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Amaiak Chilingaryan
Amayak Chilingaryan
Gary Martin, Occidental College

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Research Report

The three-dimensional detection of microvasculatory bed in the brain of white rat *Rattus norvegicus* by a Ca\(^{2+}\)-ATPase method

Amaik Chilingaryan\(^a\)*, Amayak M. Chilingaryan\(^b\), Gary G. Martin\(^a\)

\(^a\)Department of Biology, Occidental College, Los Angeles, CA 90041, USA
\(^b\)Orbeli Institute of Physiology, Armenian National Academy of Sciences, Yerevan, Armenia 375028

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**ABSTRACT**

A procedure is described which allows for the selective and non-injectional staining of the three-dimensional microvasculatory bed (MVB) in thick sections (60–140 μm) of formalin-fixed brain tissue of white rats *Rattus norvegicus*. This histochemical method detects ATPase activity and takes place between pH 10.5 and 11.2. Calcium ion is used to capture inorganic phosphate, calcium phosphate is converted to lead phosphate, and subsequently converted to black or dark brown lead sulfide. All vessels are revealed due to a precipitate on the endothelium and smooth muscle cells of arterioles. In some vessels, red blood cells also stain. The background is transparent with no staining of neurons, nerve fibers, glial cells, or nuclei. This allows for clear identification of arterioles, venules, and capillaries, which is difficult using other methods. New observations are described including the presence of webs connecting branching parts of arterioles and constrictions along vessels. This procedure should be useful in investigations of the MVB in rat brain.

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**1. Introduction**

Knowledge of the three-dimensional network of vessels in the brain is essential for understanding the functioning of this organ in normal and disease conditions. The complexity of the microvasculatory bed (MVB; arterioles, capillaries, and venules) is not appreciated using routine histology on sections typically 8 μm thick. To obtain larger views of the MVB, two basic techniques have been used: injection techniques and specific labeling of the vessels. The former relies on the injection of contrast materials, such as India ink in gelatin (Grivas et al., 2003), into vessels for light microscope (LM) observation and corrosion casting techniques for analysis by scanning electron microscopy (SEM) (Akima et al., 1986; Duvernoy et al., 1981; 1983; Nonaka et al., 2003; Rodriguez-Baeza et al., 1998). Injectional techniques have provided striking images of the vasculature of the brain, but they have three specific disadvantages (Fujimoto et al., 1996; Itoh et al., 2000; Klosovskii, 1963; Minamikawa et al., 1987): (1) arterial and venous vessels cannot be distinguished, (2) injections may result in unequal filling of some vessels and no filling of closed ones, and (3) closed vessels may artifactually be opened under pressure of perfusion. In addition, it is not possible to use any other methods on the same material.

*Corresponding author. Current address: Armine Nazaryan, M.D., Medical Clinic, 222 W. Eulalia St, Ste. 211, Glendale, CA 91204, USA. Fax: +1 818 500 0245.
E-mail address: chilingamvb@yahoo.com (A. Chilingaryan).
Detection of MVB by direct staining of structural elements of vascular walls is desirable, but developing such methods faces great challenges. Selective staining of the MVB with little background stain is essential. Standard histological methods rely on sections that are too thin to capture the three-dimensional networks, and, in thicker sections, background staining obscures the vessels. Immunohistochemical techniques used on sections up to 20 μm thick for light microscopy (Duijvestijn et al., 1992; Gallyas et al., 1993; Pardridge et al., 1990; Topel et al., 1998), and on thin sections examined by transmission electron microscopy (Farrell and Pardridge, 1991), show fragments of vessels and not expansive views of the MVB. These difficulties were partially solved by Golgi’s impregnation method on tissue blocks (Klosovskii, 1963; Marin-Padilla, 1985). It is also possible to view the capillary network and small vessels on formalin-fixed sections using a silver impregnation technique (Gallyas, 1970). Despite the capriciousness of the impregnation methods, it was possible to stain the MVB in the brain (Klosovskii, 1963; Marin-Padilla, 1985), but it was not possible to differentiate between arterial and venous vessels.

