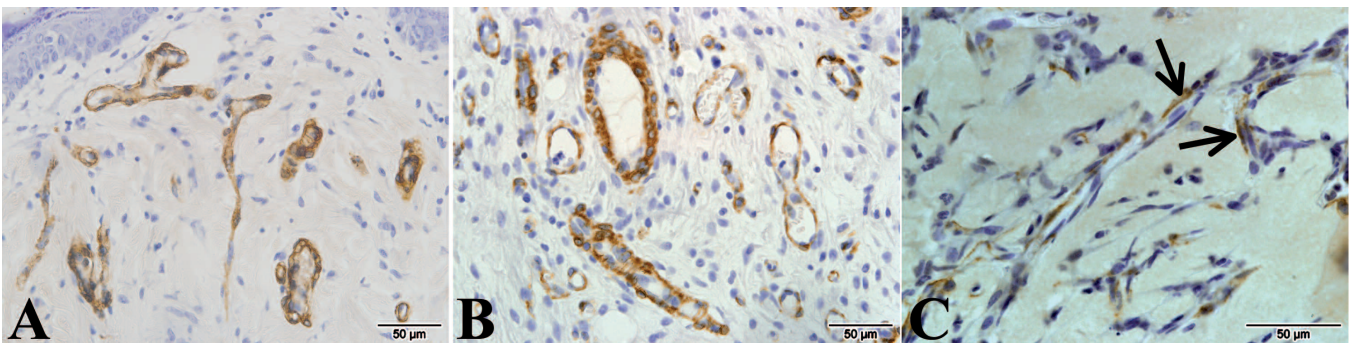


Fig. 2. Blood vessels can be identified by immunolabelling the vascular smooth muscle and pericytes. α -SMA (A, tissue: 7-day cutaneous rat wound) and desmin (B, tissue: 7-day rat cutaneous wound) are used to identify vascular smooth muscle cells, whereas NG-2 is often used to label the pericytes in the blood vessels as seen here in a tissue construct harvested from a mouse tissue engineering chamber (C).

Vascular morphometry

through graded alcohols and clearance in methyl salicylate or cedar wood oil enables visualisation of tissue neovascularisation (Hickey et al., 1998), vascularisation of implanted scaffolds (Andrade et al., 2007) and inosculation of the recipient vascular bed and implanted tissue engineering construct vasculature (Zhao et al., 2010). India Ink/gelatine is relatively inexpensive and easily processed into paraffin and methacrylate blocks for sectioning of thin and thick sections. India ink/gelatine is a space-occupying high contrast material that fills the lumen of blood vessels, but it does not stain endothelial cells, and therefore, India ink cannot visualise solid vascular cords as exist in early angiogenesis. Infused India ink/gelatine is also easily displaced through physical manipulation, evident as interrupted vascular patterns (Fig. 4). To avoid this pitfall, it is helpful to tie off the main vessels leading into the tissue and to fix the tissue at 4 degrees.

b) Dextran

Fluorescently conjugated dextrans of different molecular weights are frequently used for studying vascular permeability and the microcirculation *in vivo*

(Bellhorn et al., 1977; Rabkin et al., 1977; Smith et al., 1994) and to visualise tumour vasculature (Kurozumi et al., 2007). Dextran, a synthetic carbohydrate, is considered to be a relatively inert compound as it does not directly bind to the endothelium and as such does not label new vascular sprouts (Smith et al., 1994; Li et al., 2008). Use of dextran was initially of limited value due to fast photobleaching of FITC and rhodamine fluorochromes however conjugation of dextrane to more stable fluorochromes such as the Alexa dyes has lead to extended photostability (Panchuk-Voloshina et al., 1999).

c) Lectins

Biotinylated or fluorescently-conjugated *Bandeirea simplicifolia* (Fig. 1E) (Jiao et al., 2004) and *Lycopersicon esculentum* lectins (Ezaki et al., 2001; Matsumoto et al., 2004) have effectively been used to label blood vessels *in vivo* by injecting approximately 100 microliters of the conjugated lectin into the systemic circulation via the tail vein or injection into heart chambers 5-10 minutes prior to animal euthanasia (Bergers et al., 2003). The entire organ or tissue of

