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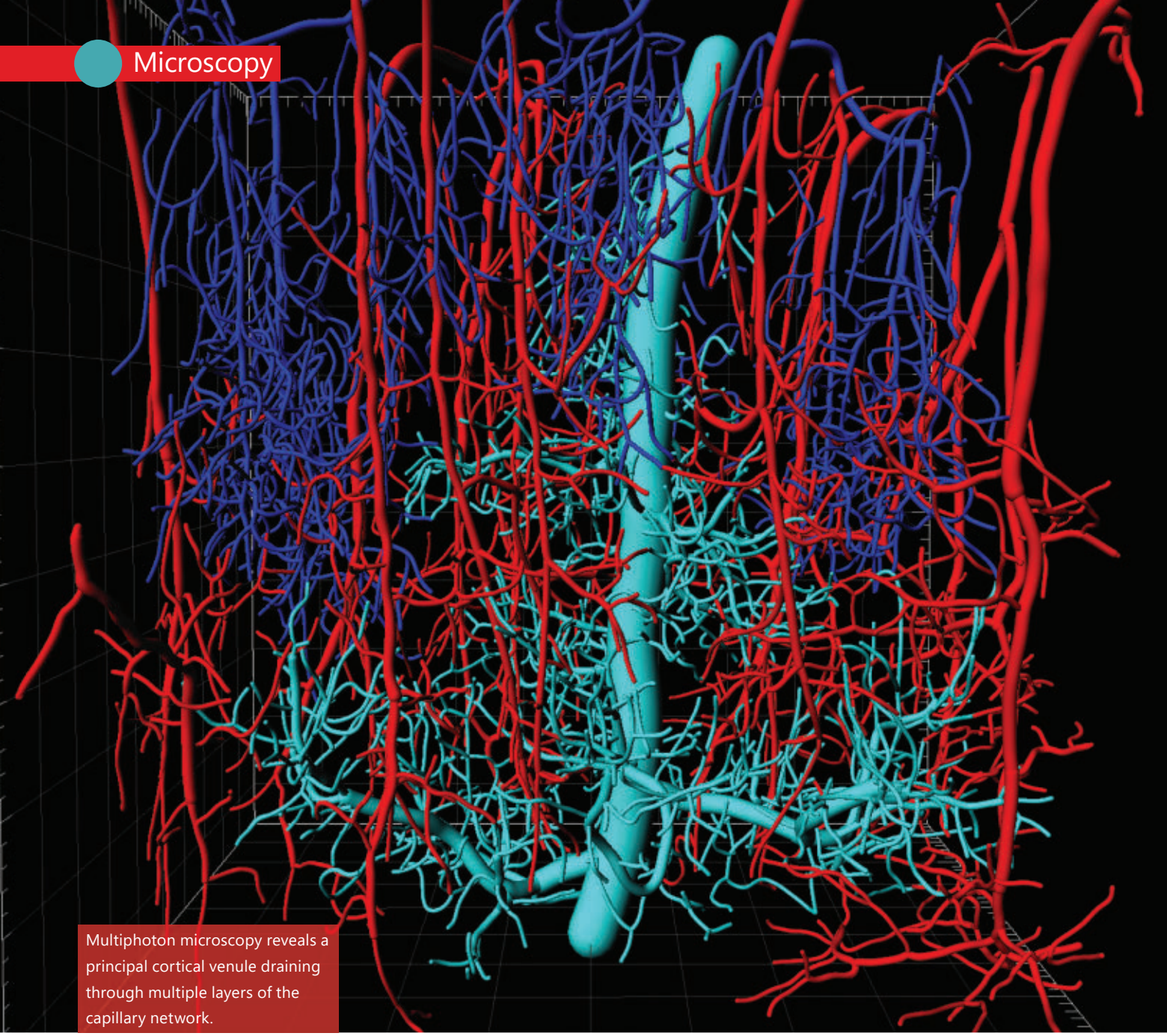

Multiphoton microscopy reveals a principal cortical venule draining through multiple layers of the capillary network.

