Video Article

Using a Laminating Technique to Perform Confocal Microscopy of the Human Sclera

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Abstract

The sclera is a dense connective tissue that covers and protects the eye. It mainly consists of dense collagen bundles (types I, III, IV, V, VI, and VII). Due to its autofluorescence, opaqueness, and thickness, it has not been found suitable for confocal microscopy. An alternative approach to the one presented here, which uses formalin-fixed sclera embedded in paraffin for immunohistochemistry, has technical challenges, especially when preheating the tissue for antigen retrieval. Since the sclera is relatively poor in both cells and vessels, the use of larger tissue samples was explored to help prevent overlooking cells and to understand their localization in relation to vessels and other anatomical sites. To allow for the analysis of larger tissue samples under the confocal microscope, a laminating technique was performed to create thin layers from the sclera. Following the analysis of results of CD31 blood vessels and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) positive cells, for which approval for scientific examination was obtained, the advantages and limitations of this method are discussed.

Video Link

The video component of this article can be found at https://www.jove.com/video/53920/

Introduction

The sclera is the rigid outer layer that covers the eye, which is made of dense connective tissue. It helps to protect intraocular structures and to maintain intraocular pressure. Thus, the sclera is essential for clear vision. It is devoid of lymphatic vessels [1,2] and thereby forms an outer lymphatic-free border between it and the lymphatic-free inner eye [3-7]. It also provides attachment sites for extraocular muscles, thereby sharing anatomical similarities with tendons. Because the sclera mainly consists of dense bundles of type I collagen and has smaller numbers of collagen types *III, IV, V, VI, VIII* [8,9] and elastin [10,11], this tissue is not easy to use for immunohistochemistry.

Anatomically, the sclera can be separated into three main layers: (1) the superficial vascularized episclera, found underneath the conjunctiva and tenon's capsule and towards the sides and the back of the eye facing the orbit; (2) the scleral stroma, the main part of the sclera; and (3) the lamina fusca, which is a thin, pigmented layer located directly above the uvea. Our anatomical knowledge about the sclera stems mainly from the first half of the 20th century. At that time, researchers studied the anatomy of vasculature mainly by using India ink injections [12] and vascular casting [13-15]. Later, it was researched in angiographic studies [16-19].

Since that time, older techniques have been improved and new ones have been developed that have allowed us to supplement previous anatomical knowledge. For example, it has only been about one decade since we have had such reliable lymphatic markers as lymphatic vascular endothelium specific hyaluronan receptor-1 (LYVE1) [20] or podoplanin [21]. Confocal microscopy offers new possibilities for studying the anatomical features of the different tissues of the eye. It allows for multiple stains to be used for differentiating markers of cells or for the localization of cells in relation to blood vessels and other anatomical structures. It provides an overview when the sample is of a larger size and allows us to scan through a sample when in search of a specific cell type. With Z-Stack technology, confocal microscopy can be used for samples up to 100-200 µm. The sclera differs in thickness between 0.3 mm behind the muscle insertions and 1 mm at the posterior pole [11]. Due to both its thickness and opaqueness, the sclera is not suitable for confocal microscopy using traditional methods.

To remedy this, scleral tissues were laminated to allow for their analysis with confocal microscopy. This technology is useful for gaining a better understanding of both physiological and pathological situations in the human sclera.

Protocol

The use of human tissues must be reviewed and approved by an institutional review board or the equivalent. The work described here was approved by the local ethics committee and had approval for scientific examination. This work was performed according to the Declaration of

Helsinki. The human scleral specimens were obtained from the eyes of globe donors (maximum post-mortem time 24 hr) at the Eye Bank of the Department of Ophthalmology, University of Cologne, Germany.

