A simple staining method for blood vessels in flat preparations of ocular tissues. Giuseppina Raviola and Thomas F. Freddo.

We describe a technique for light microscopic visualization of the vascular tree of the retina, choroid, ciliary body, and iris in flat preparations. The technique is reproducible and much simpler than other available methods in which dyes suspended in different media are injected directly into the ocular vessels.

An indispensable prerequisite for the ultrastructural analysis of the vessels of an organ is the knowledge of the general morphology of the vascular tree. The eye is an ideal object for this sort of study because its tunics can be separated and their vasculature can be independently analyzed in flat preparations. In the course of our studies on the microvasculature of the eye, we have developed a new technique for the light microscopic visualization of ocular blood vessels, and we have often used it as a prelude to our electron microscopic investigations.

Materials and methods. Six adult rhesus monkeys of both sexes were used for this study. With the animals in general anesthesia (pentobarbital), horseradish peroxidase (HRP) (Sigma, Type II, 500 mg/kg body weight, dissolved in 3 to 5 ml of phosphate-buffered saline at pH 7.2) was slowly injected into the small saphenous vein. The temperature of the eye, in situ, would not be lost in subsequent procedures. Fifteen minutes after the end of HRP injection, the animals were sacrificed with an overdose of anesthetic, the eyes were enucleated, cut at the equator, and immersed in the fixative fluid (2% paraformaldehyde-0.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4). During the first 10 min of fixation the posterior part of the eye was cut into three or four wedges, and the vitreous body was carefully removed from the surface of the retina; the retina was immediately separated from the choroid and the choroid from the sclera. The retina had to be isolated at the beginning of fixation because after a short period in glutaraldehyde-formaldehyde solutions, it became strongly adherent to the pigmented epithelium and it was easily damaged when attempts were made to separate it from the choroid. After removal of the lens, the anterior segment of the eye was cut into two parts, and the whole ciliary body and iris dissected from the sclera. The separated retina, choroid, ciliary body, and iris were fixed for 2 hr at room temperature and washed overnight in buffer. Subsequently, the tissues were processed as follows:

1. 24 hr at room temperature (20° to 24°C) and in the dark in a large volume (500 ml) of 0.05M Tris HCl buffer, pH 7.6, containing 0.05% 3,3'-diaminobenzidine (3,3',4,4'-tetraamino-biphenyl) tetrahydrochloride, grade II (DAB) (Sigma). This solution was prepared just before use.

2. 24 hr at room temperature and in the dark in a fresh solution of DAB prepared as in 1, but containing 1% of a 0.5% solution of H2O2. The 0.5% solution of H2O2 was freshly prepared from 50% H2O2 and added to the medium just before use.

3. To bleach the melanin that masked the vascular trees injected with HRP, the choroid, ciliary body, and iris were maintained in 10% H2O2 in 0.05M Tris HCl buffer, pH 7.6, for a variable length of time, from 10 to 25 days. Fresh 10% solutions of H2O2 were prepared from 50% H2O2 and changed every day. Obviously, the treatment with H2O2 was not necessary for the retina.

The degree of bleaching was controlled with a dissecting microscope. Different bleaching times were necessary, depending on the thickness of the specimens and the degree of pigmentation of the different tissues and different animals. In our experience, higher concentrations of H2O2 decreased the bleaching time, but they damaged the tissues. After bleaching, the specimens were immersed and preserved at 4°C in 100% glycerol (index of refraction 1.4729) in a tightly closed jar. For photography, the specimens were gently transferred onto glass slides and temporarily covered with a coverslip. Light micrographs were taken with either a dissecting or a light microscope.

Results and comments. The HRP injected intravenously filled the lumen of the blood vessels of the eye and, when reacted with DAB and H2O2, stained the entire vascular tree brown to black against a light yellow, transparent background (Fig. 1). The long incubation of the retina in the DAB solution at pH 7.6 demonstrated, besides the vessels, the cytochrome oxidase activity of the mitochondria. In the monkey retina, the ellipsoids of the cones are densely packed with mitochondria, and thus, with the light microscope, they appeared as brown dots in flat preparations (Fig. 2).