Multiphoton Microscopy Provides a Deeper View of the Aging Brain

by Diana Poullos, MKS Spectra-Physics

Ultrafast lasers enable the visualization of changes in the structure of the brain's white matter and capillary blood drainage, which are related to a decline in cognitive functioning.

All images adapted with permission from Reference 1.

Because deep-brain structures are key to how the nervous system communicates and maintains healthy function, understanding them is crucial for studying neurovascular dynamics, age-related changes, and neurodegenerative diseases. Multiphoton microscopy has become a widely used tool for imaging the mouse brain in neuroscience, particularly for visualizing neurons and vasculature. Its advantage compared with other technologies lies in its ability to deliver live-cell images and reveal active cellular processes as they occur, rather than only providing static details.

The introduction of ultrafast laser sources with output wavelengths longer than 1 µm in scientific instruments has extended the reach of multiphoton microscopy to greater depths within the brain. This, in turn, has enabled studies of brain white matter, which lies beyond the reach of imaging methods using shorter-wavelength laser sources. Studying this white matter is critical since changes in it have been shown to play a significant role in the deterioration of memory and cognitive function with aging.

This article reviews the importance of white matter and how it is being studied using multiphoton microscopy by researchers in the Shih Lab at Seattle Children's Research Institute, along with collaborators at the Allen Institute for Brain Science. It also presents their recently published results linking impaired capillary-venous drainage to age-related white matter loss[1].

White matter's role

While the brain is often colloquially referred to as "gray matter," that term actually refers to only one component of cerebral tissue. Specifically, gray matter is composed mainly of neuronal cell bodies and is responsible for functions such as thinking, memory, and decision-making. But various regions of gray matter in the brain, along with parts of the central nervous system, are interconnected by another type of tissue known as "white matter."

White matter consists of axons wrapped in a myelin sheath. The myelin sheath, rich in lipids, imparts the characteristic white appearance and enhances the speed and reliability of electrical signal transmission along nerve fibers. This efficient signaling is essential for integrating sensory inputs, coordinating motor functions, and supporting cognitive processes such as learning and memory. Thus, while gray matter handles processing and decision-making, white matter ensures that these signals reach the right destinations in the brain and spinal cord.

Researchers have established that brain white matter gradually deteriorates with age, reducing its ability to efficiently send and receive signals. One of the most obvious changes in white matter is a reduction in its total volume — a loss or shrinkage of myelinated axons — that makes it progressively less effective. The structural composition of white matter also degrades with age, making

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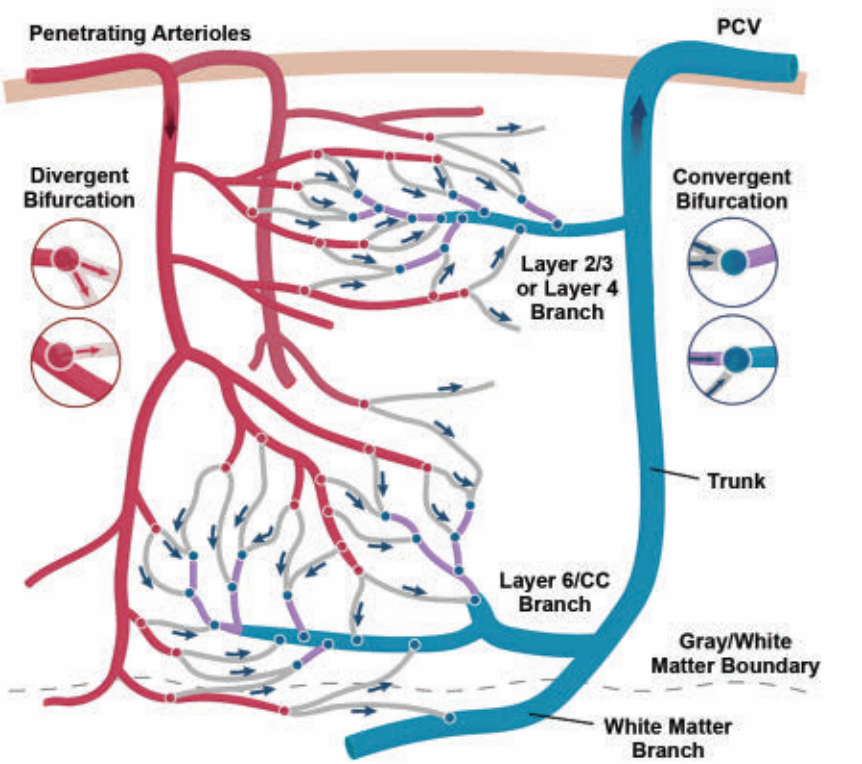


A simplified schematic summarizing the spatial relationships between principal cortical venules (PCVs) and surrounding vascular structures in a PCV drainage network. Impaired capillary-venous drainage contributes to gliosis and demyelination in mouse white matter during aging. CC: corpus callosum.

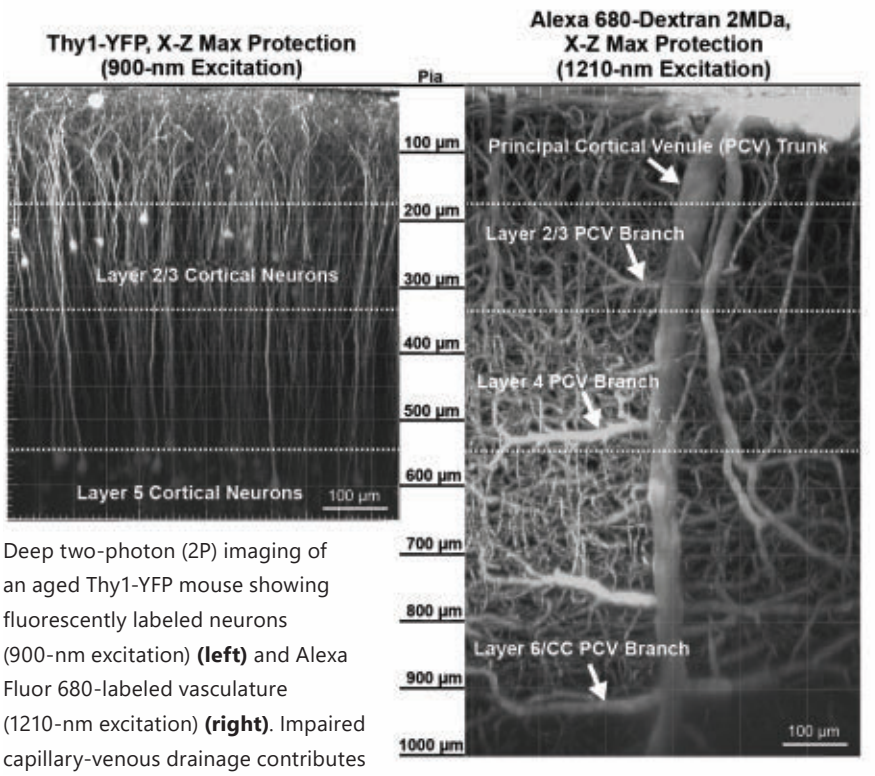
communication between brain regions slower and less reliable. Another major concern is the development of white matter lesions, which are areas of damage linked to poor blood flow. These lesions have been associated with cognitive decline, memory problems, and neurodegenerative diseases such as dementia.

Research targets

To better understand age-related changes in white matter, Stefan Stamenkovic, a postdoctoral fellow in the Shih Lab, and his colleagues studied the role of its blood supply[1]. Their focus was on small vessel disease (SVD), a range of conditions that affect the fine network of blood vessels supplying oxygen and nutrients to the brain. SVD can begin to disrupt brain function long before other hallmarks of aging, such as protein buildup or brain shrinkage, become apparent. Understanding how these changes develop could provide critical insights for identifying early warning signs of cognitive decline.

Much of the previous research on aging and brain circulation has focused on arterioles, the small blood vessels that deliver oxygenated blood to brain tissue. However, far less is known about how aging affects the veins that drain blood from the brain, even though these vessels are equally important for maintaining a healthy white matter environment. These venous drainage systems are the primary focus of the Shih Lab.

Past studies of human brain structure suggest that medullary veins, which help drain deep white matter, become twisted and narrowed with age. This could restrict blood flow, depriving white matter of the oxygen and nutrients it needs, while also preventing waste products from being cleared efficiently. The Seattle Children's Research Institute group is investigating, in mouse models, how similar structural changes might contribute to white matter degeneration, hoping to explain why these brain regions are particularly vulnerable to aging.



Deep two-photon (2P) imaging of an aged Thy1-YFP mouse showing fluorescently labeled neurons (900-nm excitation) **(left)** and Alexa Fluor 680-labeled vasculature (1210-nm excitation) **(right)**. Impaired capillary-venous drainage contributes to gliosis and demyelination in mouse white matter during aging.

A primary focus of their work is on the corpus callosum, a bundle of nerve fibers connecting the two hemispheres of the brain. It serves as a bridge between the two halves, allowing them to work together seamlessly. This structure is one of the first to show signs of decline, or atrophy, in aging and dementia. Studying it enables investigation of white matter deterioration in a controlled setting. Specifically, by better understanding how blood flow changes in aging white matter, scientists hope to uncover new ways to prevent or slow the breakdown of critical brain connections, potentially delaying the onset of age-related cognitive impairment.

Focusing on microscopy

The Shih Lab also performs its investigations using mice. While the mouse brain is smaller and less complex than the human brain, it shares a similar basic structure and cellular organization. Most importantly, structures such as the cortex, hippocampus, and white matter tracts are functionally and anatomically comparable to those in humans.

Neuroscientists employ various methods for imaging the mouse brain. These include several forms of microscopy, such as two-photon (2P), three-photon (3P), confocal, wide-field fluorescence, and light-sheet microscopy. They also use nonmicroscopy tools, including magnetic resonance imaging (MRI), positron emission tomography (PET), optical coherence tomography (OCT), and functional ultrasound (fUS).

For the type of investigations pursued by institutions such as the Seattle Children's Research Institute, both 2P and 3P microscopy have proved to be extremely useful. A key reason for their utility is their ability to perform real-time, in vivo imaging of

the mouse brain through an implanted cranial window. In addition, they deliver high spatial resolution and high-contrast images, even in thick, scattering tissue — capabilities unmatched by any other imaging technique.

While 3P microscopy can image deeper than 2P microscopy, it is experimentally more difficult due to complex laser sources and longer scanning times. Fortunately, two significant technological advancements have sufficiently extended the range of 2P microscopy to enable imaging deeper than 1 mm. The first is ultrafast laser sources, which emit farther into the infrared spectrum, and the second is new fluorophores, which take advantage of these longer wavelengths.

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Seeing deeper

The original ultrafast sources for multiphoton microscopy were Ti:sapphire lasers, which typically offer tunable output in the 690- to 1040-nm range. While still widely used, their upper wavelength limit restricts imaging depth to ~1 mm. Light scattering and absorption degrade signal quality beyond this depth.

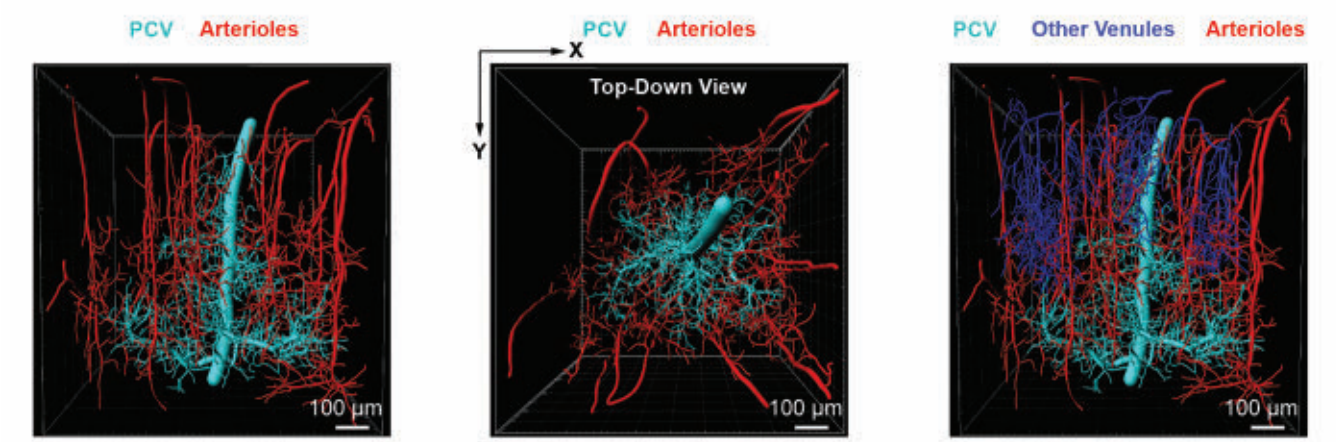
To reach farther into the infrared, MKS Spectra-Physics pioneered the development of systems that pair a ytterbium (Yb)-based femtosecond laser, which outputs at 1045 nm, with an optical parametric oscillator (OPO). The latter provides tunability from 680 to 1300 nm.

While OPO technology had been available for many years, several breakthroughs were required to transform it from a difficult-to-use, unstable system into a turnkey, field-ready light source. A key advancement was the development of periodically poled crystals, which simplified and extended the range of phase matching to yield wider tunability, higher conversion efficiency, and more compact, sealed designs. Other important design improvements included thermally stabilized and sealed modules, optimized dispersion and cavity designs, built-in dispersion precompensation, and pump lasers with high power and low jitter.

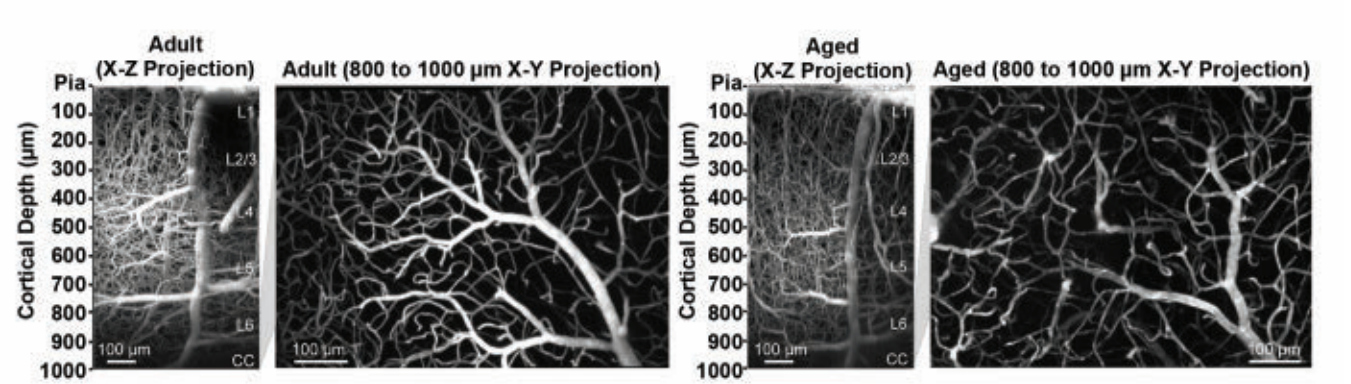
This latest generation of laser sources, with longer-wavelength output, enables researchers to use 2P microscopy at depths of up to ~1.2 mm in brain tissue. This delivers a clearer view of vascular networks in the corpus callosum and lower cortical layers. The advent of these longer-wavelength ultrafast lasers has had a significant impact on in vivo neuroscience, allowing investigators to study deep-brain structures with high spatial resolution and minimal background interference.

Taking full advantage of sources such as these requires fluorophores that can be excited by longer wavelengths. The most commonly used fluorescent dyes for bioimaging, such as FITC (fluorescein isothiocyanate) and GFP (green fluorescent protein), are excited by shorter wavelengths in single-photon and two-photon excitation, usually in the 488- to 900-nm range. This makes these dyes less useful for imaging deep blood vessels, where those shorter wavelengths cannot readily penetrate.

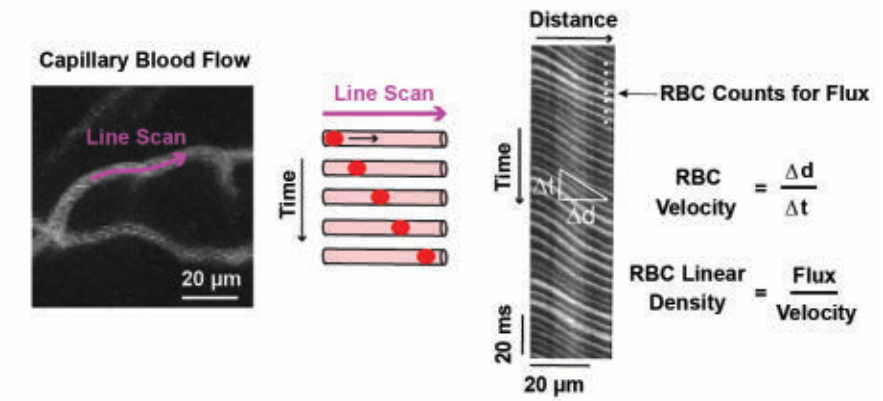
One important new fluorophore is Alexa Fluor 680, which can be efficiently excited by infrared wavelengths (≥1210 nm). In addition, the far-red



Imaging reveals a PCV draining capillary networks from multiple cortical layers. Penetrating arterioles form a ring-like arrangement around the venule, highlighting its central role in vascular outflow from deep cortical regions.



Deep 2P image stacks showing PCVs in an adult mouse **(left)** and an aged mouse **(right)**. Aged mice exhibit simplified branching and reduced vascular density in deep cortical layers. CC: corpus callosum; L: layer.



Red blood cell (RBC) motion through capillaries is tracked using repeated laser scanning along a vessel's axis. The slope of the streaks in the image reflects RBC speed, while the frequency of these streaks indicates flux. This method enabled quantification of flow deficits.

emission (680 nm) of this label reduces interference from tissue autofluorescence, which is stronger in the visible spectrum. The end result is cleaner images and better contrast, which is particularly important in dense tissue such as brain white matter.

The Seattle Children's Research Institute group used Alexa Fluor 680 conjugated to a high-molecular-weight (2 MDa) dextran, following a protocol previously developed by a collaboration of researchers[2]. This dextran molecule is too large to cross intact blood vessel walls or be taken up by cells, which ensures that the fluorophore remains confined to the bloodstream. As a result, background fluorescence is minimized, and the observed signal corresponds specifically to vascular structures.

Clear changes in the brain

The Seattle Children's Research Institute study used Thy1-YFP mice with implanted cranial windows. These genetically modified mice have fluorescently labeled neurons.

