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# The Use of Confocal Laser Microscopy to Analyze Mouse Retinal Blood Vessels

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1. Introduction

Until recently, the house mouse (*Mus musculus*) was not a prefered model to study the mammalian visual system [1]. However, the power of transgenic and knockout mice as tools to analyze the genetic basis and the pathophysiology of human eye diseases, have become the mouse one of the most used animals for the study of retinopathy [2].

In the retina there is a compromise between transparency and optimal oxygenation [3]. Thus, retinal vasculature must show special characteristics in order to minimize their interference with the light path. Retinal capillaries are sparse and small [4], representing only 5% of the total retinal mass [5]. Hence, retinal blood volume is relatively low [6]. This feature, together with an extremely active cellular metabolism, 10% of resting body energy expenditure is consumed by retinal tissue [7], makes retina very susceptible to hypoxia.

The study of retinal vasculature has an increasing relevance, since vascular alterations are one of the earliest events observed during retinopathy [8]. Vascular alterations compromise blood flow, diminish oxygen supply, and neovascularization develops in response to hypoxia. This neovascularization is the most common cause of blindness, with a growing social impact in the world [9].

The structure of the mouse retina has been extensively studied anatomically using silver impregnations [10], Nissl staining [11], electron microscopy [12, 13], differential interference contrast microscopy [14] and confocal laser microscopy [15]. More specifically, mouse retinal



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blood vessels have been analyzed by angiography using fluorescent dyes [16], vascular corrosion cast [17], trypsin/pepsin digestion [18] and confocal laser microscopy (CLM) [19-23]. However, retinal whole-mount observation by confocal laser technology is the only method that allows a three-dimensional microscopical analysis of the entire retina, combining the use of fluorescent markers for proteins, signaling molecules, etc.

The visual organ, the eye, is a structure that transforms light into electrical impulses, which are sent to the brain. The visual organ is formed by the eyeball and the accessory ocular organs. Lids, lacrimal glands and extraocular muscles provide protection and help to the visual function (Figure 1A).

The adult mouse is a very small nocturnal mammal with a relatively small eyeball having an axial length from anterior cornea to choroid of about 3.4 mm [24]. As is typical for nocturnal mammals, the mouse eyeball resembles a hollow sphere with a relatively large cornea. The eyeball is formed by three layers or tunicae, which contains the eye chambers and a very large lens that represents approximately 65% of the axial length (Figure 1B). The anterior chamber is placed between the cornea and the iris. The posterior chamber is the space situated between the iris and the lens. The vitreous chamber of the eyeball is placed behind the lens, surrounded by the retina (Figure 1B). The three tunicae of the eyeball are concentrically placed and, from most internal to most external, are: the nervous layer, formed by the retina; the vascular layer, where can be found choroid, ciliary body and iris; and the external layer, which is formed by cornea and sclera [25] (Figure 1B).

The retina is the most complex part of the eye. Its structure and function is similar to those of the cerebral cortex. In fact, retina can be considered as an outpouching of central nervous system during embryonic development. The retina comprises a blind part, insensitive to light, associated with the ciliary body and the iris; and an optical part, containing photoreceptors. In turn, optical part is formed by two sheets: the neuroepithelial stratum, composed by neurons, and the retinal pigmentary epithelium. Mouse neuroretina is composed by eight layers (Figure 1C): the nerve fiber layer, hardly distinguishable in equatorial retina; the ganglion cell layer; the inner plexiform layer; the inner nuclear layer, containing bipolar, amacrine, horizontal and the nuclei of Müller cells; the outer plexiform layer; the outer nuclear layer, formed by photoreceptors nuclei; and the layers of internal and external segments of photoreceptors. Two limiting membranes can also be distinguished: the internal limiting membrane, placed between the vitreous and the retina; and external limiting membrane, found between the outer nuclear layer and the external segment of photoreceptors [25].