This technique is a modification of the method developed by Graham and Karnovsky for the histological visualization of the injected vessels. This technique is reproducible and much simpler than other available methods in which dyes suspended in different media are injected directly into the ocular vessels.
Fig. 1. *Macaca mulatta* iris, seen from the anterior surface, in an *in toto* preparation. The pupillary margin is in the inferior part of the figure. The animal was injected intravenously with HRP; the entire iris was reacted for the histochemical demonstration of the enzyme and subsequently treated with H$_2$O$_2$ to bleach the melanin of the iridial epithelium and stroma. The small anterior vessels of the iris lie in the focal plane of this light micrograph, and more posterior vessels are visible in the background, out of focus (×71.)
Fig. 2. *Macaca mulatta*, flat preparation of retina. The animal was injected intravenously with HRP, and the retina was treated for the histochemical demonstration of the enzyme. The figure shows, at the same time, the injected retinal vessels and, on a different focal level, the photoreceptors. The treatment with DAB at pH 7.6 reveals the cytochrome oxidase activity of the mitochondria. Since the ellipsoids of the cones are more densely packed with mitochondria than the ellipsoids of the rods, they appear as dark dots. (×109.5.) Inset: Higher magnification of darkly stained cones and smaller, more numerous rods from the same preparation. (×438.)
tochemical detection of endogenous and exogenous peroxidase activities. Our procedure differs from their original method in that the tissues are maintained for a longer period of time in the incubating medium and the treatment with DAB is done in two steps—first without H₂O₂ and subsequently in DAB with a reduced concentration of H₂O₂. This latter modification was kindly suggested by Dr. M. J. Karnovsky. It permits penetration of DAB and H₂O₂ into specimens such as the choroid and iris, which are much thicker than the 40 to 50 µm tissue chopper sections for which the technique was originally developed. Prolonged exposure to low concentrations of H₂O₂ was a step that we added to unmask the injected vascular beds in heavily pigmented tissues such as choroid, ciliary body, and iris. Bleaching with H₂O₂ is a time-honored histochemical test, which was introduced for the differential diagnosis of melanin from other types of pigmented inclusions. It affects melanin and partially digests the connective tissue stroma without decreasing the color intensity of the reaction product in the lumen of the vessels.

This method represents an easy way to study flat preparations of blood vessels in the eyes of pigmented animals, and possibly in other organs. It can be applied to the study of ocular vessels in normal animals and in a variety of experimental and pathological conditions that cause alterations of the vascular tree. Furthermore, this technique can be useful in revealing leaky regions along vessels that, in normal conditions, are impermeable to HRP, and thus it can provide useful information on the integrity of the endothelial linings.

Our method has several advantages over other techniques in which dyes dissolved or suspended in solvents or resins are injected into small ocular vessels such as the anterior or posterior ciliary arteries or into the central artery of the retina. The injection of the tracer into the systemic circulation is a simple procedure; the results of the technique are highly consistent. Furthermore, postmortem injection of dyes and resins frequently results in only partial filling of the vascular tree; when HRP is injected in vivo, it circulates continuously through the vascular system, and thus the chances of filling the entire vascular bed, from its arterial to its venous end, are greatly increased. The method has two main limitations. (1) It stains only the portion of the vascular bed that is functionally open during the period of circulation of the tracer; (2) after prolonged oxidation, the tissue is no longer suitable for electron microscopic examination.

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REFERENCES

Degranulation of ocular mast cells in rats undergoing systemic anaphylaxis. MATHEA R. ALLANSMITH, ROBERT S. BAIRD, AND KURT J. BLOCH.

The percentage of degranulated ocular mast cells in five egg albumin-immunized, systemically challenged rats was compared with that in five egg albumin-immunized, saline-challenged controls. For the lid and orbit, at least 100 mast cells per rat were evaluated. For the conjunctiva, tarsus, limbus, and episclera-sclera, at least 20 were evaluated. For the ciliary body and choroid, all mast cells in 15 sections separated by at least 15 µm were examined. The morphology of degranulated and intact mast cells was the same in the experimental and control animals, except for the change in granules. There were significantly more degranulated mast cells in most of the ocular tissues of rats undergoing systemic anaphylaxis than in tissues of control rats; the increase in degranulated cells was especially striking in the choroid. We concluded that degranulation of mast cells is a measure of anaphylaxis, provided that the number of degranulated mast cells, and not simply the presence of such cells, is considered and compared with appropriate controls.