Two-photon images were acquired using a microscope equipped with a Spectra-Physics InSight X3 Tunable Ultrafast Laser. Two excitation wavelengths were used — 900 nm and 1210 nm — with attenuation to provide power at the sample ranging from 4 mW to 145 mW (higher powers for deeper imaging).

Neurons were visualized using 900-nm excitation of the fluorescent proteins expressed in the Thy1-YFP mice. Vascular structures were imaged via 1210-nm excitation of intravenously injected Alexa Fluor 680. This combination provided clear views of capillary density, venular morphology, blood flow patterns, and key anatomical landmarks.

The study compared young adult mice (five to seven months) with aged mice (22 to 24 months) to assess white matter vascular decline with age. Aged mice showed progressive capillary loss, reduced venular drainage, and signs of decreased blood flow in white matter regions.

Blood flow redistribution was particularly striking. Aging appeared to shift blood flow toward upper cortical layers at the expense of deeper white matter, essentially starving it. Without the extended imaging depth provided by longer-wavelength 2P microscopy, these significant changes would have been impossible to detect.

In vivo imaging enabled the team to analyze the microvascular structures associated with principal cortical venules (PCVs). These relatively scarce, deep-reaching vessels drain capillary networks in the deeper cortex layers and corpus callosum. Because PCVs make up only ~3% of the ascending venules, disruption of even a few could significantly impair venous outflow and compromise tissue health.

Two-photon imaging revealed how PCVs are embedded within capillary networks extending through cortical Layer 6 and into the corpus callosum. Each PCV acted as a central drainage

point, surrounded by a ring of penetrating arterioles but lacking parallel venous pathways. This configuration magnifies the impact of any obstruction.

This study also found that age-related declines in vascular density and complexity were most evident in Layer 6 and the corpus callosum. In these regions, the tributary vessels of aged mice showed fewer branches and shorter capillary lengths. The vessels were also more twisted and smaller in diameter, both of which further restrict blood flow. All these observations suggest that aging not only reduces perfusion but also reshapes the vascular architecture itself, simplifying complex drainage networks into more fragile and less efficient systems.

To quantify red blood cell (RBC) velocity and flux, the investigators used line-scan imaging. This is a 2P technique in which the microscope rapidly scans a single line (usually aligned along the axis of a blood vessel) rather than acquiring a full 2D image. The final image contains diagonal streaks representing moving RBCs, with the line slope reflecting velocity.

Using this method, the team quantified a significant drop in RBC velocity and flux in aged mice, particularly in the "preconvergence capillaries." These vessels are situated midway between arteriolar input and venular drainage and are particularly vulnerable within the network because of their narrow diameter and slower flow.

Taken together, these findings provide a clearer picture of how aging disrupts blood drainage from capillaries into venules. This breakdown in drainage plays a major role in why the brain's white matter is especially vulnerable as people age. As a final experiment, Stamenkovic and his colleagues used deep 2P together with a model of stenosis to modestly reduce cerebral blood flow and found that this was sufficient to cause some of the same changes seen in aged white matter, including tissue inflammation.

Research continues

This Shih Lab study demonstrates how deep multiphoton imaging can illuminate previously inaccessible aspects of brain aging, revealing the structural and functional decline of white matter vasculature in striking detail. By combining genetically encoded fluorescence with long-wavelength excitation of carefully selected fluorophores, the researchers were able to map changes in capillary architecture, venular drainage, and blood flow with great precision. As neuroscience continues to probe deeper into the brain, the tools that enable this exploration will be key to unlocking the mechanisms of cognitive aging and developing strategies to protect brain health.

Meet the author

Diana Poullos is senior product marketing manager for Spectra-Physics scientific lasers at MKS Instruments. She manages a product portfolio primarily used for multiphoton bioimaging. Poullos received a Ph.D. in physical chemistry from the University of Southern California, where she studied time-resolved photodetachment and electron transfer dynamics of anions and quantum dot conjugate systems in solution under the direction of Stephen Bradforth; email: diana.poullos@newport.com.

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