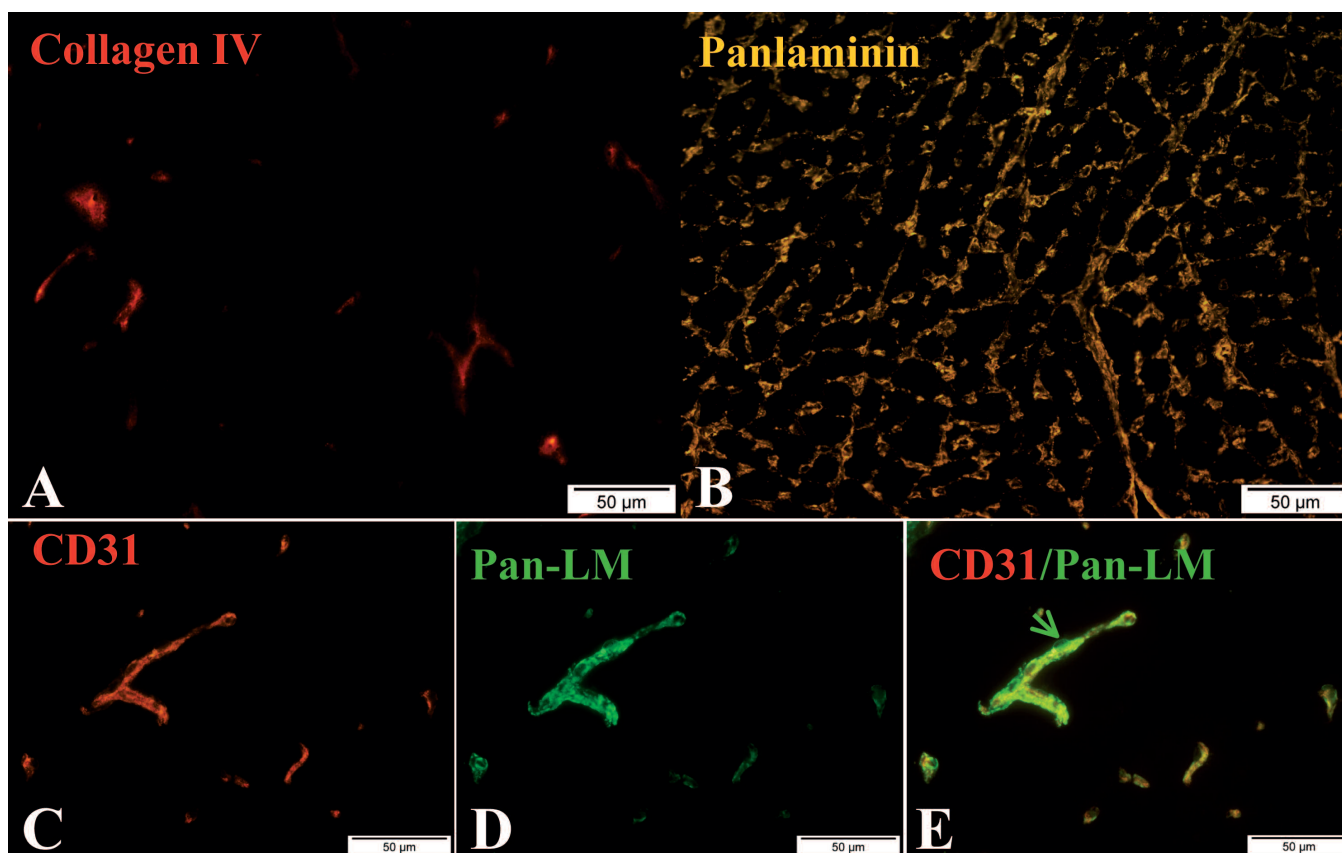


Fig. 3. Blood vessels can be identified by staining vascular basement membranes using antibodies to collagen IV (A) (Clone CIV 22) and panlaminin (B). However, since basement membranes are also present in other epithelial structures, the collagen IV and panlaminin are often combined with vascular endothelial markers such as CD31 (C, D and E, mouse brain tissue) to distinguish vascular basement membranes.