Numerous procedures have been advanced to label the MVB by taking advantage of enzymes present in or on the vessel walls to provide differentiation of arterioles, capillaries, and venules in very thick (>100 μm) sections. Chilingaryan (Chilingaryan, 1977, 1986; Chilingaryan and Chilingaryan, 1986; Chilingaryan et al., 1997) developed a technique that met these requirements on thick sections through cat organs including heart, kidney, brain, liver, and muscle. However, attempts to apply this technique to the MVB in the brain of white rats Rattus norvegicus did not give satisfactory results, and this is a major problem considering the importance of this animal in neurobiological research. It has become clear that the staining techniques and the underlying basic differences in ATPase activity vary not only with the type of organ being studied but also with the species and strain of animal being used. The purpose of this paper is to describe a technique which allows for the specific staining of the MVB in the brain of the common research rat, R. norvegicus, and compare this technique to other procedures employing the enzymatic labeling of walls in MVBs.

2. Results

Sections through the cortex, cerebellum, and brainstem were stained by both the Padykula and Herman (1955a,b) method and our procedure. Non-specific staining of thick sections (60–140 μm) processed according to the Padykula and Herman method yielded dark backgrounds which made it impossible to distinguish elements of the MVB. In thick sections (60–140 μm) through all three brain regions processed according to our technique, the three-dimensional MVB stood out clearly against a transparent background with no staining of neurons, glial cells, or nerve fibers. Fig. 1 shows the MVB in a typical section through the boundary between gray and white matter in the cortex. Arterioles penetrating into the brain parenchyma branched into pre-capillaries where the smooth muscle cells were not stained. Pre-capillaries gave rise to capillary networks. Post-capillaries emerged from capillaries and merged into venules that leave the parenchyma. The density of MVB was greater in gray matter and in locations of nuclei than in white matter. All vessels stained black or brown due to lead sulfide precipitation on the vessel endothelium. Arterial and venous vessels were easily distinguished (Figs. 1–5) because, in the former, smooth muscle cells were stained. Arterioles incubated in sections for shorter times (Fig. 3) stained lighter than those incubated for longer periods (Fig. 2). Venules and capillaries appeared as hollow tubes with thin walls.

Fig. 1 – 140 μm frontal section of the rat cortex showing capillary networks (C) with venules (V) and arterioles (A). Note pre-capillary (AC) and post-capillary (VC) vessels and the difference in vascular density in gray matter (GM) (laterodorsal thalamus nucleus) and white matter (WM). Scale bar = 50 μm.
walls. Branches from both arterioles and venules occurred at acute and obtuse angles (Figs. 2, 7). In a minority of vessels, red blood cells were clearly seen (Figs. 4 and 5). Fig. 6 shows the staining of the dense blood vessels of the choroid plexus.

The first two control experiments using heated sections and incubation solution without ATP, respectively, yielded transparent sections with no labeling (data not shown). In the third control, the addition of tetramisole hydrochloride did not affect the normal reaction described above.

In our technique, each stage in the treatment of the tissue is important for clear three-dimensional detection of MVB. ATPase activity is sensitive to fixation (Manoonkitiwongsa et al., 2000), but we saw no substantial changes in vessel staining in tissues fixed between 2 and 10 days. We found that ATPase activity at pH 9.0 requiring calcium ions (suitable for the Padykula and Herman (1955a) method) was not appropriate for vascular ATPase on thick sections. In our technique, the best calcium phosphate precipitation was achieved at a pH

Fig. 2 – Thick (140 μm) frontal section through cortex showing darker staining arterioles (A, due to smooth muscle staining) and lighter staining venules (V). Note the acute branches on the venule and the non-acute branches on the arterioles (arrow). Scale bar = 100 μm.

Fig. 3 – High magnification view of arterioles in the cortex showing branches and investing smooth muscle cells (arrows). These sections were incubated for a shorter time than the sections shown in Fig. 4 which are more densely stained. Scale bar = 20 μm.
between 10.5 and 11.2. Initially, for retention of high pH, Laski’s glycine buffer was tried, which is unique in its high molarity, but this buffer did not give satisfying results. The clarity of the results depends greatly on the incubation time. Staining of the arterial vessels occurred faster than other vessels. In longer incubation, necessary for staining of capillaries and veins, staining of arterioles often produced diffuse character, but they could still easily be distinguished from venules. Shorter incubations were useful for studying large vessels in thicker sections since they were not obscured by the staining of the capillary beds. Ammonium acetate was used as a differentiating agent with the goal of removing the adsorbed lead ions. In long washes of ammonium acetate, lead phosphate was also dissolved. Surprisingly, longer incubations or lead replacement time greatly decreased the solubility of lead phosphate in ammonium acetate.

Two new observations were made on the MVB in the rat brain using the new procedure. First, immediately following the branching of the arterioles, a film or web was seen connecting the two vessels (Fig. 7). Second, no narrowing of vessels was observed at the branching section of the vessels.

3. Discussion

The procedure described in this paper provides selective staining of the MVB in thick (60–140 μm) sections through the brain of the white rat, and arterioles, capillaries, and venules are clearly distinguished from one another. Such differentiation has not been achieved by other methods and should provide for a better understanding of the MVB in the brain of this important animal. Our technique is based on the presence of specific ATPase in the endothelium of the MVB and in smooth muscle cells lining the arterioles. In control experiments where the sections were heated or ATP was deleted, no staining occurred. This supports the enzymatic nature of the reaction leading to a precipitate on the vessels. These data greatly differ from the results of Chilingaryan (1977) that suggested that precipitate formed due to ATP hydrolysis by calcium ions since the ATPase inhibitor p-chlorolomercury-benzoate or a 6-month fixation at 4 °C did not restrict the reaction. Currently, it is unknown if the precipitation results from Ca²⁺-ATPase or ecto- and endo-ATPase activity (Manoonkitiwongsa et al., 2000), and inhibition is a complicated question because the compounds affect differently in different tissues (Meghji and Brunstock, 1995). Finally, tetramizole hydrochloride, an inhibitor of alkaline phosphatase, had no effect on the staining, suggesting that this enzyme is not responsible for the reaction.

The development of this technique follows on earlier procedures that attempted to label the MVB in various organs. The lead method of Chilingaryan (Chilingaryan, 1965; Chilingaryan and Paravyan, 1971) as well as the Golgi impregnation method provided staining of vessels in the brains of several animals, but neither technique allowed for the differentiation of arteriolar and venous vessels. There are suggestions in the literature that the Gomori alkaline phosphatase method may be useful in staining MVB (Saunders and Bell, 1971; Hunziker et al., 1974; Romanul et al., 1962; Rowan and Maxwell, 1981a,1981b; Scharrer, 1950). A modification of this method (Bell and Scarrow, 1984) provided staining of the MVB in thick sections (100–500 μm) and was widely used in several investigations (Anstrom et al., 2002a; Anstrom et al., 2002b;
Usefulness of this reaction varies in different species of rats
(Schults-Hector et al., 1993), and staining occurs only on
endothelium of arterioles and capillaries but not venules.
Labeling techniques based on the detection of various
isomers of ATPase have selectively stained endothelia of
capillaries, venules, and arterioles and arteriolar smooth
muscle cells. However, these procedures have been used
primarily in electron microscopical studies and very rarely by
light microscopy on very thin sections (Inomata et al., 1989;
Kawai et al., 1991; Kawamata et al., 1986; Nag, 1987, 1988;
Thirion et al., 1996; Vorbrodt and Trowbridge, 1991). In 1986,
Chilingaryan (1986) described a technique, based on ATPase
activity, that allowed labeling of the MVB in the brains of cats,
humans, and other animals, but no work was done on rats.
Recent use of this technique on rats did not give satisfactory
results, leading to the procedure described in the paper. The
incubation solution suitable for labeling the MVB in the brain
of rats does not stain the complete MVB in the heart and
kidney (unpublished observations). The differences in these
staining reactions illustrate the underlying differences in the
cellular activity in different organs and species.
Our technique should allow for a better understanding
of the MVB in normal and experimental conditions. For
example, changes in the constriction and dilation of
capillaries in the brain could be quantified following the
administration of vasoactive substances (Melkonyan and
Chilingaryan, 1986; Melkonyan et al., 1993). Our procedure
and injectional methods both provide three-dimensional
views of the MVB. However, our technique provides several
improvements over previous methods. First, all vessels are
stained, and not just those that are open at the time of
tissue processing. Second, the three-dimensional network
of the MVB may be examined in thick (60–140 μm) sections
by light microscopy. In previous techniques, sections had to
be considerably thinner or background staining obscured
the vasculature. Third, unlike corrosion casting methods,
which also provide detailed views of the three-dimensional
network of vascular beds, surrounding tissues are not
eliminated, and other histological methods could be
employed on the same material. Such an approach was
applied while studying the density of vascular network in
some parts of the brain by alkaline phosphatase and Nissi’s
methods (Fonta and Imbert, 2002; Tieman et al., 2004). It
may also be important that our technique stained the
erthrocytes in some of the vessels. Because it is known

Fig. 7 – A 60 μm thick section though the medulla showing a stained web at the branching of the arterioles (w). Note some branches of the arterioles (arrows) have obtuse angles. Scale bar = 20 μm.