Mice and humans have holoangiotic retinas [26]. In these species the entire retina is vascularized, in contrast with anangiotic retinas, such as the avian retinas, where there are not blood vessels inside the retina. In holoangiotic retinas blood flow is directed from the optic disc radially to the periphery of the retina, and vasculature consists of arteries, veins and a wide network of capillaries (Figures 2A and 2B). The retinal circulation develops from the hyaloid artery that regresses after birth. Hyaloid blood vessels following a template of astrocytes growth superficially and deeply forming the retinal capillary plexi [27]. In mouse retina, as happens in most of the mammals including man, blood supply is carried out by two different The Use of Confocal Laser Microscopy to Analyze Mouse Retinal Blood Vessels 21 http://dx.doi.org/10.5772/56131



**Figure 1.** Topography and structure of the retina in the mouse eye. Enucleated eye (A). Paraffin section of an eye stained with Azan trichrome (B). Hematoxylin/eosin stained paraffin section of retina (C). 1: cornea; 2: iris; 3: anterior chamber; 4: posterior chamber; 5: lens; 6: vitreous chamber; 7: retina; 8: choroid; 9: sclera; 10: optic nerve; 11: extraocular muscles; 12: inner plexiform layer; 13: outer plexiform layer; 14: photoreceptor inner segment; 15: photoreceptor outer segment; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; RPE: retinal pigmentary epithelium; arrow head: internal limiting membrane; arrow: external limiting membrane. Scale bar: 34 µm.

vascular systems: retinal vessels, which irrigate from the internal limiting membrane to the inner nuclear layer; and choroidal vessels that supply the rest of the retina [25] (Figure 2F).

The main source for retinal blood supply is the internal carotid artery that gives rise to the ophthalmic artery. This artery goes along with the optic nerve and internally is the origin of the central retinal artery [25, 28, 29]. At the level of the optic disc, the central retinal artery branch in four to eight retinal arterioles, depending on mouse strain. Arterioles run towards retinal periphery, where retinal venules are originated. (Figures 2C and 2D). Retinal arterioles are the origin of precapillary arterioles, which give rise to a capillary network settled between retinal arterioles and venules (Figure 2E). Capillaries are placed in the retina forming two plexi: internal vascular plexus, at the level of ganglion cells and inner plexiform layers; and external vascular plexus, between inner and outer nuclear layers (Figure 2F). The figure 3 shows a



**Figure 2.** Blood vessel distribution in mouse retina. To show the topography of blood vessels in the mouse retina, scan laser ophtalmoscope images (A and C), collagen IV antibody immunohistochemistry (green) of whole-mount (B,D and E) and paraffin embedded (F) mouse retinas are presented. Nuclei counterstained with ToPro-3 (blue). A: arteriole; V: venule; OD: optic disc; GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; arrowhead: blood vessels. Scale bars: 108 µm (D), 122 µm (E) and 34 µm (F).

The Use of Confocal Laser Microscopy to Analyze Mouse Retinal Blood Vessels 23 http://dx.doi.org/10.5772/56131



**Figure 3.** Representative schema of vascular architecture and blood flow in mouse retina. Blood flows from arterioles to venules through a capillary network. Capillaries are placed forming two plexi, the most superficial situated in the outer plexiform layer and the deepest localized in the ganglion cell and inner plexiform layers. Hypoxic blood is collected from capillaries by retinal venules. A: arteriole; V: venule; arrows: blood flow direction.

schematic representation of capillary retinal plexi (adapted from [19]). Retinal capillaries converge into retinal venules, which course parallel to arterioles and drive hypoxic blood to the central retinal vein (Figures 2C and 2D).

The structure of retinal blood vessels is similar to other localizations of the body. The blood vessel wall can be divided in three layers or tunicae: the adventitia layer, the most external, is formed by connective tissue; the media layer, where can be found smooth muscle cells; and the intima layer, consisting in a monolayer of endothelial cells [30]. Retinal arterioles show a tunica adventitia, mainly formed by collagen IV, surrounding all cellular components of blood vessel wall (Figure 4A). Retinal arterioles have a well-developed tunica media, formed by one layer of smooth muscle cells placed perpendicularly to vascular axis (Figures 4B and 4C). Smooth muscle cell number diminishes when arterioles branch in precapillary arterioles, forming a non-continuous layer of sparse smooth muscle cells. Finally, the tunica intima is made of endothelial cells placed parallel to the vessel axis (Figure 4D).