interest is then removed, processed into frozen blocks and cut at 50-100 micron thickness for visualisation of the vascular network by confocal microscopy and subsequent stereological measurement. Similarly, *Ulex europaeus* (Fig. 1F) has been used for intravital perfusion of human umbilical cord, vena saphena magna and *ex-situ* intravital perfusion of human rectal wall (Debbage et al., 2001). In addition to being relatively inexpensive, the value of lectin perfusion labelling has been improved by conjugating lectins to Alexa dyes which provides extended photostability (Panchuk-Voloshina et al., 1999) and therefore enables better confocal microscopy image acquisition with high signal intensities.

d) DiI

Recently, a protocol has been described to visualise blood vessels by cardiac perfusion of an aqueous solution of sodium chloride, glucose and DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), a lipophilic carbocyanine dye, which incorporates into endothelial cell membrane, angiogenic sprouts and pseudopodial processes of the angiogenic endothelial cells (Li et al., 2008). Intensely fluorescent DiI-stained vessels can then be visualised by conventional and confocal microscopy thus enabling not only three-dimensional reconstruction of the vascular tissue network but also its quantification via stereological methods. The disadvantage of this technique is that DiI is a lipophilic molecule, thus use of lipophilic solvents (such as ethanol, xylene and histolene) will result in loss of DiI labelling.

e) Vascular corrosion casting

Perfusion of a low viscosity resin such as methacrylate (Bonner-Weir and Orci, 1982) or Mercox resin into an animal, via the vena cava (Wagner et al., 2006) or the main artery supplying a tissue engineered construct (Lokmic et al., 2007) or organ (Fig. 4), generates a cast of the vascular network. While the cast can be visualised by light microscopy, in our experience, following potassium hydroxide tissue maceration, the resin casts of the AVL construct vasculature become fragile and break easily resulting in loss of fine detail. In addition, we found that blue Mercox resin is incompatible with methyl salicylate whereby a loss of resin rigidity and colour was observed. Thus visualisation of the entire vascular network of the whole mount under the dissecting light microscope was not feasible (Z. Lokmic, unpublished data). It is only when casts are processed for scanning electron microscopy, that the vascular casts enable quantification of vessel size, shape, distribution, branching, arterio-venous anastomosis and coiling, and, visualisation of detailed internal surface vessel morphology (Hessler and Douglas, 2001; Wagner et al., 2006). Detailed description of vascular corrosion casting techniques have

been previously published (Verli et al., 2007).

Recently, a quantification method, using confocal z-stacking through a corrosion cast of the glomerulus and computer modelling for estimation of total capillary length, number of segments and branching points of the resin-cast capillary system, has been reported. To achieve this, the study employed a mixture of blue and clear Mercox resin (1:1 ratio) to obtain a cast that was fluorescent when excited with 633 nm confocal microscope laser light (Wagner et al., 2006). Overall, while a tremendous amount of information can be obtained through vascular casting, accessibility to the scanning electron microscope and the time-consuming process of cast preparation and processing for SEM, make this technique a poorly utilised option.

Visualization of lymphatic vessels

The lymphatic circulatory system plays a vital role in draining the excess extracellular fluid from the tissue into the systemic circulation as well as in the immune defence system whereby it facilitates transport of antigen-presenting cells and antigens into the lymph nodes where the appropriate immune responses will be initiated. Histologically, the initial lymphatic capillaries are blind ending with a large and irregular diameter, being composed of an internal endothelial lining (Alitalo et al., 2005; Baluk et al., 2007; Pflücke and Sixt, 2009). However, in routine histological staining of paraffin embedded tissue, due to tissue processing, sections of lymphatic capillaries are often difficult to visualise

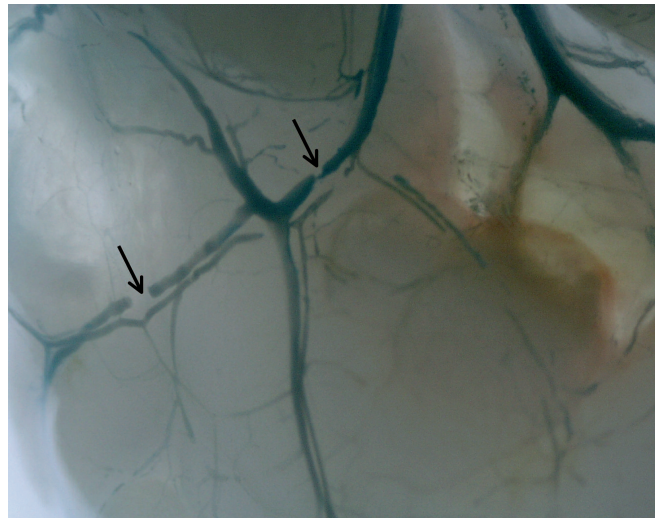


Fig. 4. Blood vessels can be visualised in three dimensions by perfusing the vasculature with space occupying compounds such as low viscosity resins, an example is blue Mercox resin. However, in the case of blue Mercox resin, such visualisation is compromised by disintegration of the cast once the tissue is processed for maceration leading to breakage of the cast, such as seen here where Mercox resin was used to perfuse rat intestinal vasculature (arrow). x 2.4

because they readily collapse. Therefore, it is necessary to employ lymphatic endothelial cell specific markers to visualise such vessels as well as to reliably distinguish the lymphatic vessels from small blood vessels.

Visualisation of lymphatic vessels in two-dimensions

While a variety of cell surface molecules have been proposed as specific for lymphatic endothelium, none is an ideal choice (Baluk and McDonald, 2008; Clasper and Jackson, 2009). Most reports have employed VEGFR-3, podoplanin (Fig. 1G,H) or LYVE-1 to visualise lymphatic vessels. The use of these markers for the immunohistochemical analysis of lymphangiogenesis in both frozen and paraffin-embedded tissue sections has been described recently (Clasper and Jackson, 2009) and will be only briefly discussed here.

a) Podoplanin

Podoplanin, an integral 43 kDa transmembrane mucoprotein was first described in podocytes (Breiteneder-Geleff et al., 1997) but was later described in follicular dendritic cells and follicular dendritic sarcomas (Xie et al., 2008), myoepithelial cells of the breast and salivary glands, myofibroblasts of the prostate, peritoneal mesothelial cells, osteocytes and stromal reticular cells of lymphoid organs (Schacht et al., 2005), as well as Kaposi's sarcoma and a subset of angiosarcomas (Kahn et al., 2002). D2-40 is a commercially available monoclonal antibody directed against human podoplanin and is commonly used to label human lymphatic vessels (Fig. 1G) (Schacht et al., 2005; Zeng et al., 2005). Commercially available antibody to gp 38 (clone 8.1.1) is used to detect mouse lymphatic vessel podoplanin (Farr et al., 1992; Schacht et al., 2003). In rat tissue the lymphatic vessels have been detected with anti-podoplanin antibody clone HG19 (Fig. 1H).

b) LYVE-1

Lymphatic Vessel Endothelial Receptor 1 (LYVE-1) is a cell surface receptor expressed on both the luminal and abluminal surface of lymphatic endothelial cells and normal liver blood sinusoids (Mouta Carreira et al., 2001; Prevo et al., 2001). A homologue of CD44, LYVE-1 is a transmembrane receptor for hyaluronan, an anionic, non-sulfated extracellular matrix glycosaminoglycan distributed abundantly throughout the body (Jackson, 2009). Immunohistochemical staining of LYVE-1 permits visualisation of the lymphatic capillaries and their subsequent morphometric assessment and morphologic description. Recently it has been reported that while initial lymphatic vessels are positive for LYVE-1 and gp 38, the LYVE-1 staining is absent in the collector lymphatic vessels (Makinen et al., 2005; Pflücke and Sixt, 2009).