Fig. 8 – High magnification view of an arteriole in a 60 μm thick section through the medulla showing multiple constrictions on the stem of the vessel from a normal diameter of 18 μm at Z, to 10 μm at A, 12 μm at B and D, and 13 μm at C. Scale bar = 20 μm.
that biomicroscopic studies are based on erythrocyte movement, the simultaneous staining of vessels with the blood elements may provide new opportunities to investigate intraorgan microcirculation.

While developing this new technique, two new observations were made on the MVB in the rat brain. First, there is a web which connects two arterioles for a short distance after they branch. This could be an extension of smooth muscle cells that stretch across the branch. Second, one or more constrictions were observed along the main axis of vessels distal to the branch point, and, in fact, no narrowing of vessels was observed at the branching section of the vessels. Over the past 40 years, Chilingaryan has observed constrictions only on the branching point in the brain sections of newborn human brains with post-natal trauma (unpublished data).

In conclusion, the importance of a healthy MVB in the brain cannot be overstated, and the non-injectional technique described in this paper can provide reproducible results on the three-dimensional architecture of the MVB in the brain of white rats R. norvegicus. Our research demonstrates that arterial staining differs substantially from venous and capillary staining, vessels in different organs stain differently, and vessels in different species will also require adjustments in the incubation solutions to achieve proper staining. However, this procedure provides excellent visualization of the MVB in the brain of the white rat.

### 4. Experimental procedure

A total of seven white rats R. norvegicus, 200–250 g, of both sexes were acquired from Occidental College Psychology Department. They were overdosed by an I.P. injection of sodium pentobarbital (Nembutal), decapitated with a guillotine, and the brains were removed and placed in 4% formalin. The cerebellum and brainstem were cut from the cortex, which was also bisected in the frontal plane. After 48 h, the tissues were stored in 0.9% saline for up to 60 days. Frontal sections of the brain were sliced on a Cryostat (Tissue-Tek II) at 60–140 μm. Sections could be stored in 0.9% saline with a small thymol crystal in the refrigerator for about a month and still show clear staining of the MVB. Sections were rinsed in dH2O for 5 min and processed by either the Padykula and Herman (1955a,b) method or our new technique described below.

For our technique, sections were incubated for 60–80 min in a solution at pH 10.5–11.2, containing 5 ml dH2O, 10 ml 2 M ammonium hydroxide, 0.6 ml 1 M CaCl2, 3 ml 0.5% ATP (Sigma #A3377), and dH2O added to a total volume of 20 ml. After incubation, the sections were washed in dH2O for 5 min and then transferred into a lead replacement solution (20 ml 0.2 M ammonium hydroxide, 3 ml 5% sodium citrate, and 2 ml 0.2% lead acetate) for 30–60 min. If a precipitate formed, more sodium citrate was added. Sections were then washed in dH2O for 1 min, and 60 and 140 μm thick sections were transferred into a 2 N ammonium acetate solution for 30–60 s and 40–120 s, respectively. Thinner sections required less time to remove the adsorbed lead ions than thicker sections. Sections were then washed in dH2O for 1 min, transferred into a 5–10% sodium sulfide solution (concentration is not critical) for 10 s, or until darkening was completed. Finally, the sections were washed in dH2O and placed on slides. After excess fluid from the transfer was removed, a drop of glycerin gelatin solution was added as a coverslipping agent (Lillie, 1965).

Three controls were used to determine the enzymatic nature of the staining reaction. First, enzymatic activity in some sections was inhibited prior to incubation by preheating the sections in 90°C water for 15 min. Second, some sections were incubated without the substrate, ATP. Third, to make sure that alkaline phosphatase was not contributing to the staining, for some sections, 1–1.5 mM tetramisole hydrochloride (Sigma #L97526), a specific inhibitor (Stoward and Pearse, 1991), was added to the incubation solution.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainres.2005.11.059.

### References


Schults-Hector, S., Balz, K., Bohm, M., Ikehara, Y., Rieke, L., 1993. Cellular localization of endothelial alkaline phosphate...
reaction product and enzyme protein in the myocardium. J. Histochem. Cytochem. 41, 1813–1821.