1. Experimental Preparation

1. Prepare 96% ethanol and phosphate buffered saline (PBS) in different tubes. Prepare the primary antibodies in the recommended dilution of PBS containing 2% bovine serum albumin (BSA) and keep all solutions on ice. Prepare 100 µl per sample.
2. Clean all instruments and use sharp scalpels. After repeated cutting with the same scalpel, change the scalpel.
3. Obtain 1-2 colibri forceps, straight micro dissecting scissors, a #10 scalpel or ophthalmic scalpel micro feather, and four 26 G needles.
4. Wrap aluminum foil around a polystyrene plate or use a cork plate to affix the tissue. Work underneath a binocular stereo microscope. Work sterile underneath a laminar airflow if needed.

2. Prepare the Sclera

1. Gently hold the bulb and perform a perforating trepanation on the corneoscleral part, then rotate the trepan. Rotate the trepan carefully and evenly onto the surface to perform an equally round cut. Use a 15.5 mm sized trepan. Cut the remaining attachments with curved scissors. Remove the corneoscleral trepanation. This will result in an anteriorly opened bulb.
    1. Put the sclera on a swab with the open part upwards. Remove the retina and uvea, using colibri forceps to pull off both layers (retina and uvea) from the sclera. Do not leave the pigmented uvea on the inner sclera. Use curved scissors to remove the retina and uvea from the papilla. Remove the remaining conjunctiva, extraocular muscles, and tenon's capsule from the superficial sclera.
2. Remove the scleral sample in the required size by using straight-type scissors and the colibri forceps. Take about 2 cm^2 sized scleral samples from different locations. Because of the small size, hold the sclera gently and cut out the required size as squares using the straight-type scissors. The corneoscleral trepanation defines the anterior margin of the scleral samples.
    1. Avoid repeated grabbing with the forceps as the area where the tissue was grabbed is not suitable for confocal microscopy analyses. Prepare the required number of samples depending on the experiment type (*e.g.* anteriorly vs. posteriorly, compare **Figure 1**) and the amount of antibodies used.
3. Put the samples in 1.5 ml tubes for later use. Fix the samples in 1.5 ml 96% ethanol for 15 min, and then wash them three times each for 5 min in 1.5 ml PBS on the shaker. If working with frozen tissue, perform this step before snap freezing.
4. Use fresh tissue. However, if this is not possible, snap freeze the sclera in liquid nitrogen and keep it at -20 °C for later use. If working with frozen tissue, thaw before use. Avoid repeated thawing and freezing cycles.
5. Keep each sample in PBS containing 1.5 ml 5% BSA at room temperature for 2 hr. This step induces a swelling of the sample that is helpful for the laminating and also prevents unspecific binding.
6. After the samples have swelled, prepare the scleral squares for lamination. Work underneath a microscope, *e.g.* a binocular stereo microscope. Affix the posterior scleral samples to the polystyrene membrane wrapped in aluminum foil or the cork plate using the 26 G needles.
7. Bend the edges of the needles so they do not interfere with the visual field underneath the microscope.
8. Laminate the full-thickness scleral samples.
    1. First, hold the anterior scleral edge using colibri forceps. Then carefully cut a thin layer of the superficial sclera using the #10 scalpel, the ophthalmic scalpel micro feather, or a spatula blade. Hold the scalpel horizontally and cut the superficial layer as thinly as possible from the underlying layer.
    2. Dissect a thin scleral layer from the scleral square. This method is comparable to standard surgical techniques to prepare a flap during trabeculectomy and should result in 30-80 µm thick layers (compare **Figure 2**).
9. Prevent drying of the sclera by adding small amounts of PBS to the tissue, *e.g.* 50 µl PBS using a pipette.
10. Put the cut layers into 100 µl of PBS in the 96 well plate and label both the orientation (towards extern and intern) and the layer (superficial and profound) *e.g.* with a waterproof color.
11. Repeat steps 2.7-2.9 until the tissue is fully laminated.

3. Perform Immunohistochemistry

1. Add 100 µl of the required primary antibodies into the recommended dilution. Here, *e.g.* use CD 31 (monoclonal mouse anti human) and LYVE1 antibody (rabbit anti human), both in a dilution of 1:100 in PBS (containing 2% BSA) and incubate at 4 °C overnight. To change the medium in the 96 well plate, hold the pipette to the wall of the well and remove the fluid.
2. The next day, wash the samples three times each for 5 min with 200-300 µl PBS on the shaker. Add 100 µl of the corresponding secondary antibodies; here use goat anti mouse fluorescein isothiocyanate (FITC) and goat anti rabbit cyanine dyes 3 (Cy3), and incubate the samples at room temperature for 1-2 hr. Dilute the secondary antibodies 1:300 in PBS containing 2% goat serum.
3. Rinse the samples again three times each for 5 min in 200-300 µl PBS on the shaker.
4. Perform nucleus staining, *e.g.* 4',6-diamidino-2-phenylindole (DAPI) (dilution 1:2,000, 100 µl in each well and incubate 10 min at room temperature), then wash again 2 times in 200-300 µl PBS on the shaker.
5. Transfer the samples onto microscope slides, embed them in 1-2 drops of fluorescent mounting medium, add a coverslip, seal it by covering the edges with transparent varnish, and store at 4 °C for later use or directly examine the slides with the confocal microscope.
6. Use a confocal microscope (or equivalent) to analyze the samples. Use the desired magnification, *e.g.* 10-40X magnification.
7. To verify the thickness of the samples, measure with confocal microscopy using Z-Stacks. The amount of possible scleral sections depends on the location of the sclera, as the thickness differs equatorially and posteriorly (compare introduction).

Representative Results

In the representative experiments performed here, there are demonstrable benefits derived from the use of this particular laminating technique. The first experiment illustrates the diverse network of the episcleral blood vessel plexus in three representative pictures (**Figure 3**). The vessels are positive for CD31.

The second experiment shows immune cells, in particular LYVE1 + cells of the episclera and their relationship to the CD31 positive blood vessels. Here Z-Stack technology was used at 10X magnification to scan through the tissue and understand three-dimensional relations of blood vessels and immune cells (**Figure 4**).

The sclera, as well as the enclosed extraocular muscles, can be analyzed for the presence of blood vessels or immune cells. **Figure 5** shows LYVE1 positive cells and CD31 positive blood vessels.