c) VEGFR-3

VEGFR-3, also known as Flt-4, is a tyrosine kinase receptor for VEGF C and VEGF-D. During murine embryogenesis, VEGFR-3 is widely expressed and it is believed to play a role in remodelling of the primary vascular plexus, haematopoiesis and cardiovascular development (Pajusola et al., 1992; Kaipainen et al., 1995; Dumont et al., 1998). However, in adulthood this expression becomes confined mainly to lymphatic endothelium (Kaipainen et al., 1995). Endothelial VEGFR-3 expression in all lymphatic vessels, including the thoracic duct, indicate that VEGFR-3 is a reliable marker of all lymphatic vessels, however, in adult human tissues, VEGFR-3 is also detectable on fenestrated blood capillaries in the spleen, liver, bone marrow, kidney glomeruli and endocrine glands (Partanen et al., 2000) and should therefore be used in combination with a reliable vascular endothelial cell marker.

Visualisation of lymphatic vessels in three dimensions

Specific visualisation of lymphatic vessels in small animals is a challenging task since this system is not a continuous circuit (as the arterio-capillary-venous circuit is) but rather drains into the vena cava and originates in the tissues as small uni-directional blind-ending capillaries. A common approach for visualisation of lymphatic vessels in mouse is by injection of fluorescently labelled dextran molecules or specific large and small molecular weight antigens into the tissue distal to the draining lymph node. However, the predominant aim of these experiments is not to visualise the lymphatic vessels but to specifically track antigen movement from peripheral lymphatic vessels to lymph nodes. These molecules are picked up by the dendritic cells located in resident tissue, close to the site of injection and carried to lymph nodes for immune surveillance. Therefore, visualisation of lymphatic vessels is indirect and high-resolution light microscopy and multiphoton-intravital microscopy are needed to visualise antigen trafficking (Halin et al., 2005; Sixt et al., 2005; Roozendaal et al., 2009).

While the lymphatic vessels of organs and tissues are difficult to visualise, whole mount immunostaining of mouse ear skin by lymphatic endothelium specific markers permits visualisation of lymphatic vessels in three-dimensions (Shayan et al., 2007; Pflücke and Sixt, 2009). Such vessels can then be quantified using imaging software such as Image J (Abramoff et al., 2004) combined with a plug-in designed specifically for quantifying lymphatic vessel branches, loops, vessel diameter, density and inter-lymphatic vessel distance (Shayan et al., 2007). In humans, a range of non-invasive imaging techniques such as X-ray imaging, computer tomography, positron emission tomography, ultrasound, magnetic resonance imaging and

fluorescence lymphangiogram are used to visualise lymph nodes and larger lymphatic vessels (Sharma et al., 2008). However, such approaches to visualising the human lymphatic system are in the early stages of development and are yet to be adapted for use in small animals and for morphometric quantification.

Stereology

Following selection of the most appropriate method for visualising blood vessels and lymphatic vessels, the next step is to quantify these structures. Such quantification is performed through stereological methods, a science concerned with obtaining quantitative information about three-dimensional microstructure from observations made on two-dimensional (i.e. planar tissue sections) or three-dimensional tissues (i.e. whole mounts) (Carpenter, 1979; Howard and Reed, 2005). The following section will describe commonly used stereological methods to estimate vessel parameters. While in the text the term 'vessel' is used, it is important to note that the same method applies to both lymphatic and vascular vessel stereology.

