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Beyond observation: Microscopy with ultrashort laser pulses to probe and manipulate cortical vasculature

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Understanding the microscopic details of cortical blood supply is fundamental both to clinical progress in the treatment of stroke and neurovascular disease, and to building a more comprehensive interpretation of blood flow based brain-imaging techniques, such as functional magnetic resonance imaging. We have developed several novel ultrashort pulsed laser based methodologies that allow us to map microvascular morphology, probe blood flow dynamics, query the oxidation state of hemoglobin, and induce and monitor microvascular disruption. Here, we provide a brief overview of this ongoing work.

Fundamental quantitative questions regarding brain architecture such as the cortical volume dedicated to blood supply or the codistribution of microvasculature and neuron cell bodies are extremely difficult to address with standard histological techniques. We have pioneered an all-optical approach to histology that mitigates many of the difficulties of conventional methods (patent pending) (13, 14). Volumetric histological data is obtained iteratively by the alternate acquisition of successive optical sections through a depth of $\sim 100 \mu\text{m}$ and photoablation of the previously imaged tissue. Our images are collected using a Ti:Sapphire oscillator based two-photon laser scanning microscope (4, 15). Ablation makes use of a Ti:Sapphire multi-pass amplifier based on the

design of Murnane and Kapteyn (1) with ~ 100 -fs, 800-nm, 1 to 10- μ J pulses. We can cut fixed and fresh tissue with $\sim 1 \mu\text{m}$ tolerance and minimal collateral damage. Figure 1 shows a volume of fluorescently tagged cortical vasculature which has been reconstructed using this iterative all-optical approach. This project involves colleagues at UCSD (Roger Tsien Laboratory), the Colorado School of Mines (Jeffrey Squier), and Science Applications International Corporation (Augustin Ifarraguerri and Beverly Thompson).

Dynamic blood flow measurements can be made *in vivo* in rodents prepared with a window in the animal's skull (16). Our two-photon microscope, shown schematically in Figure 2, can be used to image vasculature and blood flow in single vessels in neocortex (8). Red blood cells stand out as dark spots against fluorescently labeled plasma and their flow velocity can be measured in real time (figure 3). This approach has been used by us (7, 9) and others (3) to quantify changes in blood flow driven by sensory stimulation.

In addition to enabling three dimensional optical sectioning, nonlinear microscopy has the potential advantage of providing intrinsic functional images. In collaboration with our colleagues in Colorado, we are investigating the nonlinear optical properties of hemoglobin as a means of determining its ligand binding state concomitant with optical sectioning. In the first measurements of its kind, we found that oxygenated, deoxygenated, and carboxylated hemoglobin solutions have significantly different third harmonic spectral features in the wavelength range of 770 to 1000 nm (2). We have also begun measuring two-photon absorption cross-sections in hemoglobin and other non-fluorescent biomolecules.

Lastly, we have launched a series of investigations into the blood flow changes that result from vascular insult. This work is motivated by suggestions that the highly interconnected cortical vascular network is robust to multiple blockages (6). We induce intravascular clotting in microvessels without the application of

any exogenous agents through the use of focused amplified 800 nm laser pulses (patent pending) (10, 11). Since the absorption of ultrashort pulses in the vessel lumen is a highly nonlinear process, localized damage can be produced up to ~ 500 μm below the surface of the brain. Adjusting the energy of the amplified laser pulses between 1 and 5 μJ allows us to create a range of vascular insults ranging in severity from blood plasma extravasation, *i.e.* small hemorrhages, and ischemic clots, to gross hemorrhages (figure 4). Concurrent two-photon imaging allows us to monitor the consequences of accumulated vascular defects. For the case of single point ischemic clots in surface vessels, related work has shown a reestablishment of flow at the first downstream branch point due to a flow reversal in one of the branches (5, 12). Although the reestablished flow is, on average, at 50 % of its initial flux, this is likely to be sufficient to maintain tissue oxygenation at physiologically viable levels. This work involves colleagues at UCSD (Patrick Lyden Laboratory) and Vanderbilt University (Ford Ebner).

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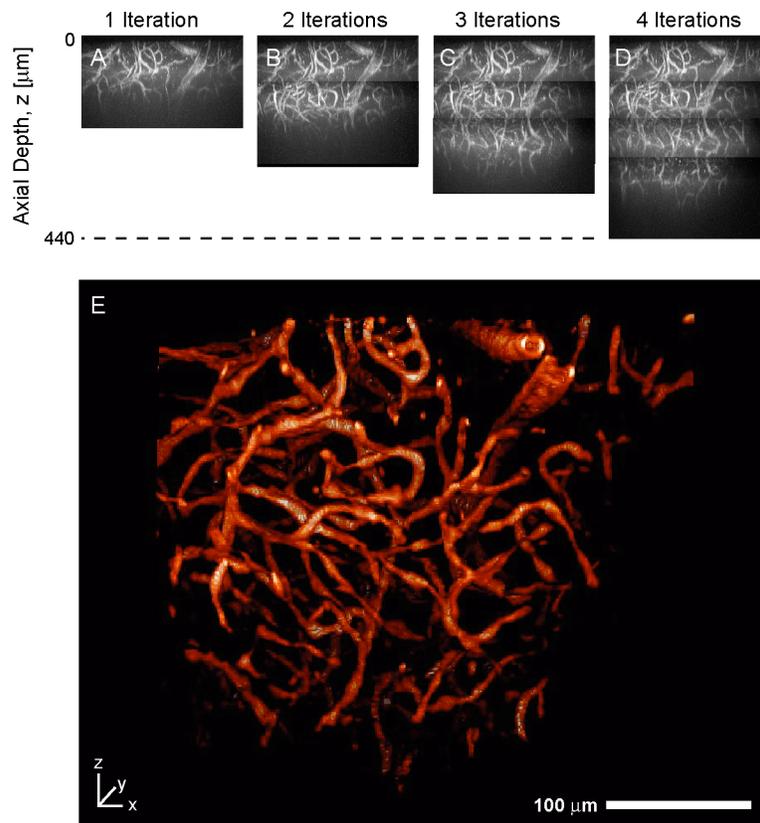
Captions

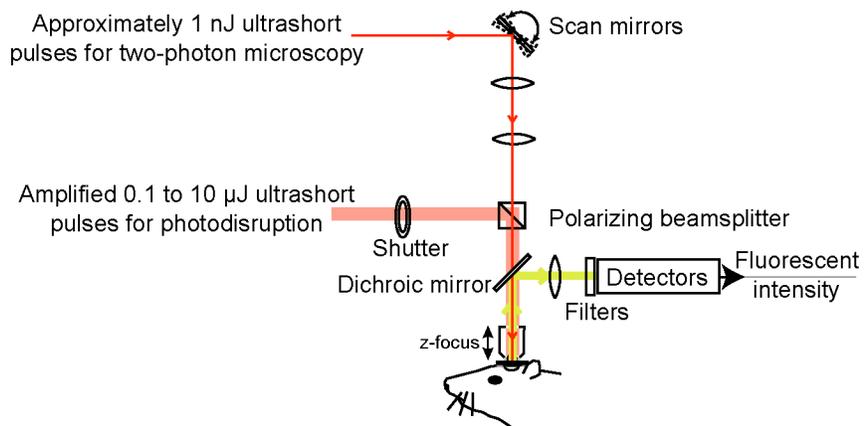
Figure 1. All-optical histological processing. (A to D) Serial reconstruction of vasculature in a block of neocortex in a cyan fluorescent protein labeled transgenic mouse. **(E)** