

## ARTICLE

# Demonstrating Cerebral Vascular Networks: A Comparison of Methods for the Teaching Laboratory

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One challenge of neuroscience educators is to make accessible to students as many aspects of brain structure and function as possible. The anatomy and function of the cerebrovasculature is among many topics of neuroscience that are underrepresented in undergraduate neuroscience education. Recognizing this deficit, we evaluated methods to produce archival tissue specimens of the cerebrovasculature and the “neurovascular unit” for instruction and demonstration in the teaching lab. An additional goal of this project was to identify the costs of

each method as well as to determine which method(s) could be adapted into lab exercises, where students participate in the tissue preparation, staining, etc. In the present report, we detail several methods for demonstrating the cerebrovasculature and suggest that including this material can be a valuable addition to more traditional anatomy/physiology demonstrations and exercises.

*Key words:* neurovascular unit; cerebral blood flow; perivascular cells, blood vessels.

Blood vascular networks constitute a major component of the structure of the brain in addition to neurons, glia, and the extracellular milieu. Despite its crucial role in brain oxygen supply and nutrient regulation, the cerebral vascular system is often underrepresented in introductory neuroscience curricula. Moreover, although the gross anatomy of CNS vascular networks are commonly found in neuroscience textbooks (e.g. blood-brain barrier, Circle of Willis), the intimate relationship between blood vessels, glia, and neurons is often under-emphasized. In the present report we detail our effort to generate histological material, implementing a variety of different methods, for use in a laboratory-based class with the goal of providing students with a greater understanding of the “neurovascular unit” composed of blood vessels, perivascular cells, glia, and neurons. In addition, we describe a web-based approach for demonstrating cerebral vasculature and neurovascular units.

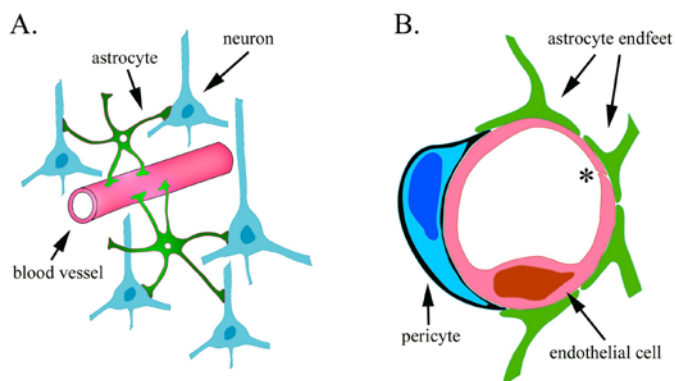
## The Importance of the Neurovascular Unit

The neurovascular unit is comprised of the endothelial cells which make up the vessels as well as several other associated cell-types including astrocytes and perivascular cells such as pericytes and smooth muscle cells. Figure 1 contains a schematic representation of the neurovascular unit.

Pericytes wrap around vessels and are in direct contact with endothelial cells via gap junctions (Figure 1B; reviewed in Bergers and Song, 2005). These cells provide structural support for vessels and also participate in vasomotion (vaso-constriction and dilation) thereby affecting cerebral blood flow. Pinocytosis and phagocytosis have been observed in pericytes, suggesting that these cells also play macrophagic roles.

Blood vessels in the brain are also surrounded by the endfeet of astrocytes (Figure 1). Thus, astrocytes constitute a cellular bridge between neurons and blood vessels. Astrocyte endfeet located on vessels interact

directly with endothelial cells and are capable of up-take and/or release of a number of molecules (e.g. amino acids, growth factors; reviewed in Abbott, 2002; Abbot et al., 2006). For example, astrocytes take-up glucose and convert it to lactate. Astrocytes are then able to release lactate into the extracellular milieu, providing neurons with the lactate necessary for energy production via glucose metabolism (reviewed in Tsacopoulos and Magistretti, 1996). Thus, astrocytes play an important role in the metabolic function of neurons.



*Figure 1.* Schematic illustration of the neurovascular unit composed of endothelial cells, pericytes, astrocytes, and neurons. Asterisk in B is adjacent to tight junction formed by endothelial cell.

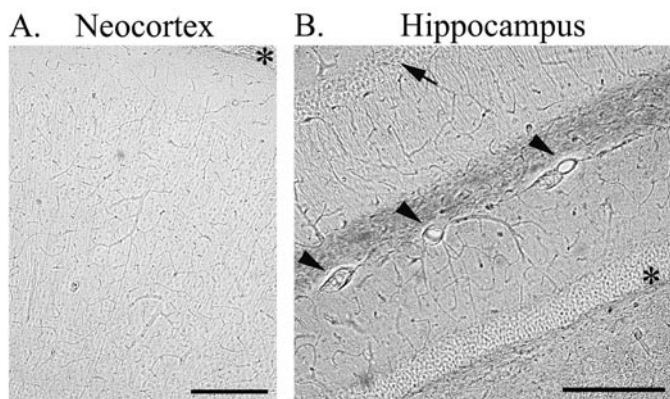
There are a number of reasons why we have included instruction and demonstration of the cerebral vasculature and the neurovascular unit in undergraduate neuroscience lecture and lab-based courses. The first and most obvious is that the brain (neurons, glia, etc.) requires oxygen. Natural extensions of this fact include the neural and functional consequences of ischemia (e.g. stroke) or decreased oxygen content (e.g. due to high altitude). Second, neuronal activity (action potential generation) comes at a high metabolic cost, requiring glucose

metabolism. This fact relates to the hemodynamic correlates of neural activity which is measured by blood-oxygen-level dependent function magnetic resonance imaging (BOLD-fMRI; Thompson et al., 2003; Pasley et al., 2007). Finally, changes in vascularity (e.g. vessel loss, angiogenesis) have been associated with disease such as brain tumors (Jain et al., 2007), Alzheimer's disease (Iadecola, 2004; Girouard and Iadecola, 2006) and epilepsy (Schwartz, 2007).

### Preparation of Histological Material for Demonstration of Neurovascular Networks.

Our goal was to create a collection of histological material demonstrating cerebral vasculature and cell-vascular interactions for use in a laboratory-based class for undergraduate neuroscience majors. In the process, we identified a number of different methods/preparations which varied in time and resource investment as well as in the fine anatomical details that were demonstrated. A natural extension of this work was evaluating which methods were suitable for student participation with the goal of incorporating one or more of these methods into lab exercises.

Rat and mouse brains were used for all of the preparations described below. In some cases brains were removed following deep anesthesia and cardiac perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PB. In other cases, brains were removed following deep anesthesia without perfusion. Unperfused brains were fixed by submersion in 4% paraformaldehyde. Brain sections of varying thickness (40-200 $\mu$ m) were prepared on a vibratome or freezing-stage sliding microtome.



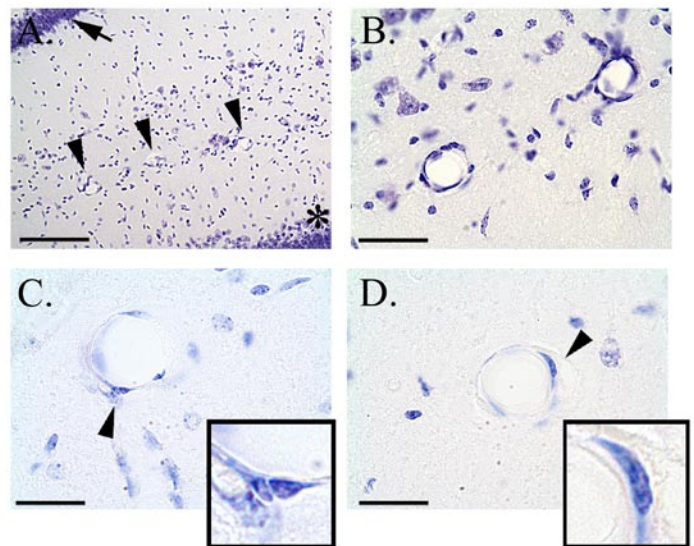
**Figure 2.** Demonstrating cerebral vasculature in unstained sections from mouse neocortex (A) and hippocampus (B). Asterisk in A refers to pial surface. Arrow and asterisk in B refer to the CA1 pyramidal cell and dentate gyrus granule cell layers, respectively. Arrowheads in B point to large vessels at the hippocampal fissure. Scalebars in A & B: 250 $\mu$ m.

#### Visualizing vessels in unstained tissue.

We adapted methods described in Zheng et al., (1991) for visualizing vessels in unstained sections. Perfused and unperfused sections were mounted and coverslipped in 2% gelatin in order to prevent dehydration and preserve vessel structure. With the microscope condenser at its lowest

position, providing sufficient contrast, vessels can be readily identified. Figure 2 contains photomicrographs of unstained sections with visible blood vessels in neocortex (Figure 2A) and hippocampus (Figure 2B). Branching in vessels is also clearly distinguishable.

An obvious advantage to using this method is that no additional reagents are required in order to demonstrate cerebral vasculature. Students can mount and coverslip the tissue themselves and can view structures at relatively high magnification. When possible, students can also participate in the tissue sectioning. Cell somata are visible in addition to vessels making it possible to link the association of vascular networks embedded within the parenchyma. Thus, this method is ideally suited for laboratory classes with access to rat/mouse brains but with limited budgets for additional reagents.



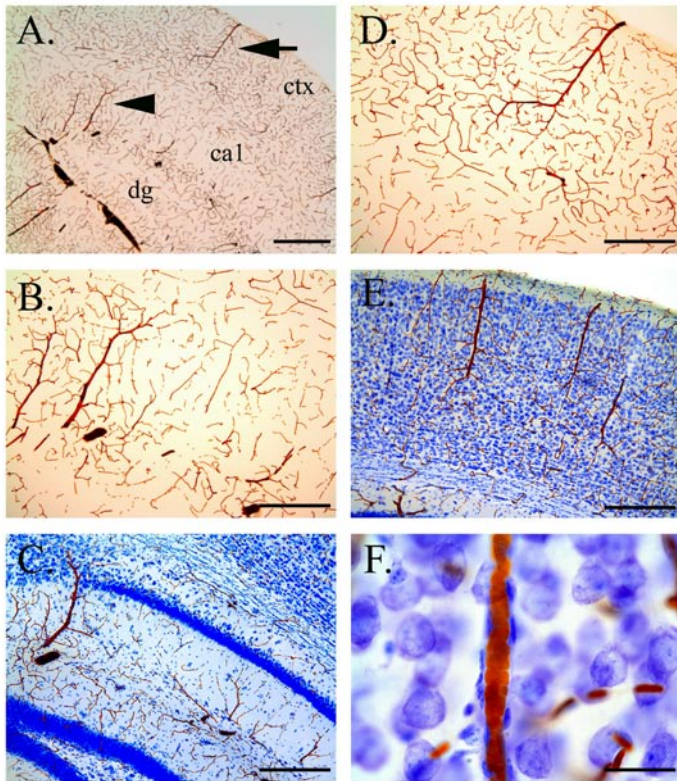
**Figure 3.** Demonstrating cerebral vasculature in Nissl-stained sections from mouse hippocampus. Arrow and asterisk in A refer to the CA1 pyramidal cell and dentate gyrus granule cell layers, respectively. Arrowheads in A point to large vessels at the hippocampal fissure. B-D, Examples of putative pericytes in close association to vessels found at the hippocampal fissure. Arrowheads in C-D refer to pericytes shown in insets. Scalebars in A-D in microns: 125, 42, 25, 25, respectively.

#### Visualizing vessels in Nissl-stained tissue.

Nissl-staining is frequently employed in the teaching lab. This simple stain allows for visualization of cell somata with the ability to distinguish neurons from glia based on soma size and color. Nissl stained sections can be used to illustrate some important aspects of cerebral vasculature. Following Nissl staining, dehydration, and coverslipping, visualization of vessels using the method described for unstained tissue is not possible. However, in addition to staining of neurons and glia, perivascular cells are stained, allowing for visualization of many vessels. In particular, vessels cut along the transverse axis are especially demonstrative since putative pericytes can be seen in close contact with vessels. Figure 3 contains representative photomicrographs of Nissl-stained sections where vessels found in the hippocampal fissure can be

seen (arrowheads in A). Closer examination of these vessels clearly reveals individual pericytes.

An advantage of the above method is that demonstrations can be done on tissue that was already intended to be stained as part of the planned course exercise. Additionally, this type of demonstration can be done on archival tissue which has previously been stained. Given the minimal additional expense required to illustrate the relationship between pericytes and blood vessels (i.e. the cost of staining materials) this method is advantageous for use in courses with limited budgets.

