

Review Article

Intravital microscopy in a dorsal skinfold chamber: Hemodynamics, tumor angiogenesis and inflammation

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Abstract

We review the dorsal skinfold chamber technique as used in microvascular studies, focusing on hemodynamics, tumor angiogenesis and inflammation. We discuss dorsal skinfold chamber implantation, intravital microscopy and microvascular visualization. Implications of vascular diameter, blood flow velocity and functional capillary density are discussed in the context of pathological models, such as resuscitation from hemorrhagic shock and acute anemia. We also review the process of dynamic vascularization of tumor angiogenesis. We further discuss oxygen and nitric oxide transports, as well as platelet activation and inflammation during the wound healing process.

Keywords: dorsal skinfold chamber, microcirculation, hemodynamics, angiogenesis

1. Introduction

A dorsal skinfold chamber is a widely accepted technique which has been implemented for the microvascular study for over 30 years. A dorsal skinfold chamber has been used as a tool to study the microcirculation in both healthy and acute or chronic diseased situations. This technique has a unique advantage for hemodynamic and angiogenesis studies providing the insight of blood flow and vascular changes in a microscale. To view a dorsal skinfold chamber, intravital microscopy is used. A dorsal skinfold chamber is particularly done in small animals such as mice, hamsters and rats. In addition, a dorsal skinfold chamber model has several benefits compared to other models for the in vivo study of microcirculation, for example, in case of chronic implantation or minimum acute surgical trauma or unanesthetized conditions. Using an intravital microscope in combination with a dorsal skinfold chamber provides the quantitative study for microvascular functions such as vascular diameter, blood flow velocity, functional capillary density and oxygen transport. Furthermore, vascular angiogenesis and remodeling can also be qualitatively observed during a prolonged period of time by this model with assisted intravital fluorescence microscopy.

2. Dorsal skinfold chamber implantation

The dorsal skinfold chamber consists of two symmetrical frames (Figure1). Generally, animals implanted with a dorsal skinfold chamber should have a body weight ranging between 22-25 g in mice, 60-80 g in hamsters and 150-200 g in rats. The back of the anaesthetized animals is carefully shaven and chemically depilated avoiding micro-injuries to the skin. Subsequently, the hair-free back is cleaned under 37 °C warm water ensuring a complete removal of the depilatory cream,

which may otherwise induce inflammatory irritations. Then, the animal is put in a prone position. A sandwiched skinfold is then cranially and caudally affixed at the midline with two 5-0 silk sutures. The first chamber frame is fixed by 5-0 silk sutures on its superior edge to the back side of the skinfold and two openings are carefully prepared at the base of the skinfold that is closed to the body, through which the connecting screws are passed from the back to the front side. After marking the circular area of the later observation chamber, the animals are placed in a lateral position under an operation microscope. Using microsurgical instruments, one layer of skin is completely removed from a circular area of 15 mm in diameter, and the remaining layer, consisting of the epidermis, subcutaneous tissue, and a layer of thin striated skin muscle, is covered with a coverslip incorporated in one of the frames. The basic principle of this model is to provide a chronic access to exposed tissues in the implanted chamber for microscopic imaging through an observation chamber. The dorsal skinfold chamber model such as this has been established in rats ¹, mice ², nude mice ³⁻⁶, as well as in hamsters. ⁷⁻¹¹ The dorsal skinfold chamber can easily be positioned under an upright microscope for intravital microscopic analyses of the microcirculation.

Figure 1 Two symmetry titanium frames used for a dorsal skinfold chamber.

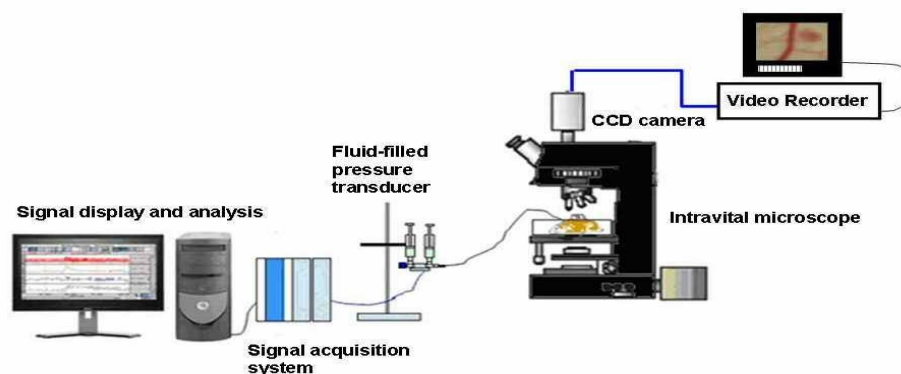


One of titanium frame has a circular cover glass slip with a retaining ring. Both titanium frames are connected with small stainless steel bolts and nuts.

3. Intravital microscopy and visualization from a window chamber

Intravital microscopic measurements in the dorsal skinfold chamber are performed in living animals, which enables the qualitative and quantitative assessment of functional parameters such as tumor angiogenesis, microvascular diameter, hemodynamic changes, leukocyte adhesion and platelet activation. ^{1, 5, 6, 12} Figure 2 illustrates the experimental set up for intravital microscopy in a dorsal skinfold chamber hamster model. For this purpose, the microscopic images are stored on videotape, DVD or hard disc for the subsequent analysis by means of a computer-assisted offline analysis system. The animal is fixed on a stage, which allows for horizontal positioning of the observation window under the microscope objectives.

Figure 2 Experimental setup for intravital microscopy of animal with a dorsal skinfold chamber.



Animal is put into a restrain tube and fixed on a stage of intravital microscope. An intravital microscope connects with a camera to capture images and video of microvasculature. Fluid-filled pressure transducer is connected to animal's arterial catheter to measure blood pressure. Blood pressure signals are acquired and amplified with a signal acquisition system before signal display and analysis.

A rodent dorsal skinfold chamber has been used by many researchers to enable them to undertake a close examination of the details of microcirculation. As shown in Figure 3, the arterial (arteries and arterioles) and venous (veins and venules) vascular network can be easily seen by the unaided eye. However, it is much more difficult to identify which vessels are arterioles or venules without using a high magnification microscopy. Figure 4 shows an image under 200x magnification from an intravital microscope. With intravital microscopy, we can identify arterioles and venules by observing the direction of blood flow at branches or bifurcations. For example, if we see blood flow going away from a big line to small lines which means that the observed vessel and its branches are arterials as graphically demonstrated in Figure 5A. On the other hand, when we see blood flow going into a large vessel from small vessels that represents venous as illustrated in Figure 5B. For capillaries, we can see a single file flow of red blood cells as shown in Figure 6. A dorsal skinfold chamber provides more advantageous for a long term microvascular study than other microvascular visualization techniques in other tissues such as mesentery or cremaster tissues.

Figure 3 Dorsal skinfold chamber in place (A) and the vascular networks as viewed by the unassisted eye (B).

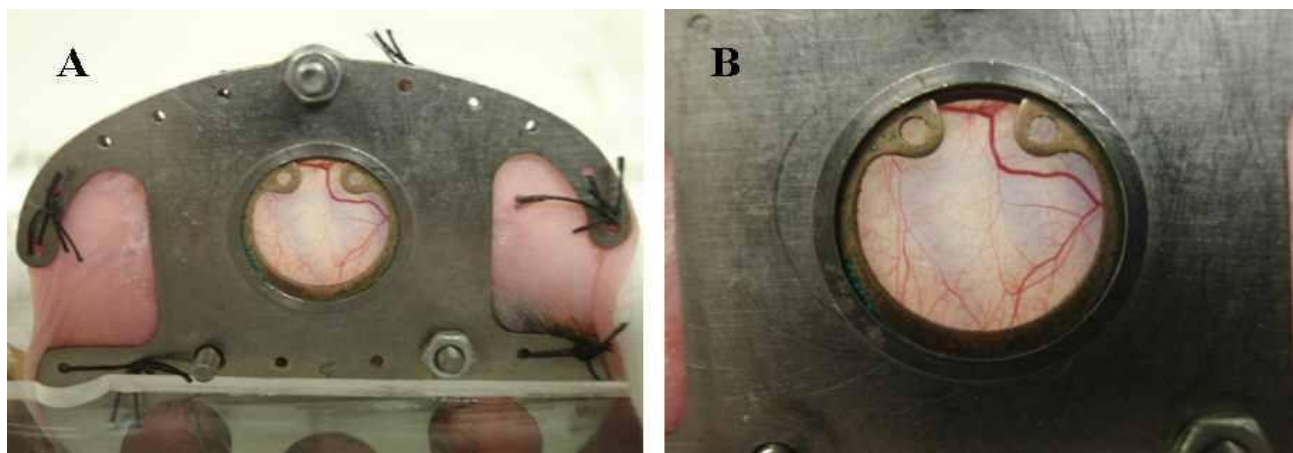


Figure 4 Images under 200x magnification from an intravital microscope (A and B). Vascular network can be observed as well as blood flow

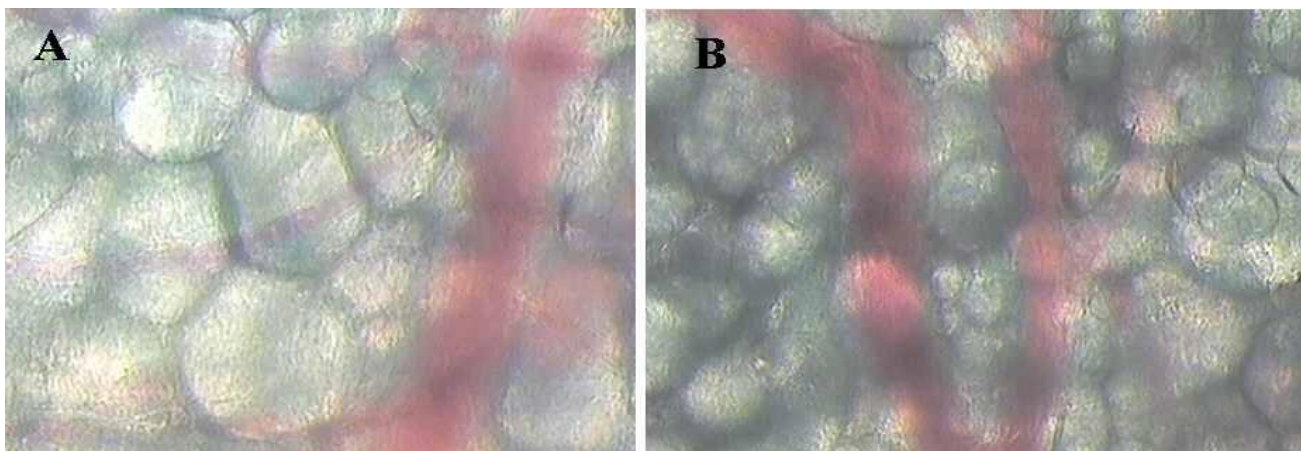
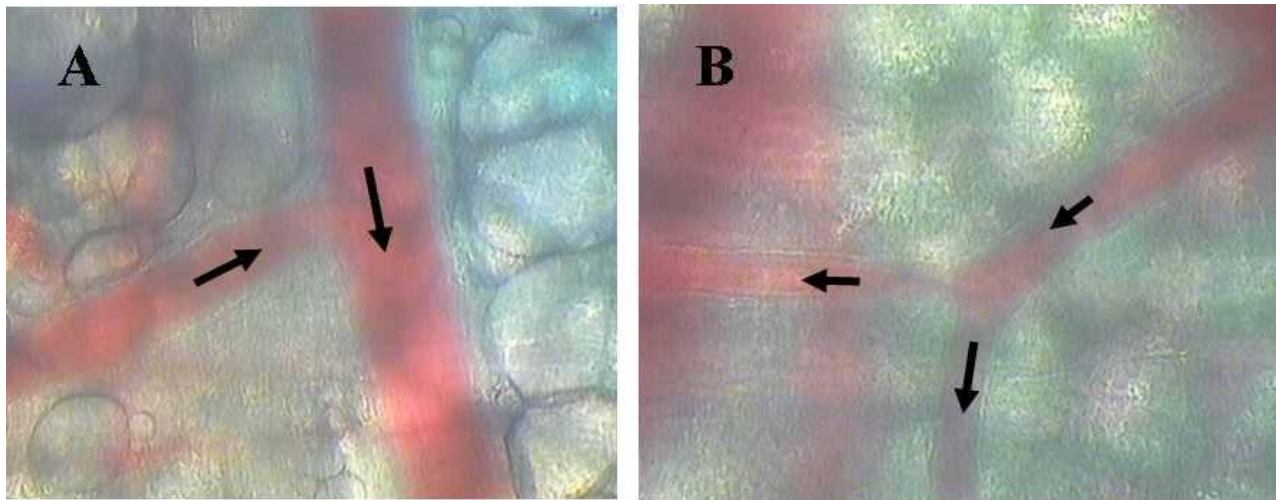
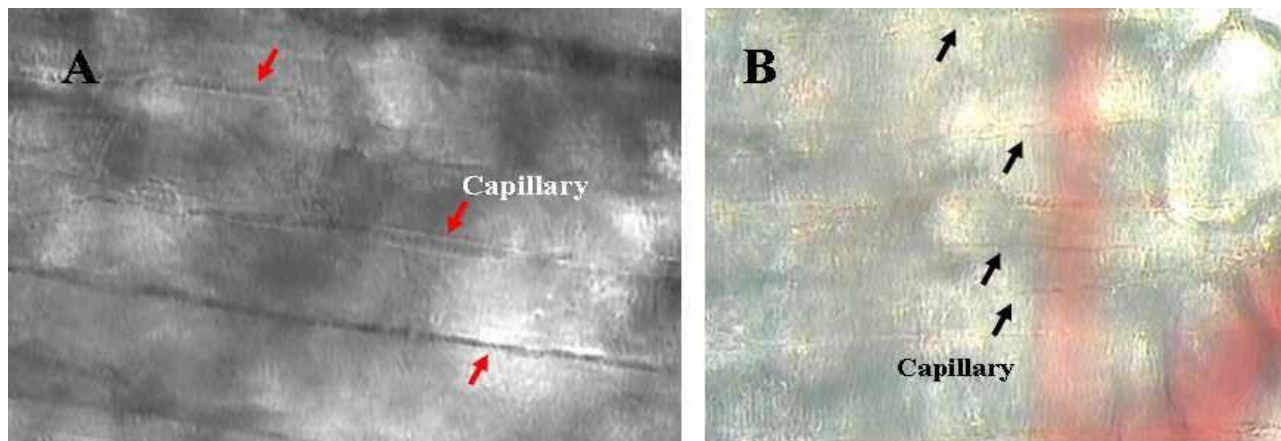


Figure 5 Venous (A) and arterials (B) showing the flow direction at vascular braches.



In venous network, blood flows from small lines into larger lines in order to transport wastes back into the circulation. In arterial network, blood flows from large lines to smaller lines in order to deliver oxygen and nutrient to cells.

Figure 6 Capillaries viewed through a dorsal skinfold chamber under an intravital microscope (A and B).



Single file flow of red blood cells is observed in capillaries as pointed by arrows. Due to no nucleus, red blood cells easily deform themselves to pass through capillaries which have a diameter less than their diameter.

It has been reported that a dorsal skinfold chamber can only be used for up to 2-3 weeks to monitor microvasculature because the elasticity of the dorsal skinfold decreases over time.¹³ This can lead to a tilting of the chamber, which may affect the perfusion of the prepared tissue. However, the period of viewing through the window and the quality of image viewed from an intravital microscopy depend on the quality of surgical preparation especially during subcutis with the panniculus carnosus removal. Not only hemodynamic studies, but tumor angiogenesis and host tissue with biomaterial interaction studies also use a dorsal skinfold chamber model as a visualization tool of microvessels.¹⁴ Combining the fluorescent dyes with a dorsal skinfold chamber, under a fluorescence intravital microscope, leads to a technique that enhances microvasculature and blood components visualization. Algenstaedt et al. applied fluorescein isothiocyanate (FITC)-dextran as a plasma tracer to measure functional vascular density, diameter and blood flow in diabetic mice with a dorsal skinfold chamber.¹⁵ They also labeled leukocytes with Rho-6G to investigate about leukocyte endothelial interactions (LEIs). Similarly, Dellian and colleagues used Rho-6G stained leukocytes to study LEI in tumor microvessels.¹⁶ Laschke et al. applied similar techniques to study about microvascularization and microcirculation of freely transplanted ovarian follicles in hamster dorsal skinfold chamber.¹⁷ These studies demonstrated the various potentials of enhanced visualization through a dorsal skinfold window in many aspects.

Recently, swept-source optical coherence tomography (SS-OCT) has been applied with a dorsal skinfold chamber to provide an alternative method for microvascular visualization.¹⁸ The SS-OCT images have been compared to images obtained from the intravital fluorescence microscopy technique. This comparison has showed a good agreement between these two techniques. Furthermore, Farhat et al. performed dynamic light scattering with OCT to measure the intracellular motion in a mice tumor model with a dorsal skinfold chamber.¹⁹ Therefore, a dorsal skinfold chamber is still useful for researchers to visual microvasculature and blood components although novel visualized techniques have been developed.

4. Diameter, velocity and functional capillary density

Vascular diameter is a mandatory parameter for the determination of vasodilation and vasoconstriction. There are many methods to measure vascular diameter which can be used in conjunction with intravital microscopy images such as the shearing image technique²⁰ and offline measurements using image analysis software (i.e. ImageJ, ImagePro).²¹ The shearing image technique has been used intensively with a dorsal skinfold chamber because of its ease of use and ability to measure real time values. This technique uses a video signal combined with sync separation and adjustable delay to determine horizontal displacement. A microvascular diameter measurement has been performed before and after intervention in many experimental conditions such as hemorrhagic shock resuscitation and acute hemodilution.^{10, 22-24} By measured microvascular diameter, the vasomotor response can be observed as well as calculated microvascular resistance.²⁵ In addition, to estimate blood flow rate, for which it is necessary to know the vascular diameter and blood flow velocity.

On-line blood flow velocity measurements have been applied with a dorsal skinfold chamber using photodiode cross-correlation method.^{7, 22, 26} This technique measures the time delay between the photonic signals from two specific windows along the centerline of a vessel. Another technique used to measure red blood cell (RBC) velocity is an optical Doppler velocimeter.²¹ Blood flow (Q) is then determined using the equation $Q = V \times \pi(D/2)^2$ where V is the mean RBC velocity and D is the vessel diameter. Another recent study applied a polymeric micro-optical interface for flow monitoring in a hamster dorsal skinfold chamber model.²⁷ Although this concept of optical monitoring has a somewhat noisy signal, this development has shown a possible application in flow monitoring in an in vivo experiment.

Functional capillary density (FCD) is an important indicator of tissue perfusion that can be obtained by intravital microscopy.²⁸ FCD defines as capillary segments or lengths that have RBC flow through per investigation area. FCD also implies a capability of oxygenation in capillary network.^{11, 29} FCD can be determined either by manual counting with intravital microscopy or calculation with a computer-assisted program. Many microvascular function studies have used a dorsal skinfold chamber to assess FCD in animal models experiencing hemorrhagic shock or acute anemia conditions.³⁰⁻³⁷

In addition, using a dorsal skinfold chamber model revealed the impairment of FCD during a severe experimental malaria study.⁹ Ischemia-reperfusion conducted with a skinfold chamber demonstrated that FCD was higher when post-ischemia infusion was performed with a perfluorocarbon emulsion compared to infusion before ischemia.³⁶

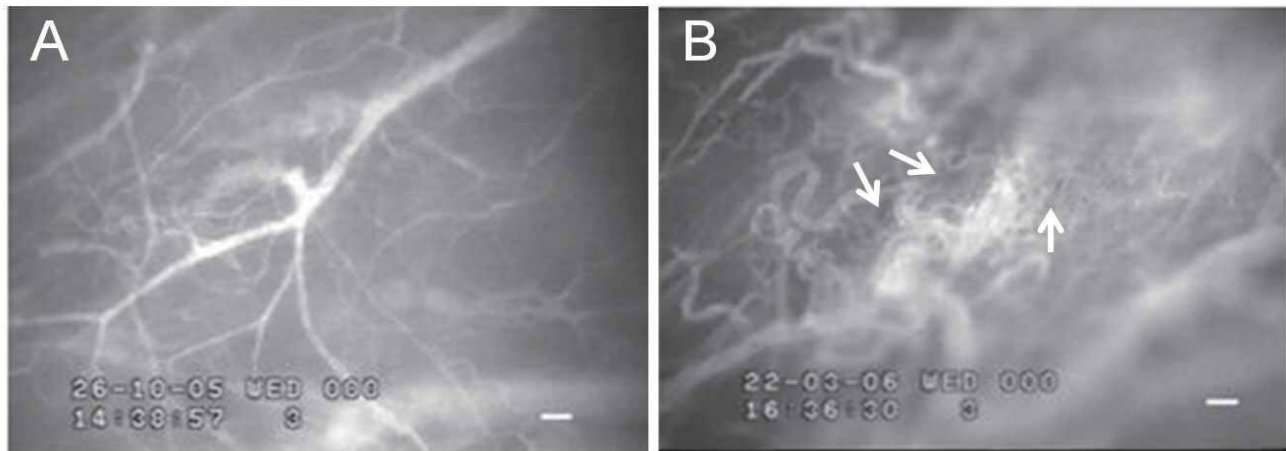
5. Tumor angiogenesis

A cancer requires nutrients, in particular tumors greater than 1–2 mm³ in volume needs their own blood supply for continued growth.³⁸ The new vessels which provide this blood supply are usually the result of angiogenesis, a process defined as the formation of new blood vessels from existing vasculature. Inadequate blood supply in the tumor leads to hypoxia as oxygen diffusion is limited. When the center of the tumor is lack of oxygen, hypoxia results, which in turn leads to the expression of several angiogenic factors, for instance, vascular endothelial growth factor (VEGF).³⁹

The dorsal skinfold chamber model has been used extensively with BALB/c-nude mice or SCID immunodeficient mice for the study of microcirculation and angiogenesis.^{6, 12, 13} An implanted tumor, blood vessels, and host tissue are observed directly through a transparent window. Thus, tumor growth can be visualized and quantified over time along with the development of the vasculature, at high resolution and without tissue damage. BALB/c-nude mice or SCID immunodeficient mice are used to implant human cells, whereas syngeneic mice may be used for the implantation of mouse cancer cells, such as tumor cell lines derived from transgenic models of cancer.^{3, 6, 12, 40} This offers the possibility to visualize in vivo angiogenic sprouting and network formation, and to quantify in vivo morphological (vascularized area, microvessel density) parameters of the endometrial microcirculation (Figure 7). Thus, this novel experimental approach can give new insights into the dynamic angiogenic process and can be used to directly test the effects of anti-angiogenic agents. Furthermore, treatment with the selective cyclooxygenase-2 inhibitor NS398⁴¹ and rapamycin⁴² induces the regression of

ectopic endometrium due to the inhibition of vascularization and cell proliferation. Interestingly, this vascularization is not solely driven by VEGF, but depends on the cross-talk between VEGF, fibroblast growth factor and platelet-derived growth factor, suggesting that the combined inhibition of these growth factors is a more effective regimen for the anti-angiogenic treatment of endometriosis.⁴³ Recently, the successful treatment of tumor angiogenesis in mice by curcumin has been reported.¹²

Figure 7 Intravital fluorescence microscopy of newly developing blood vessels within a dorsal skinfold chamber of control (A), and at day 14 after transplantation of hepatocellular carcinoma (HepG2) (B), with newly formed capillaries (arrows). Bar indicates 50 μ m.