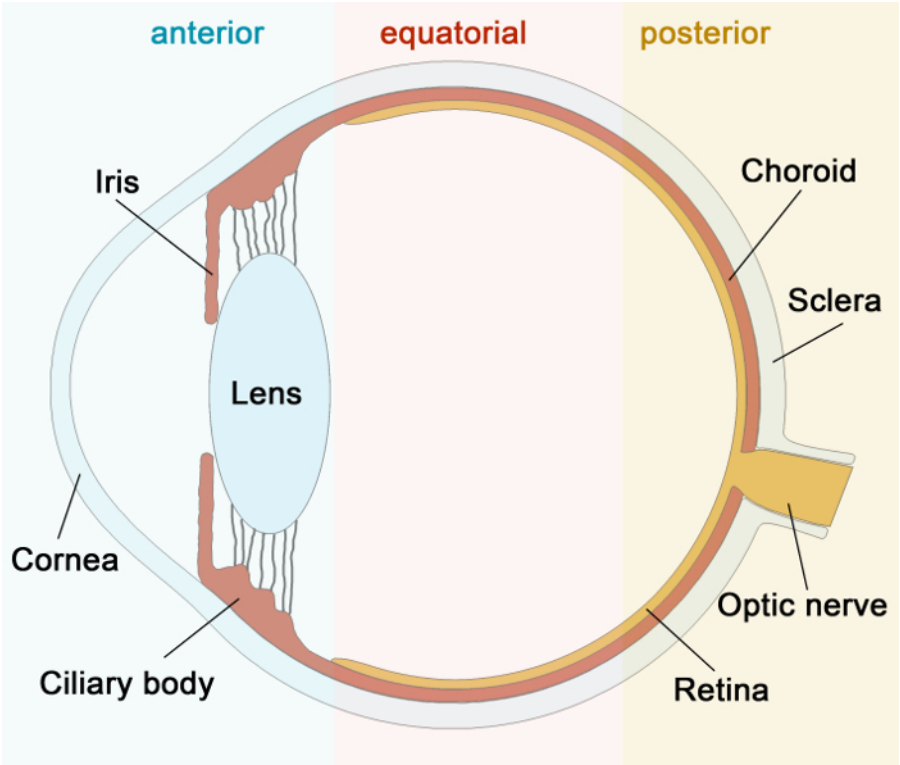

Figure 1: Schematic Picture of the Human Eye. Here is a schematic of the cross-section of the human eye. Please click here to view a larger version of this figure.

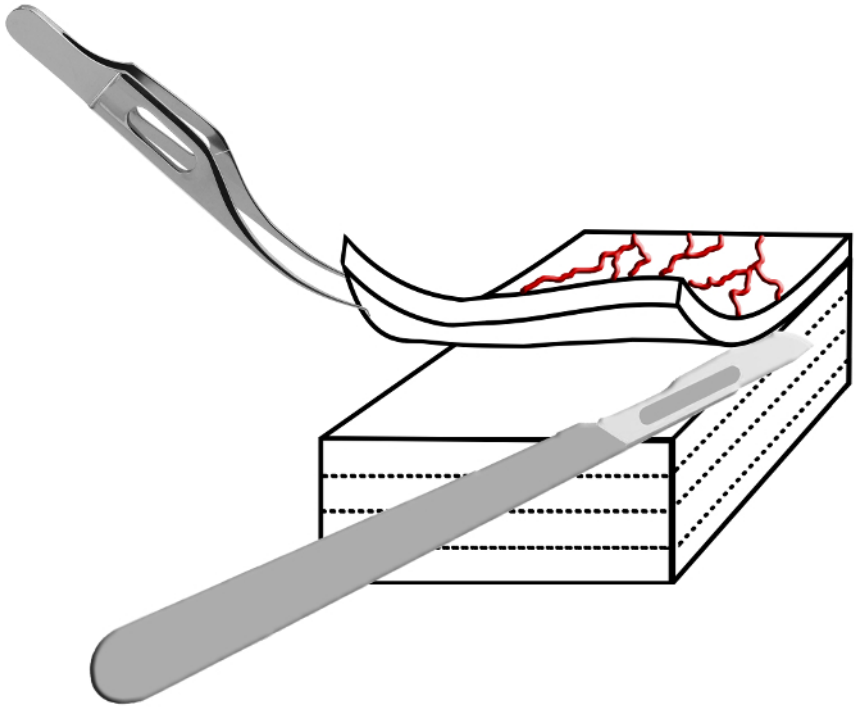

Figure 2: Schematic Picture of the Laminating Technique. The sclera is carefully held with colibri forceps and laminated into fine layers using a scalpel. Repeating this step leads to thin tissue slides that can be used for confocal microscopy. Please click here to view a larger version of this figure.

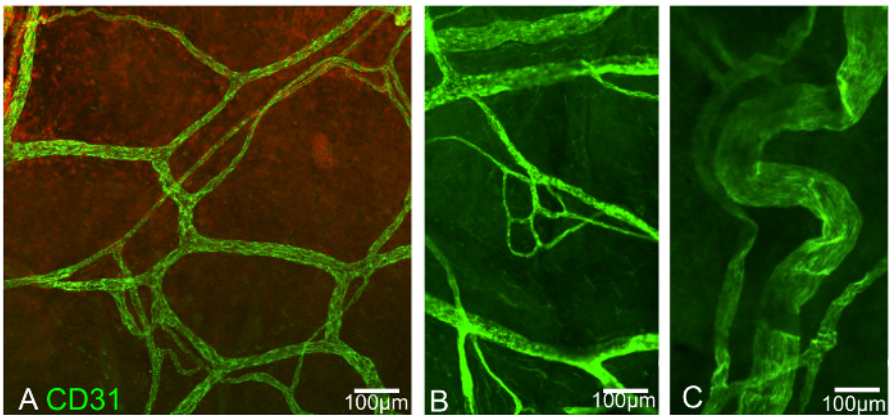

Figure 3: Episcleral Blood Vessel Plexus, Immunopositive for CD31. A and B are derived from the anterior episclera, while C is from the posterior location. Scale bar indicates 100 µm. Please click here to view a larger version of this figure.

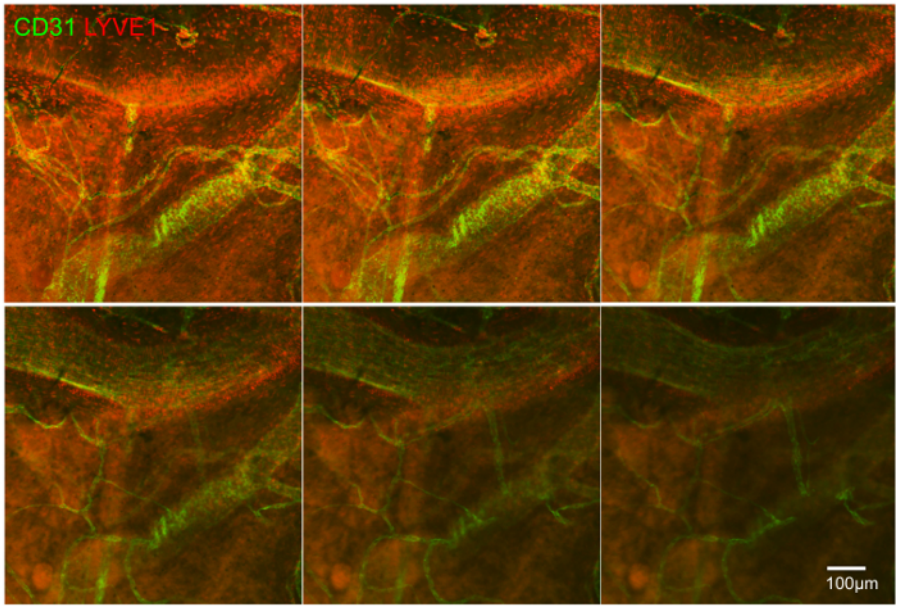

Figure 4: CD31 + Blood vessels and LYVE1 + Cells in a z-stack, Showing the Anatomical Relations Between Them. Small vessels and larger ones may overlap. Scale bar indicates 100 µm. Please click here to view a larger version of this figure.

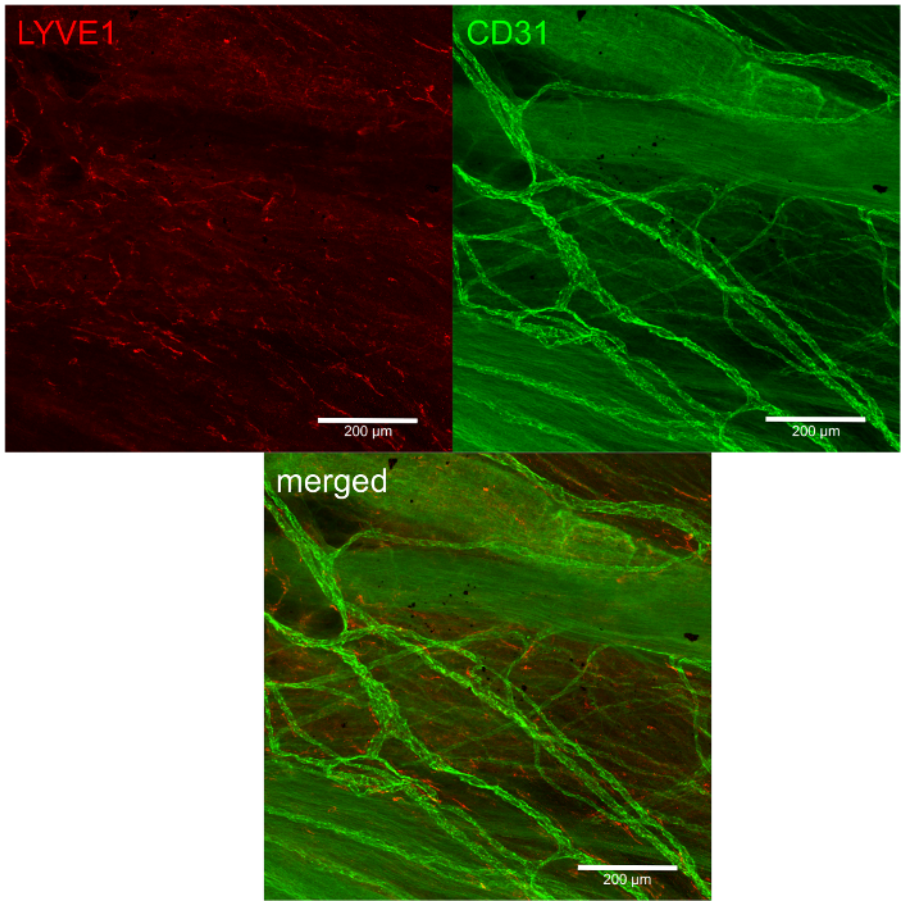

Figure 5: Enclosed in the Sclera are the Extraocular Muscles. They contain a similar fine blood vessel network and some LYVE1 + cells. Scale bar indicates 200 µm. Please click here to view a larger version of this figure.

Discussion

Laminating the human sclera is a method for performing confocal microscopy on this tissue. A critical step in this process is the use of ethanol instead of formalin for affixing the tissue. In our experience, better results are obtained when using ethanol instead of formalin for fixation.

Blunt scalpels aggravate the procedure and should be avoided. Similarly, the drying up of the sclera should be avoided, as it complicates the procedure and reduces the quality of the images.

Regarding the immunohistochemical staining, other antibodies than the ones applied here can be used. In general, collagen shows more auto-fluorescence and background in the green channel, therefore red, blue, and far-red channels show better results with less background.

However, there are several limitations of the technique. Laminating the sclera requires a lot of practice, as it is a microsurgical procedure performed under a microscope. Because this technique is performed manually, the single laminated slices are not exactly the same size and differ in their thickness within one piece of tissue. To have tissues of exactly the same size, trepanation might be an alternative, although the thickness of the layers will still vary. To achieve an exactly defined thickness for the scleral sections, an automated microkeratome might be an alternative for future experiments. If lamination is performed with layers that are too thick, the cover slide may dislocate, and the tissue can potentially dry out. The exact borders between episclera and stroma may be hard to differentiate once the laminations are performed.

Despite this, laminating the sclera has several advantages in the use of immunohistochemistry, particularly when compared to formalin-fixed paraffin embedded immunohistochemistry, which is the standard method in the current clinical situation. Laminating the sclera allows confocal microscopy to be performed, larger sized areas of sclera to be analyzed, different layers of the sclera to be examined, as well as the screening and scanning of tissue. In contrast, when working with formalin-fixed paraffin embedded samples, the sclera changes its consistency in an unhelpful way during preheating for antigen retrieval. Throughout the heating process, the collagen fibers shrivel and the tissue loses contact with the slide. Previously, the vessel plexus in the episclera have only been visible by using angiographic techniques or in the whole mount view, but not in cross sections.

In addition to the technical difficulties, the sclera is relatively avascular and relatively poor in immune cells compared to other tissues in the eye, such as the retina. Therefore, when using formalin-fixed paraffin embedded slides, which are usually 4 µm thick, multiple slides are needed to detect positive cells in the sclera. Recently, it was shown using this laminating technique that the human sclera is not acellular, but contains plenty of LYVE1+ macrophages to accumulate around blood vessels [1].

To summarize, performing confocal microscopy on laminated scleral sections is a promising tool to investigate pathological disorders in the future, such as scleritis, uveitis, or trauma.