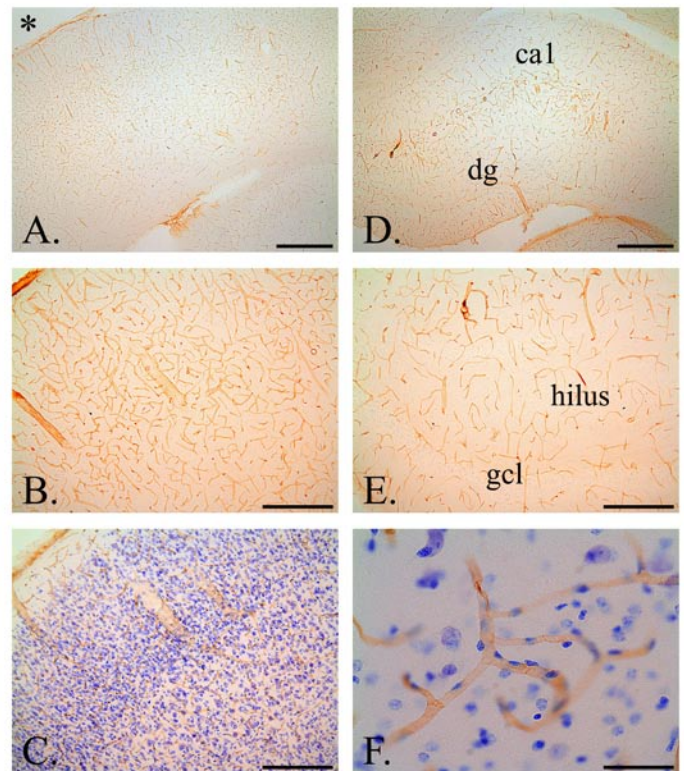


**Figure 4.** Peroxidase staining of RBCs in unperfused sections. Arrowhead and arrow in A refer to vessels shown at higher magnification in B and D, respectively. Nissl-counterstained sections shown in C (hippocampus), E and F (neocortex). High magnification of stained RBS in a vessel and associated perivascular cells shown in F. Scalebars in microns: A, 500; B-E, 250, F, 25.

#### *Visualizing vessels in unperfused tissue stained with 3, 3 diaminobenzidine (DAB).*

We adapted methods described by Sherman and Paull (1985) for use with unperfused tissue sections. This method stains the endogenous peroxidase found in red blood cells (RBCs). Sections from unperfused brains are placed in a solution of 1% DAB (Fluka) in PBS for 3-5 mins. Next 30% H<sub>2</sub>O<sub>2</sub> (Fisher Scientific) is added to the DAB solution to a final concentration of 0.06% H<sub>2</sub>O<sub>2</sub>. Within seconds of adding the H<sub>2</sub>O<sub>2</sub>, vessels can be seen turning a dark brown color. Sections are left in this solution for 3-5 mins. and subsequently washed three times in PBS for 10 mins. each. Sections are mounted on gelatin-coated slides in PBS and coverslipped after drying with Permount (Fisher Scientific; cat. no. SP15-100). Some slides were counterstained with Nissl.

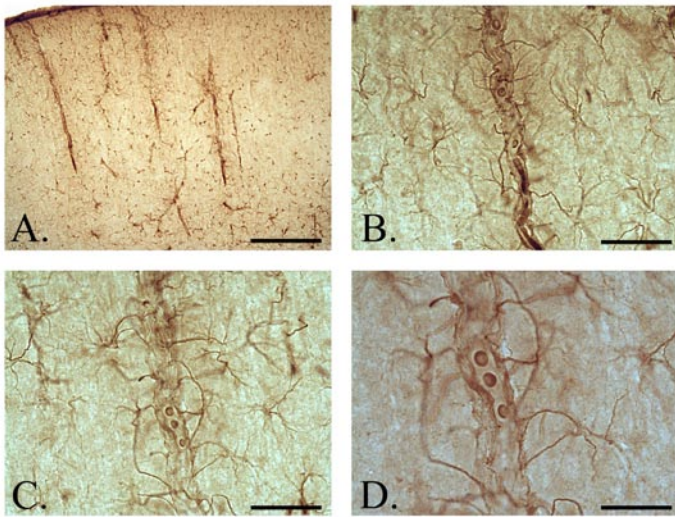
Results from our staining of unperfused sections are shown in Figure 4. Staining with DAB resulted in dark brown staining of RBCs found in blood vessels with little background staining. Fine branches were also visible containing individual RBCs in single-file. Visualization of vessels with this technique varied depending on section thickness, with thicker sections (80-200µm) revealing greater detail of individual vessels and branches as well as vessels from the pial surface which penetrate into neocortex all the way to deep layers. Some DAB-stained sections were also Nissl counter-stained. This material provided visualization of stained vessels as well as stained somata of neurons and glia (Figure 4C,E,F). High magnification of double-stained sections allowed for clear identification of labeled vessels with stained pericytes and nearby neurons (Figure 4F).



**Figure 5.** Immunocytochemistry against rat endothelial cell-specific antigen (RECA). Representative micrographs of rat neocortex A-C, and hippocampus D-F. Asterisk in A refers to pial surface. Nissl counter-staining in C & F reveal stained vessels and associated perivascular cells. Scalebars in microns: A, 500; B-E, 250, F, 42.

DAB staining of unperfused sections is a simple method for fine visualization of cerebrovascular networks. When combined with Nissl counter-staining, several components of the neurovascular unit can be visualized, making the additional reagents (and the cost of those reagents) and preparation time (only about 45 mins. – 1 hr.) worthwhile. Nevertheless, there are two important factors to consider when deciding to use DAB stained sections in the teaching laboratory. First, all histochemical steps requiring DAB must be done wearing gloves and safety goggles while under a fume hood, making this preparation useful only when a fume hood is available. Second, DAB waste

including the tissue washes in PBS must be disposed of according to institutional guidelines for hazardous waste. These issues might prohibit student participation in the staining process; however, once slides are stained and prepared they can be used repeatedly over many years.



**Figure 6.** Immunocytochemistry against astrocyte-specific protein GFAP. Representative micrographs of rat neocortex reveal the extensive network of glial cells and their processes which make contact and surround blood vessels. Small, round, stained cells in B-D are red blood cells non-specifically stained from the DAB chromagen. Higher magnification of panel C is shown in panel D. Scalebars in microns: A, 250; B-C, 42; D, 25.

#### *Visualizing vessels and components of the neurovascular unit with immunocytochemistry.*

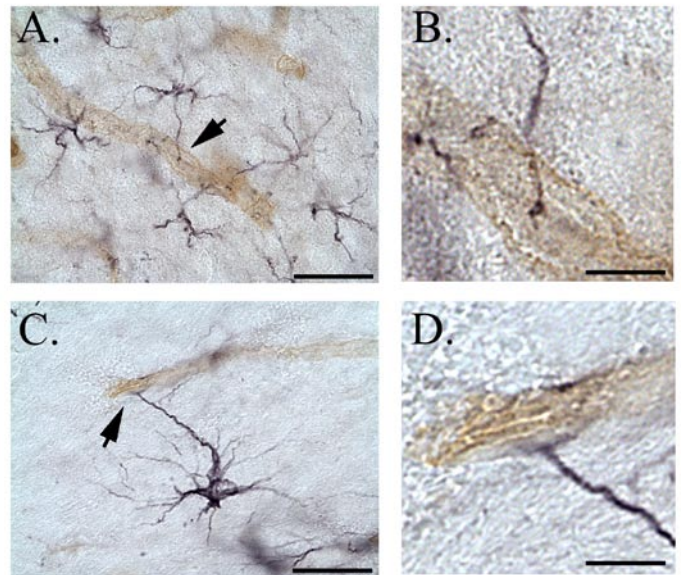
Immunocytochemical (ICC) methods constitute the most targeted method for labeling the cerebral vasculature as well as one or more components of the neurovascular unit. This approach is based on the principal that unique components of the neurovascular unit show molecular specificity and that there exist primary antibodies to those molecules. Thus, antibodies that label molecules found only in endothelial cells will exclusively label cerebral blood vessels while antibodies against molecules found only in astrocytes will only label astrocytes. Fortunately, such antibodies exist and are available for all components of the neurovascular unit. A list of commercially-available antibodies we have used in the past for research purposes can be found in Croll et al. (2004; see also Kasselman et al., 2007).

With this in mind, we used a commercial primary antibody against an unknown antigen found only in rat endothelial cells (rat endothelial cell antigen, RECA; Serotec; raised in mouse; dilution 1:1000). Detailed methods and reagents required for ICC can be found in Ramos et al. (2006).

Immunocytochemistry performed with the anti-RECA antibody resulted in the labeling of the cerebral vasculature with no background. Endothelial cells were labeled on vessels of all sizes as well as fine branches. Figure 5 contains representative photomicrographs of stained sections containing the neocortex (A,B) and hippocampus

(C,D). Unlike DAB staining of unperfused tissue, ICC with anti-RECA resulted in clear labeling of the vascular tube formed by endothelial cells. Counterstaining with Nissl revealed labeled endothelial cells as well as stained pericytes (Figure 5F).

We also used ICC against the astrocyte-specific molecule *glial fibrillary acidic protein* (GFAP; Dako; raised in rabbit; dilution 1:10,000). ICC with a primary antibody against GFAP resulted in excellent visualization of astrocytes and astrocytic processes (Figure 6). Blood vessels were evident due to the dense surrounding plexus formed by the labeled astrocytic processes. Higher magnification revealed putative sites of contact between astrocytic endfeet and vessels.