Retinal capillaries are formed by pericytes and endothelial cells surrounded by basement membrane (Figure 5A). Pericytes are a contractile cell population positive in retina for  $\beta$ -actin (Figure 5B), nestin (Figure 5C), NG2 (Figure 5D) and PDGF-R $\beta$  (Figure 5E), among others [31-34]. Endothelial cells are placed in the most internal part of capillaries, in direct contact with blood stream. These cells show an elongated morphology with a big nucleus that protrudes to the



**Figure 4.** Morphology and composition of retinal arterioles. Different markers were used in order to show the components of the arteriole wall. (A) collagen IV antibody (green) was used to specifically stain basement membrane. Smooth muscle cells (red) were evidenced by means of  $\alpha$ -smooth muscle actin antibody (B) and phalloidin (C). (D) Lectin from *Lycopersicon sculentum* allowed the analysis of both endothelial glycocalyx and microglial cells (arrowhead). Scale bars: 7  $\mu$ m (A,B and C) and 8  $\mu$ m (D).

vascular lumen (Figure 5). Different markers stain specifically endothelial cells, among others: Von Willebrand factor (Figure 6A), PECAM-1 (Figure 6B) and CD34 (Figure 6C). As happens in the brain, endothelial cells are connected by tight junctions (*zonula ocludens*) (Figure 6D). These tight junctions are an important component of blood-retinal barrier, which prevents the free pass of blood borne molecules to the retinal parenchyma [8]. Retinal venules show, as arterioles do, three concentrically placed layers: a tunica adventitia mainly formed of collagen IV (Figure 7A); a tunica media, consisting of a non-continuous layer of sparse smooth muscle cells (Figure 7B); and a monolayer of endothelial cells, the tunica intima (Figure 7C).



**Figure 5.** Morphology and composition of retinal capillaries. (A, D and E) Blood basement membrane was marked with anti-collagen IV antibody (green). (B) Capillary morphology in a  $\beta$ -actin/EGFP (green) transgenic mouse. Note that pericytes expressed more  $\beta$ -actin than endothelial cells. (C) Pericytes expressed nestin in the retinal capillaries of a nestin/EGFP (green) transgenic mouse. Specific pericyte markers, such as NG2 (D) and PDGF-R $\beta$  (E), has also been employed to show the distribution and morphology of pericytes. Nuclei counterstained with ToPro-3 (blue). E: endothelial cell; P: pericyte. Scale bars: 9.5  $\mu$ m (A and B) and 7.3  $\mu$ m (C,D and E).

In addition to neurons, retinal blood vessels are surrounded by glia that seems to play a role in the formation of blood-retinal barrier [5, 35-38] and the control of retinal blood flow [38]. The term glia encloses two components: neuroglia and microglia. Retinal neuroglia is formed by astrocytes and Müller cells (Figure 8). Astrocytes are only placed in the internal part of the retina, nerve fiber and ganglion cell layers, in close relation with arterioles and venules [39] (Figures 8A and 8B). The principal markers for astrocytes are glial fibrillary acidic protein



**Figure 6.** Morphology and composition of retinal capillaries. (B,C and D) Blood basement membrane was marked with anti-collagen IV antibody (green). Endothelial cells were specifically marked with anti-Von Willebrand factor (red) (A), anti-PECAM-1 (red) (B) and anti-CD34 (red) (C) antibodies. (D) Endothelial cell contribution to blood retinal barrier was evidenced using anti-ocludin antibody, a specific marker for endothelial tight junctions. Nuclei counterstained with ToPro-3 (blue). Scale bars: 5.5 µm (A,B and C) and 6.8 µm (D).

(GFAP) (Figure 8A) and desmin (Figure 8B). The nuclei of Müller cells are localized in the inner nuclear layer and their cytoplasmic prolongations extend practically to the entire retina forming the inner and outer limiting membranes (Figure 1C). Müller cells are very easily distinguished using the PDGF-R $\alpha$  (Figure 8C). Cytoplasmic prolongations of neuroglia, called vascular end-feet, contact with retinal blood vessels (Figures 8A, 8B and 8C).