6. Gas transport measurement

As blood carries and delivers oxygen to tissues and cells, it is necessary to measure how oxygen transportation occurs as well as oxygen extraction, which is related to cell metabolism. Oxygen delivery is determined from arteriolar oxygen saturation of RBCs, microvascular blood flow and microvascular pO_2 measured by phosphorescence quenching microscopy with a dorsal skinfold chamber.^{44, 45} In addition, oxygen extraction is measured using a similar method to determine oxygen delivery but based on the arteriolar-venular difference in oxygen saturation of RBCs instead of arteriolar oxygen saturation of RBCs. Oxygen transport is widely studied in the field of blood substitute as a major concern of this field.^{46, 47} Furthermore, in tumor research, microvascular oxygenation has been intensively investigated using the noninvasive oxygen-dependent phosphorescence quenching technique in a dorsal skinfold chamber model.⁴⁸⁻⁵⁰ Another technique which used a dorsal skinfold chamber to determine vascular oxygenation is the phosphorescence lifetime image (PLI) which is related to the phosphorescence signal intensity.⁵¹ The PLI has been reported to provide an accurate measurement of oxygen in less than 40 mmHg, hypoxia-like conditions.

Nitric oxide (NO) is a bioactive molecule which plays a significant role in the cardiovascular system. In vivo, NO is available in low concentration and it has a short half-life and high reactivity with other biological molecules.⁵² Therefore, it is very challenging to perform the NO real-time measurement in vivo. Tsai and colleague used microelectrodes coated with Nafion to measure perivascular NO at the end of microhemodynamic measurement by removing a coverglass of dorsal skinfold chamber.¹⁰ In their process, perivascular tissue was prepared before penetrating the micropipette with microelectrode. However, this study was done at the end of study, not during the experiment, which can not provide information at other time points.

7. Platelet activation

Activated blood coagulation has been linked to the process of tumor angiogenesis since relevant clotting factors were found to exert potent regulatory functions on endothelial proliferation. Particularly tissue factor, expressed in tumor cells, regulates synthesis of the endothelial cell-specific growth factor VEGF by its cytoplasmic domain.⁵³ Tumor-derived VEGF induces tissue factor expression in endothelial cells, resulting in enhanced thrombin formation. Thrombin is a potent mitogen of endothelial cells, which are induced to secrete growth factors and express both VEGF receptors, fetal liver kinase 1 (flk-1) and fins-like tyrosine kinase 1 (flt-1).⁵⁴ Besides the plasmatic factors of blood^{55, 56}, microhemodynamic

parameters and platelets were investigated at consecutive stages of tumor angiogenesis in comparison with normal tissue. Intravital microscopy in a dorsal skinfold chamber was performed on days 1, 3, 8, and 14 after implantation of LLC-1 and BFS-1 tumor cells. Platelet interaction with the vessel walls in established tumor microvessels was indistinguishable from that observed in microvessels of non-tumor-bearing subcutaneous tissue.⁵⁷

8. Inflammation

Wound healing is a complex and dynamic biological process that proceeds through three distinct and temporally overlapping phases: inflammation, proliferation and remodeling.⁵⁸ Paramount to successful healing is wound closure, and the replacing of injured or necrotic tissue with granulation tissue. Formation of granulation tissue is, in turn, dependent on angiogenesis (i.e. the growth of blood vessels from pre-existing post-venular microvasculature). The local physiological characteristics that are changed in injured tissue, such as lower oxygen tension, high lactate levels, acidic pH and cytokines released by macrophages⁵⁹, stimulate sprouting angiogenesis. Upon implantation of a dorsal skinfold chamber in mice, a layer of skeletal muscle is responsible for skin. In the wound healing-induced angiogenesis model presented here, the number of macrophages (the major phagocytic and inflammatory mediator cell) peaked at 3 days post-injury, coinciding with the period when the wounded tissue began to revascularise.⁶⁰ In the dorsal skinfold chamber, between 5 and 8 days post-injury, numerous angiogenic, tortuous and disorganized flowing vessels and blind-ended vessels (BEVs) were observed.⁶¹ BEVs are the proximally perfused segments of newly formed angiogenic sprouts and their temporal development in relation to wound closure regulates the initial patterning of the regenerating vascular plexus.

Conclusion

We show that a dorsal skinfold chamber in combination with intravital microscopy provides a suitable method which enables the qualitative and quantitative assessments of functional parameters such as tumor angiogenesis, microvascular diameter, hemodynamic changes, functional capillary density and leukocyte adhesion, including platelet activation and inflammation. Accordingly, this model has been increasingly used during the last three decades for *in vivo* studies.

Authors' contributions

ND and SC equally conceived and prepared this review. All of the authors read and approved the final manuscript.

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