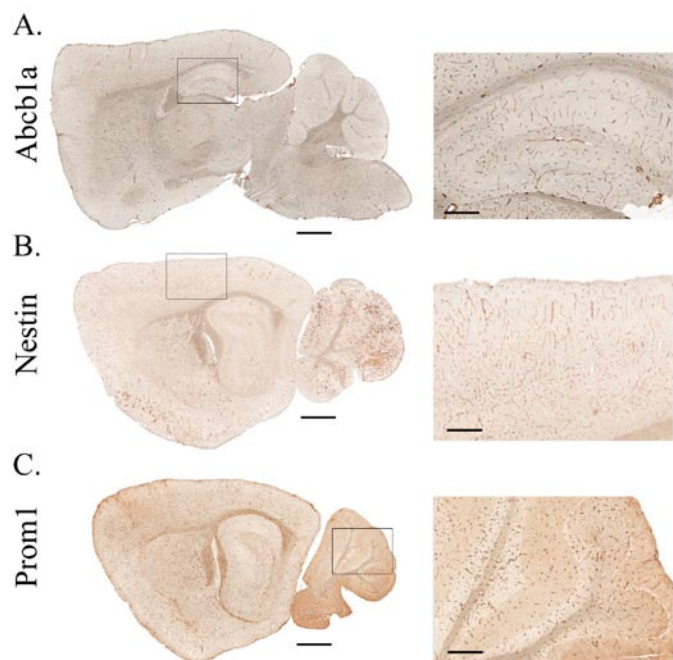
**Figure 7.** Double immunocytochemistry against astrocyte-specific antigen GFAP (black staining) and the rat endothelial cell-specific antigen RECA (brown staining). A-D, Representative micrographs of rat neocortex. GFAP endfeet in A and C (arrows) are shown at higher magnification in B and D, respectively. Scalebars in microns: A, 250; C, 25; B and D 6.25.

We next used double-ICC with anti-RECA and anti-GFAP antibodies in order to determine whether greater visualization of these components of the neurovascular unit would be possible. ICC against GFAP was first performed followed by ICC with anti-RECA.

Results from double-labeled sections are shown in Figure 7. Black labeled astrocytes were visible in close contact with brown labeled vessels. Examination of this tissue at high magnification revealed putative contacts made by astrocytes directly onto vessels (Figure 7B,D).

There are a number of advantages to using ICC for demonstration of cerebral vascular networks and the neurovascular unit. First, discrete cellular elements of the neurovascular unit can be directly targeted with antibodies to cell-specific molecules. Using this approach, we were able to specifically label endothelial cells (anti-RECA) and astrocytes (anti-GFAP). Second, using double ICC (or triple ICC) multiple cellular elements can be revealed. Using this approach we were able to demonstrate contacts made by GFAP+ astrocytes onto RECA+ endothelial cells.

We discovered several disadvantages to using ICC. First, primary and secondary antibodies come at a financial cost that can be prohibitive for use in many courses. Second, ICC is time consuming, and in our experience requires at least two days. It may be difficult to incorporate ICC into student exercises in the teaching lab, as most courses generally meet only one day a week for several hours. Therefore, in some cases, the financial expense and time requirements of ICC might outweigh the increased resolution provided by ICC. This might especially be true when comparing DAB staining of unperfused tissue with the results obtained following ICC for endothelial cells with anti-RECA. Both methods are valuable in demonstrating cerebral vasculature but the former can be performed with substantially less time and resources. Third, because ICC for conventional light microscopy uses DAB as a chromagen, concerns related to access to a fume hood and hazardous waste disposal also apply as described above for DAB staining of unperfused tissue. Finally, single and especially double ICC requires some level of histology expertise. Often numerous staining attempts are required before optimal antibody dilutions are determined.

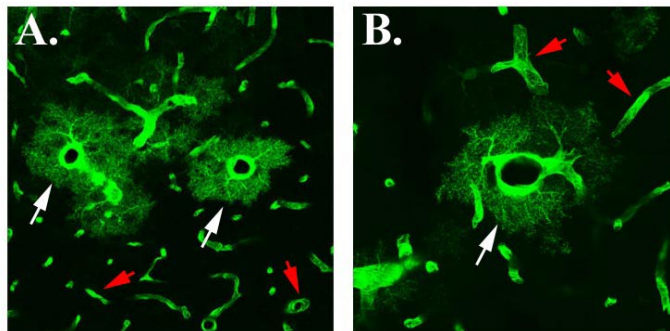


**Figure 8.** GENSAT database search for GFP expression in vascular cells. Representative micrographs from 3 distinct transgenic mouse lines with staining of cerebral vasculature. Transgene promoter for each transgenic mouse line indicated on left. Scalebars in microns: all left panels, 500; all right panels, 250.

### Visualizing cerebral vasculature and the neurovascular unit “*in silico*” for use in the teaching laboratory and lecture hall.

Previously we demonstrated the versatility of the Allen Brain Atlas (ABA; [www.brain-map.org](http://www.brain-map.org)) for use in the teaching lab and lecture hall as a tool for demonstrating cerebral cytoarchitecture, cellular diversity, and area-specific gene expression (Ramos et al., 2007). We

extended this approach and made an extensive database search for genes with expression in components of the neurovascular unit. For example, a search for genes containing the term “*vascular*,” “*endothel*,” and “*angio*” results in an output of 5, 48, and 35 genes, respectively. We reviewed micrographs (>200) from each of these genes but did not identify staining profiles which revealed the cerebral vasculature. Note that for many genes, micrographs from more than one brain/case were available. For reasons that are not known, perhaps due to the methods used for *in situ hybridization* (Lein et al., 2007), the ABA is not well-suited for demonstrating the cerebral vasculature.



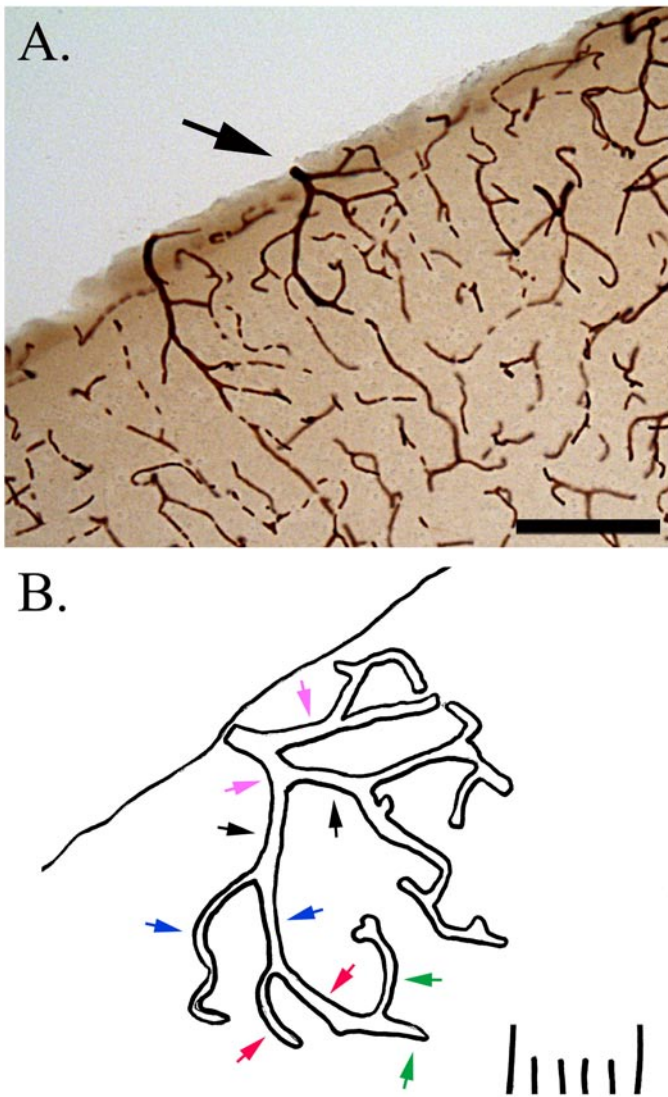
**Figure 9.** GENSAT database search for GFP expression in endothelial cells (red arrows) and astrocytes (white arrows). Representative micrographs from the ID3-GFP transgenic mouse line (A: cerebral cortex, B: caudate putamen).

We next searched for other anatomical/genetic databases and identified the GENSAT database (<http://gensat.org/index.html>) as a useful tool for demonstrating cerebral vasculature and the neurovascular unit *in silico* (Gong et al., 2003; Heintz 2004). The GENSAT database contains photomicrographic atlases of brain sections taken from green fluorescent protein (GFP) transgenic mice which have been stained using ICC (anti-GFP antibody). Moreover, histological data can be found from animals of various ages (embryonic day 15.5, postnatal day 7, adult). Thus, micrographs found in this database reveal the expression profile in those cells/tissues where GFP expression is driven by transgene insertion. For example, the GENSAT database contains photomicrographs from transgenic mice where GFP is driven via insertion into the GFAP gene. As expected, GFP expression is found exclusively in astrocytes, making these micrographs useful teaching tools for demonstrating the distribution and morphology of astrocytes in the brain.

We used the search tools of the GENSAT database and searched for “*vascular*” which resulted in links to histological photomicrographs from 131 transgenic mice lines where GFP immunostaining is observed in the cerebral vasculature. We present photomicrographs taken from the GENSAT database for three such mouse lines where GFP expression is clearly visible in the cerebral vasculature in Figure 8. An additional resource of the GENSAT database is the ability to zoom-in on photomicrographs and view different structures at higher magnification. Figure 8 also contains higher magnification

micrographs of vasculature in hippocampus, neocortex, and cerebellum (right panels in A-C, respectively).

We also performed a GENSAT database search for “vascular neuron,” “vascular glial,” and “vascular neuron glial” which resulted in links to photomicrographs from 88, 59, and 39 (respectively) transgenic mouse lines where GFP is found in these multiple cell-types. Representative micrographs from the Id3-GFP transgenic mouse line where GFP expression can be found in endothelial cells as well as astrocytes are found in Figure 9.



**Figure 10.** Camera lucida reconstruction of DAB-stained neocortical blood vessels. Photomicrograph (A) and camera lucida drawing (B) of a blood vessel. Arrows point to vessel branches of varying branch order. Pink: 2<sup>nd</sup> order, Black: 3<sup>rd</sup> order, Blue: 4<sup>th</sup> order, Red: 5<sup>th</sup> order, Green: 6<sup>th</sup> order. Scalebars in microns: A, 125, B, each division 10.

There are only advantages to using the GENSAT database for demonstrating the cellular components of the neurovascular unit. First, because the GENSAT database is publicly available, there is no financial commitment necessary, making its use possible at any institution. The option to use the GENSAT database is, therefore, ideally

suited for use in laboratory classes with no budget available for the additional resources required for the histological demonstrations we have described above. Moreover, histological material from the GENSAT database can be used in the lecture hall where it is becoming more common to have available internet access and associated LCD projectors. Thus, demonstrating the cerebral vasculature and the cellular components of the neurovascular unit in the lecture hall requires only internet access and a video projector.

**Conclusions: Multiple methods for revealing the cerebral vascular and the neurovascular unit are accessible to nearly all institutions.**

The anatomy of the cerebral vasculature and the relationship between cells in the brain and blood vessels are very important topics in neuroscience. Despite its importance in brain function, the cerebral vasculature has been under-represented in neuroscience curriculum. Recognizing this deficit, we evaluated several methods for use in the preparation of histological material detailing the cerebral vasculature and the neurovascular unit, which we have used in a laboratory-based course (part of our neuroscience curriculum).

In the present report, we detail histological methods which reveal the cerebral vasculature and one or more cellular components of the neurovascular unit. Moreover we have identified the strengths and limitations of each preparation. Based on these details, we believe that there exist one or more preparations which can be used in most laboratory-based courses. In instances where there is no budget for the resources required for the preparations described, we detail how the GENSAT database can be used. Finally, identification of the GENSAT database as a means for demonstrating the cerebral vasculature has also introduced a novel teaching tool for use in the lecture hall.

The present report describes our efforts to prepare histological material for students to examine with conventional compound microscopes. This material expands our collection of materials for demonstration and instruction of neuroanatomy, which also includes whole brains from various mammalian species, models of human brains, and computer software. An additional goal of assessing these various methods was to determine which could be incorporated into a “lab exercise,” where students participate in tissue preparation and staining. Individual instructors and departments wishing to develop lab exercises for students using one of the methods described above will have to determine which preparation will be most suitable for their respective course. Moreover, to what extent students participate in the histology should be carefully determined. Mounting and visualizing unstained tissue as well as Nissl staining are two preparations ideally suited for hands-on student participation. In contrast, DAB-staining and ICC are preparations requiring significant supervision and training.

In addition to having students participate in the histology, there are exercises that can be implemented where students *analyze* several features of the neurovascular unit. Tata and Anderson (2002) provide

both methods and examples of several important features of the neurovasculature that can be quantified. These measures include vessel diameter, capillary segment length, branch point number, and capillary tortuosity. In their report, Tata and Anderson (2002) use computer-assisted camera lucida where many measures are automatically calculated by the computer. Equipped with microscopes with drawing tubes in our teaching lab (Olympus BX41; 4X, 10X, 40X objectives), we wondered whether one or more of the measurements described by these authors could be determined using conventional camera lucida. Figure 10, contains a representative camera-lucida drawing of a neocortical blood vessel. As can be seen, branches of multiple order (e.g. 2<sup>nd</sup>, 3<sup>rd</sup>, etc.) can be easily traced and their lengths measured. Branch point number and number of endings can also be determined. More sophisticated measurements such as vessel tortuosity and diameter prove difficult to accurately measure using this technique. However, drawings can be scanned with conventional flatbed scanners and digitally imported into the free image analysis software ImageJ which is distributed by the NIH (<http://rsb.info.nih.gov/ij/>). Using this software package, a number of measures can be obtained including total area, length, and volume of the drawn vessels.

One challenge for neuroscience educators is to make as many aspects of neuroanatomy and neurophysiology accessible to students. We hope that the present description of methods for demonstrating cerebral vasculature and its relationship to glia and neurons will inspire greater discussion of the neurovascular unit and brain hemodynamics (Moore and Cao, 2008). Although there are additional methods for revealing the cerebral vascular which are not described here (Fonta and Imbert, 2002; Bovetti et al., 2007; Chuquet et al., 2007), the methods described above represent realistic preparations that can be incorporated for use in laboratory courses.

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