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A FACED lift for cerebral blood flow imaging

Bruno Weber[a,b,1] and Franca Schmid[a,b,c]

Living cells require energy—some of them more than others. Some cells change their metabolic rates from minimal to maximal within seconds, while others are bottomless pits with an unchecked continuous demand. The supply of energy substrates and oxygen and the removal of metabolic waste are maintained through a complex vascular network in which glucose-rich plasma and oxygen-charged red blood cells (RBCs) are transported. The fact that alterations in energy metabolism are a common proxy to diagnose and monitor disease across tissues further underlines the relevance of an in-depth understanding of the energy supply.

The brain is no exception yet comes with many special features and unsolved mysteries. The energy requirements are approximately an order of magnitude greater than the body's per volume average. And, most importantly, as the brain has a limited energy storage capacity, a constant supply of oxygen and glucose is mandatory. Disruptions in supply of just a few minutes cause irreversible damage to brain cells. As a consequence, the brain uses an intricate regulatory system, involving mural cells as well as neurons and glial cells, to control its energy supply. A clearer understanding of blood flow changes at the level of individual vessels and across the entire vasculature is indispensable to unlocking how this interconnected system orchestrates its adaptations. In PNAS, Meng et al. (1) introduce a powerful ultrafast method to improve in vivo measurement of cerebral blood flow in microvascular networks, which will significantly advance the applicability of two-photon microscopy for quantifying microvascular perfusion.

Even though we have known, since the end of the 19th century, that the brain locally regulates blood flow to meet local increases in energy demand (2, 3), the underlying hemodynamic processes and intercellular and intracellular signaling pathways remain largely undiscovered (for recent reviews, see refs. 4 and 5). And, to be highlighted in the present context, methods that permit blood flow measurements with high spatial and temporal resolution are limited, but they are crucial to generate novel insights into the microvascular aspects of blood regulation.

Because of its significance, researchers have incessantly developed and applied a wide array of methods to measure cerebral blood flow. These are based on diverse modalities, such as radiolabeled diffusive compounds, hydrogen diffusion and microelectrode technology, magnetic resonance imaging, optical spectroscopy, optical coherence tomography, laser speckle imaging, and, more recently, focused ultrasound and optoacoustic imaging. Some of these methods have achieved gold standard status, whereas others have disappeared from the map.

In 1998, Kleinfeld et al. (6) introduced two-photon microscopy to track individual RBCs. In anesthetized mice that received intravenous injections of a fluorescent dextran to stain the blood plasma, kilohertz line scans through short segments of capillaries were used to quantify the displacement of RBCs, resulting in an RBC velocity in millimeters per second. This method has been widely adopted hitherto and is, in fact, the only procedure that allows for quantitative blood flow measurements at the level of a single capillary (7, 8). Unfortunately, this technique also suffers from severe limitations: 1) The speed of galvanometric scanning restricts line scan repetition rates to the low kilohertz range, 2) only single and straight segments can be monitored, and 3) the vessel must lie within the focal plane of the microscope.

Meng et al. (1) have overcome the first two of these three limitations. The authors apply their recently introduced technology, named FACED (free-space angular chirp enhanced delay), in two-photon microscopy for cerebral blood flow measurements. FACED provides an elegant yet simple way to boost the imaging speed to capture fast dynamics (8). In FACED, a pulsed femtosecond laser beam is focused with a converging angle $\Delta\theta$ and directed into a pair of nearly parallel mirrors having a separation S and a misalignment angle α (Fig. 1*A*). The beam is multiply reflected by these mirrors, which results in multiple subpulses ($N = \Delta\theta/\alpha$) of distinct propagation directions and a temporal delay between pulses of $2S/c$ (c, the speed of light). These spatiotemporally dispersed subpulses at the output of the FACED module serve as an array of excitation sources. In combination with high-rate data acquisition, FACED-enhanced two-photon microscopy allows line scanning at megahertz repetition rates (Fig. 1*B*). In combination with a galvanometric scanner, kilohertz frame rates can be achieved to obtain two-dimensional (2D) images for velocity mapping of larger vessels with multifile flow or at vascular bifurcations (Fig. 1 *C* and *D*).

FACED-enhanced two-photon microscopy opens the door for kilohertz frame rate calcium imaging, as has been previously demonstrated by Wu et al. (9). In PNAS, Meng et al. (1) now use this approach to acquire ultrafast line scans tracking the movement of RBCs in the cerebral cortex of mice (Fig. 1*B*). With FACED-enhanced two-photon imaging, two major limitations of the classical two-photon line-scanning approach (6) can be overcome: 1) The repetition rate of classical 2D galvanometric line scanning is

Author affiliations: [a]Experimental Imaging and Neuroenergetics, Institute of Pharmacology and Toxicology, University of Zürich, 8057 Zürich, Switzerland; [b]Neuroscience Center Zürich, ETH Zürich and University of Zürich, 8057 Zürich, Switzerland; and [c]Institute of Fluid Dynamics, ETH Zürich, 8092 Zürich, Switzerland

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[1]To whom correspondence may be addressed. Email: bweber@pharma.uzh.ch.

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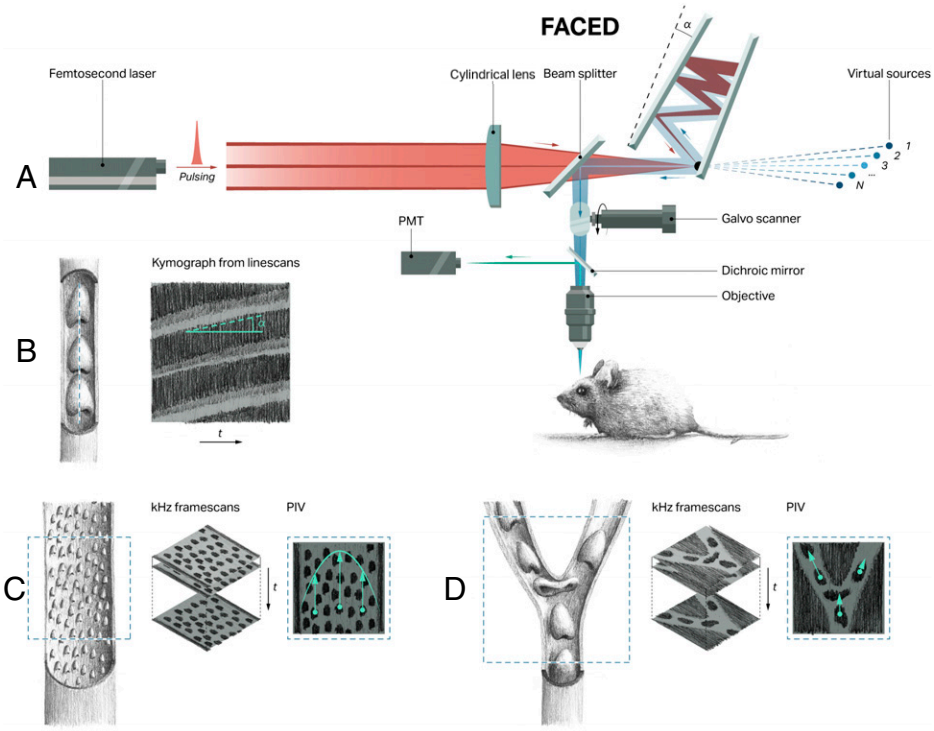

Fig. 1. (*A*) Schematic showing the integration of FACED in a two-photon microscope. A cylindrical lens and a nearly parallel pair of mirrors split a laser pulse into multiple subpulses and form spatiotemporally separated virtual excitation light sources at the focal plane of a microscope objective. PMT: Photomultiplier tube. (*B*) Line-scanning method to track the displacement of RBCs over time. This can be achieved with classical kilohertz or FACED-enhanced megahertz line scanning. The angle α in the kymograph is determined by the RBC velocity. (*C* and *D*) FACED-enhanced kilohertz frame rate imaging of a larger vessel and a vascular bifurcation plus analysis using particle image velocimetry (PIV) to quantify the velocity field. This method can be applied to measure radial RBC velocity profiles and RBC behavior at vascular bifurcations.

limited to ~2 kHz, which limits measurements to slow-flowing capillaries with RBC speeds below 10 mm/s (Fig. 1*C*). 2) Kilohertz frame rates allow for measurements of a single larger or tortuous vessel or of a set of vessels (Fig. 1*D*). The FACED-enhanced and the classical approach share, however, the limitation that the monitored vascular segment(s) must be located within the microscope focal plane.

FACED-enhanced two-photon microscopy will very likely become a widespread tool and might be a turning point for the investigation of some of the most pertinent open questions in cerebral microcirculation. These include, for example, rheological phenomena at vascular bifurcations where the unequal partitioning of RBCs is a heavily studied topic (10) that is relevant for local RBC distribution and, in consequence, for local oxygen supply (11). In the same vein, and thanks to the faster scanning rates, FACED offers the opportunity to generate novel and quantitative insights into well-established RBC-related flow phenomena such as the Fåhraeus and the Fåhraeus–Lindqvist effects (12). Importantly, fast scanning rates are also relevant for velocity measurements in vessels with medium flow speeds. This is because the angle of the RBC streak in the resulting kymographs will be less steep, which increases the robustness and accuracy of the commonly applied analysis algorithms (13, 14).

Measuring flow speeds of up to 50 mm/s and across all vessel types will provide a more complete picture of cortical microvascular perfusion and goes hand in hand with the increasing size of microvascular network reconstructions (15). Due to the hierarchical structure of the cortical vasculature (16), accurate velocity measurements at the feeding vessels of the capillary bed, that is, pial and penetrating arteries, are necessary to advance our understanding of how blood supplies reach the capillary bed. Additionally, such measurements will allow more-informed in silico studies, where high-resolution velocity measurements can be incorporated by inverse modeling (17) and are key for model validation.

Even though we know that capillary flow is constantly fluctuating, a precise quantification of these fluctuations is still lacking, and average flow speeds are used for most analyses of microvascular flow. However, in order to properly understand, for example, flow changes observed during neurovascular coupling, an in-depth quantification of baseline fluctuations is essential. This also holds for the topic of temporary and lasting capillary stalls, which has received much attention over the last few years and proves to be highly relevant in pathologies such as Alzheimer's disease (18) or in poststroke scenarios (19).

Quantifying RBC dynamics in the complete microvascular network of a volume of at least 1 mm^3 remains impossible, to date. However, it is only a matter of time before optoacoustic or focused ultrasound-based (20, 21) in vivo imaging methods will overcome their limitations in spatial resolution and approach the required submicrometer range required to track single RBCs and measure capillary diameters. Until then, two-photon microscopes in many cerebrovascular laboratories might get a FACED lift!

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