Retinal microglia originates from hemopoietic cells and invade the retina from the blood vessels of the ciliary body, iris and retinal vasculature [40]. Resting microglial cells are scattered troughout the retina forming a network of potential immunoephector cells, easily marked with Iba1 (Figure 8D). Several studies show that microglial cells have characteristics of dendritic antigen-presenting cells, while others resemble macrophages [41]. During retinopathy, activated microglial cells participate in phagocytosis of debris and facilitate the regenerative processes. Microglial cells are also in contact with blood vessels, forming a special subtype of



**Figure 7.** Morphology and composition of retinal venules. Different markers were used in order to show the components of the venule wall. (A) Collagen IV antibody (green) was used to specifically stain basement membrane. (B) Smooth muscle cells were evidenced by phalloidin binding (red). (C) Immunohistochemistry against Von Willebrand factor (red) showed endothelial cell morphology. (D) Lectin from *Lycopersicon sculentum* allowed the analysis of both endothelial glycocalyx and microglial cells (arrowhead). Scale bars: 14.5 µm (A) and 11.5 µm (B,C and D).

perivascular microglial cells, localized in the perivascular space of Virchow-Robin (Figures 4D and 7D).

During the examination of retinal vasculature labeled with two different fluorescent markers, emission signals can often overlap in the final image. This effect, known as colocalization,



**Figure 8.** Perivascular glia in mouse retina. Different cell markers were used in order to show perivascular neuroglia and microglia. GFAP (A) and desmin (B) mark astrocytes (arrow), PDGF-Rα (C) stain Müller cells (arrow) and Iba1 (D) is expressed by microglial cells (arrow). (A,B,C and D) Blood basement membrane was marked with anti-collagen IV antibody (green). Nuclei counterstained with ToPro-3 (blue). Arrowhead: vascular end-foot. Scale bars: 23 µm (A,B and C) and 20 µm (D).

occurs when fluorescent dyes bind to molecules residing in a very close spatial position in the tissue [42]. Although colocalization is getting more relevance in modern cell and molecular biological studies, it is probably one of the most misrepresented and misunderstood phenomena. In this way, proteins continue to be described as more or less colocalized with no quantitative justification. This lack of information prevents researchers to analyze protein dynamics or protein-protein interactions [43].

In Figure 9 we can observe a retinal arteriole with the blood vessel basement membrane stained with anti-collagen IV (green) and anti-matrix metalloproteinase 2 (MMP2) (red). MMP2 is a constitutive gelatinase protein that can be observed in a wide variety of healthy mice tissues [44]. One of the main substrates of MMP2 is collagen IV, so MMP2 colocalize with collagen IV in retinal arterioles (yellow). An accurate colocalization analysis is only possible if fluorescent emission spectra are well separated between fluorophores and a correct filter setting is used (Figure 9). When a high degree of emission spectra overlap and/or filter combinations are not well defined the resulting colocalization will be meaningless [42].



**Figure 9.** Colocalization of collagen IV with MMP2 in the arteriolar basement membrane. Double inmunohistochemistry was performed in retinal paraffin sections using antibodies against collagen IV (green) and matrix MMP2 (red). (A) Digital images of green (left image) and red (central image) channels showing colocalization (arrowheads) in the right image. Nuclei counterstained with ToPro-3 (blue). (B) Graphic representation in a scatterplot, where pure red and green pixels are between abscissa/ordinate and white lines. Colocalizating pixels are found inside white elliptic region. (C) Pearson's correlation coefficient, where S1 and S2 are pixel intensities in channels red and green respectively; and S1<sub>aver</sub> (S2<sub>aver</sub>) is the average value of pixels in the first (second) channel. (D) Overlap coefficient, with  $k_1$  being sensitive to the differences in the intensities of channel 2 and  $k_2$  depending on the intensity of channel 1 pixels. Scale bar: 10.2 µm.

Graphical display for colocalization analysis is well represented by a fluorogram: a scatterplot which graphs the intensity of one color versus another on a two-dimensional histogram (Figure

9B). Along the y-axis is plotted green channel, while red channel is graphed on the x-axis. Thus, having each pixel a pair of fluorescent intensities in a Cartesian system. In the scatterplot, pixels having lower fluorescent intensities are close to the origin of abscissa and ordinate, while brighter pixels are dispersed along the graph (Figure 9B). Pure green and red pixels cluster close to the axes of the graph, while colocalized pixels are localized in the center and in the upper right hand of scatterplot (Figure 9B).