Disclosures

The authors declare no financial disclosures. The contents of this article are solely the responsibility of the authors.

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References

1. Schlereth, S. L. *et al.* Enrichment of lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1)-positive macrophages around blood vessels in the normal human sclera. *Invest Ophthalmol Vis Sci.* **55** (2), 865-872, (2014).
2. Schlereth, S. L. *et al.* Absence of lymphatic vessels in the developing human sclera. *Exp Eye Res.* **125** 203-209, (2014).
3. Hos, D., & Cursiefen, C. Lymphatic vessels in the development of tissue and organ rejection. *Adv Anat Embryol Cell Biol.* **214** 119-141, (2014).
4. Hos, D., Schlereth, S. L., Bock, F., Heindl, L. M., & Cursiefen, C. Antilymphangiogenic therapy to promote transplant survival and to reduce cancer metastasis: what can we learn from the eye? *Semin Cell Dev Biol.* (2014).
5. Streilein, J. W. Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. *Curr Opin Immunol.* **5** (3), 428-432 (1993).
6. Streilein, J. W., & Niederkorn, J. Y. Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. *J Exp Med.* **153** (5), 1058-1067 (1981).
7. Streilein, J. W., Yamada, J., Dana, M. R., & Ksander, B. R. Anterior chamber-associated immune deviation, ocular immune privilege, and orthotopic corneal allografts. *Transplant Proc.* **31** (3), 1472-1475 (1999).
8. Keeley, F. W., Morin, J. D., & Vesely, S. Characterization of collagen from normal human sclera. *Exp Eye Res.* **39** (5), 533-542 (1984).
9. Lee, R. E., & Davison, P. F. Collagen composition and turnover in ocular tissues of the rabbit. *Exp Eye Res.* **32** (6), 737-745 (1981).
10. Moses, R. A., Grodzki, W. J., Jr., Starcher, B. C., & Galione, M. J. Elastin content of the scleral spur, trabecular mesh, and sclera. *Invest Ophthalmol Vis Sci.* **17** (8), 817-818 (1978).
11. Foster, C. S., Sainz de la Maza M. *The sclera.* Springer, (2012).
12. Kiss, F. Der Blutkreislauf des Auges. *Ophthalmologica.* **106,** 225 (1943).
13. Ashton, N. Anatomical study of Schlemm's canal and aqueous veins by means of neoprene casts. Part I. Aqueous veins. *Br J Ophthalmol.* **35** (5), 291-303 (1951).
14. Ashton, N., & Smith, R. Anatomical study of Schlemm's canal and aqueous veins by means of neoprene casts. III. Arterial relations of Schlemm's canal. *Br J Ophthalmol.* **37** (10), 577-586 (1953).
15. Ashton, N. Anatomical study of Schlemm's canal and aqueous veins by means of neoprene casts. II. Aqueous veins. *Br J Ophthalmol.* **36** (5), 265-267; contd (1952).
16. Hayreh, S. S., & Scott, W. E. Fluorescein iris angiography. II. Disturbances in iris circulation following strabismus operation on the various recti. *Arch Ophthalmol.* **96** (8), 1390-1400 (1978).

17. Virdi, P. S., & Hayreh, S. S. Anterior segment ischemia after recession of various recti. An experimental study. *Ophthalmology.* **94** (10), 1258-1271 (1987).
18. Bron, A. J., & Easty, D. L. Fluorescein angiography of the globe and anterior segment. *Trans Ophthalmol Soc U K.* **90** 339-367 (1970).
19. Ikegami, M. [Fluorescein angiography of the anterior ocular segment. Part 1. Hemodynamics in the anterior ciliary vessels (author's transl)]. *Nihon Ganka Gakkai Zasshi.* **78** (7), 371-385 (1974).
20. Banerji, S. *et al.* LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol.* **144** (4), 789-801 (1999).
21. Breiteneder-Geleff, S. *et al.* Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol.* **154** (2), 385-394,(1999).