Together, a combination of stereology and microscopy enables quantitative objective analysis of experimental procedures on the structure of interest. In biological sciences, stereology is commonly known as morphometry. Based on fundamental principles of geometry and statistics, stereology utilises random, systematic sampling with geometric probes to provide unbiased and quantitative data (Weibel, 1969, 1975;

Cruz-Orive and Weibel, 1990; Howard and Reed, 2005; Nyengaard and Gundersen, 2006; Dockery and Fraher, 2007; Weibel et al., 2007). In the context of blood vessels, most frequently estimated parameters include densities of vessel number, length, vessel volume and surface area (Dockery and Fraher, 2007). For statistically valid stereological extrapolation from plane sections to three-dimensional tissues, it is necessary to select a section at random and to assume that the tissue section is a representative of the whole tissue sample (Weibel, 1969, 1975; Cruz-Orive and Weibel, 1990; Howard and Reed, 2005; Nyengaard and Gundersen, 2006; Dockery and Fraher, 2007; Weibel et al., 2007).

Tissue sampling

The quality of vascular parameter quantification in terms of accuracy and unbiasedness is dependent on the appropriate sampling. In particular, the samples used for measurement should be of a uniform size and randomly selected so that any part of the tissue for examination should have an equal chance of being selected for the measurement (Howard and Reed, 2005). Selecting tissues based on its appearance or presence of 'hot spots' of vascularisation as seen in tumours (Weidner, 2008) leads to sampling bias and inaccurate estimation. This type of measurement only represents an estimate in a portion of the tissue examined and not the entire tumour. In addition to sampling bias, systematic bias, which is not detectable from observations, occurs when the method used to quantify the object of interest is biased (i.e. incorrect instrument calibration, tissue shrinkage,

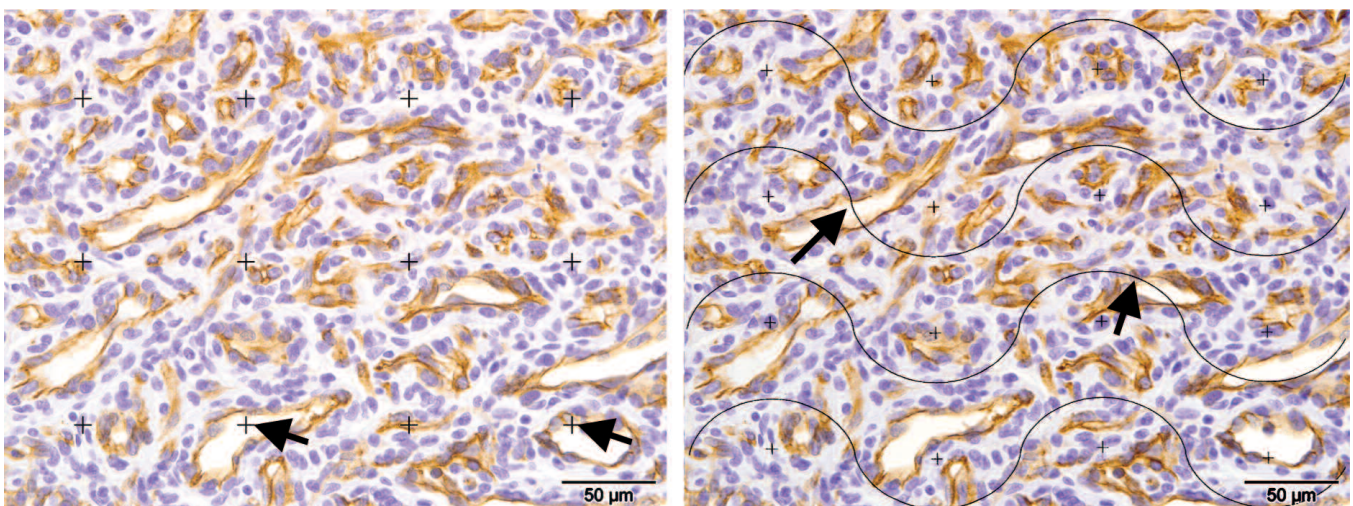


Fig. 5. Geometric probes can be used to estimate various vascular parameters. For example, percent vascular volume can be estimated by counting a number of points falling on blood vessels (arrow) divided by the number of points falling on the total reference compartment (A). Similarly surface area of blood vessel per unit volume of the reference volume can be estimated by counting the number of intersections between the line (such as cycloid line test system composed of parallel lines and separated by a known distance,) and the blood vessel surface (B, arrow). Blood vessels in this tissue sample (infantile haemangioma) have been labelled with anti-human CD31 antibody (clone JC70A). Geometric probes were interposed over the captured photographs of tissue samples using the ImageJ system (version 1.43, NIH, Washington DC). x 60

incorrect optical set up of microscopes and inconsistent staining uniformity) which can also influence the accuracy of the parameter estimate (Howard and Reed, 2005; Dockery and Fraher, 2007). When deciding how much to sample, the final decision will depend on the type of tissue examined and the target object within that tissue. However, it has been recommended that if point and intersection counting are used to analyse the micrographs, the total count for any object of interest should not exceed 200 (Cruz-Orive and Weibel, 1990; Garcia et al., 2007). In addition, Cruz-Orive et al suggest that in order to obtain statistically meaningful results, where nothing is known about variation for the material of interest, five animals should be used to estimate the parameter of interest since the probability that the estimate is due to chance is $P=(1/2)^{n-5}<0.05$ (Cruz-Orive and Weibel, 1990).

Geometric probes

Two-dimensional grids consisting of various dimensions (points, lines, planes, etc) have been designed for use on tissue sections. These grids are randomly applied to tissue sections (Fig. 5A,B) and the intersection of the geometric probe (such as line or point) and the tissue is counted in order to estimate parameters of interest. The type of geometric probe used for estimation depends on the type of parameter the researcher is estimating. Since stereology is a geometric quantification in three-dimensions, the sum of the dimension probe and feature must sum to three (Howard and Reed, 2005). For example, to estimate total volume which is a three-dimensional feature, a point grid is used, which is a zero-dimensional probe. In contrast, an optical dissector, a three-dimensional stereological probe, can be used to estimate the number of particles (i.e. cells, nuclei, nucleoli) in thick sections. Basically, to perform particle number estimation a reference point is defined, followed by a 'lookup plane' a point just above the reference plane. Particles that are not present in the look up plane will then be counted in three-dimensions as they will appear in the volume bound by two-dimensional space and the measured distance between the planes (Cruz-Orive and Weibel, 1990; Garcia et al., 2007). By counting only particles that appear in one plane and not the other ensures uniform sampling.

Stereological parameters in assessment of tissue vasculature

a) Vascular volume density

Vascular volume density (or percent vascular volume) is the volume proportion of blood vessels within a reference volume, for example a wound bed or a tissue engineered construct. The vascular volume density parameter does not account for number, surface area or spatial arrangement of blood vessels within the tissue examined. The most important aspect of

estimating vascular volume is defining the reference area, a compartment to which the parameter being estimated can be compared. Therefore, the vascular volume density equals the number of points falling on blood vessels divided by the number of points falling on the total reference compartment. The percentage is obtained by multiplying that number with 100%. This approach has been used to estimate percent vascular volume density in the rat *in vivo* AVL model of tissue engineering (Lokmic et al., 2007, 2008a) and the mouse flow-through chamber model (Rophael et al., 2007).

b) Absolute (total) vascular volume

The absolute (total) vascular volume can be estimated using the Cavalieri method (Hyde, et al., 2007; Michel and Cruz-Orive, 1988) or by organ displacement volume (Scherle, 1970). Therefore, the vascular volume density is multiplied by the volume estimated for the reference compartment to give the absolute vascular volume in the reference compartment (Lokmic, et al., 2008a).

c) Surface density

Surface density is surface area of vessels per unit volume of the reference volume, for example the area of gas exchange surface (capillary-alveolar interface) per unit volume of lung. Surface density is a two-dimensional feature and therefore geometric probes containing lines (one-dimensional) need to be used to estimate this parameter by counting the intersections between the line and the vessel surface (Howard and Reed, 2005). Absolute surface area can also be estimated by multiplying the surface density fraction with the volume of the reference compartment (Dockery and Fraher, 2007).

d) Vessel length

Vessel length density is the length of a blood vessel within a unit reference volume for example, length of capillaries per mm^3 of tumour tissue. Length density can be estimated by multiplying the number of intersections of the probe and blood vessels, counted on a tissue section per unit area, multiplied by two. Multiplication of this value by the volume of reference space yields an estimate of total vessel length in the reference compartment (Dockery and Fraher, 2007). As length is a one-dimensional feature it requires a two dimensional plane-probe (Howard and Reed, 2005) or test system comprised of a series of cycloid and test points whereby the vessel length is measured by counting the number of intersections between cycloids and borders of labelled vessels within the pre-determined reference compartment (Dockery and Fraher, 2007).

It is also possible to estimate the number of vessel segments in three-dimensional space, vessel segment length (i.e. length of capillaries between branching

points), vessel diameter, cross-sectional area, diffusion distance, thickness of blood vessel walls (Weibel et al., 1973; Weibel, 1975; Howard and Reed, 2005; Dockery and Fraher, 2007; Hyde et al., 2007) and vessel network branching (Parsons-Wingerter et al., 1998). However, these estimates, while valuable, are not routinely used in the general assessment of tissue vascularisation.

Automated user-interactive software for blood and lymphatic vessel network measurement

A number of software tools are available for the quantification of blood vessel and lymphatic networks. These greatly assist in routine measurement protocols. Computer packages that assist in counting any morphological feature can be used to stereologically assess blood vessels (Rophael et al., 2007). One such package is the Computer Assisted Stereological Toolbox (CAST system, Olympus, Denmark). This package has the significant advantage that it is capable of providing systematic random sampling of an entire tissue section, that is using digital video imaging, the operator selects a percentage of the section to be counted and after outlining the section perimeter the package randomly and uniformly selects fields of view for analysis that are the requested percentage area of the section. This feature eliminates operator bias in the selection of fields for counting. Not all stereological software packages include this important feature.

Shayan et al. (2007) described the Lymphatic Vessel Analysis Protocol (LVAP) plug in as part of the ImageJ program (Abramoff et al., 2004), which enables measurements such as lymphatic vessel density, vessel width, branch points, and lymphangiogenic sprout length measurements. The operator selects a parameter to be measured, a specific grid is applied to the section being viewed microscopically and the operator then locates the mouse cursor on the parameter (or 'event'), the computer mouse button is clicked, which records the event electronically and marks the site so that it will not be counted again.

New software packages specific for blood vessel and lymphatic networks are becoming available. Vickerman et al. (2009) describe the Vessel Generation Analysis (VESGEN) system an automated, user-interactive software package which requires only a binary (white/black) vascular image for analysis and can measure vessel diameter, fractal dimension, tortuosity, avascular spacing, density of vessel area, length, number, and branch points.

Conclusion

Stereology is a powerful tool used to evaluate the formation of vascular and lymphatic networks in any research model, including tissue engineered constructs, embryological and adult tissues and pathological specimens. To distinguish vessels from the surrounding tissue it is necessary to use different cellular markers to

identify blood and lymphatic vessels, particularly since lymphatic vessels tend to collapse during tissue processing into paraffin blocks and so morphologically resemble blood capillaries and post-capillary venules. Many of the cell markers are shared by both the blood vascular and lymphatic endothelium, for example CD31. Therefore, in some experimental circumstances when the identification of both the lymphatic vessels and blood vessels is needed, a combination of both blood vascular and lymphatic markers is employed. In order to ensure unbiased quantitative data, when performing stereology, it is important to be aware of sampling and systematic bias and to follow the fundamental principles of stereology, namely that of randomly selecting samples.

In the future, with the advent of new medical imaging technologies and improvement in their resolution of the microcirculation both in humans and animals, it will be possible to stereologically examine an organ, a pathological site or a developing tissue engineered construct *in vivo* and *in situ* as a function of time and observe the effects of various factors to either stimulate angiogenesis, stabilise the already formed vessel network or inhibit angiogenesis without removing the tissue *ex-vivo*.

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