As discussed above, a quantitative assessment of colocalization is important in order to analyze protein dynamics and association. Using the information given by the scatterplot several values can be generated. Pearson's correlation coefficient ( $R_r$ ) is used as standard technique for image pattern recognition. This coefficient is employed to describe the degree of overlap between two images and can be calculated according to the equation seen in Figure 9 C. Values of this coefficient ranges from -1 to 1. The value -1 correspond to a complete lack of overlap between images and 1 a total match of pixels in the two images. Pearson's coefficient takes into account only similarity among pixels in the two images, and does not consider information of pixel intensities. Thus, Pearson's correlation coefficient can overestimate colocalization when the degree of colocalization is low [45].

Another standard value used to quantify colocalization is the overlap coefficient ( $R^2$ ) (Figure 9D). This coefficient uses two values ( $k_1$  and  $k_2$ ) in order to characterize colocalization in both channels. This coefficient avoids negative values, which have a harder interpretation. Some authors find this coefficient less reliable than Pearson's correlation coefficient, since overlap coefficient is only applicable in images with similar intensities in the two channels [45].

Diabetic retinopathy is a common and specific microvascular complication of diabetes, and remains the leading cause of blindness in working-aged people [46]. Recent metadata studies established that in the world there are 93 million people with diabetic retinopathy [47]. Nearly all individuals with type 1 diabetes and more than 60% of individuals with type 2 diabetes have some degree of retinopathy after 20 years of disease. There are two phases in diabetic retinopathy. Early phase is known as non-proliferative diabetic retinopathy, and is characterized by thickening of capillary basement membrane, pericyte and vascular smooth muscle cell loss, capillary occlusion and formation of microaneurysms [48]. Proliferative retinopathy, the second phase of the disease, is characterized by the formation of new vessels that pas through the inner limiting retinal membrane and penetrate in the vitreous chamber. New vessels are surrounded by fibrous tissue that may contract, leading to retinal detachment and sudden visual loss. Neovascularization is a consequence of retinopathy appears in approximately 50% of patients with type 1 diabetes and in about 15% of patients with type 2 diabetes [49].

Confocal laser microscopy allows the study of retinal blood vessels in diabetic mouse models (Fig. 10). Non-obese diabetic (NOD) mice develop type 1 diabetes by autoimmune destruction of pancreatic  $\beta$  cells [50].

The analysis of 8 months-old NOD mice whole-mount flat retinas marked with anti-collagen IV antibody showed basement membrane alterations in venules (Fig. 10). Similarly, db/db mice also showed alterations in basement membrane of retinal venules (Fig. 10). Db/db mice are homozygous for a mutation in the leptin receptor, and spontaneously develop type 2 diabetes

The Use of Confocal Laser Microscopy to Analyze Mouse Retinal Blood Vessels 31 http://dx.doi.org/10.5772/56131



**Figure 10.** Venule basement membrane alterations in diabetic retinopathy mouse models. Compare the morphology of basement membrane (green) between wild type (WT) mice and Non-obese diabetic (NOD) mice, and db/db mice. Nuclei counterstained with ToPro3 (blue). Blood basement membrane was marked with anti-collagen IV antibody. A: arteriole, V: venule. Scale bars: 21,2 µm (WT), 15 µm (NOD) and 16,2 µm (db/db).

[51]. These result suggested that, both in diabetes type 1 and 2, normal functions of blood basement membrane are altered in venules during diabetes. Venule basement membrane separate endothelial cells and smooth muscle cells from underlying connective tissue provid-

ing structural support, a selective barrier of filtration, and a substrate for molecular adhesion that modulates cells of the vascular wall.

By the above, we can conclude that the analysis of whole-mount retinas by laser confocal technology is a reliable method that allows a complete three-dimensional microscopical analysis of retinal blood vessels in health and disease. Combining the use of fluorescent markers for proteins, signaling molecules, etc, it will be possible study the topography and the structure of blood vessels. Thus, the use of confocal laser microscopy together with new mouse eye disease models may provide basis to fully understand the alterations of retinal vasculature during retinopathies.

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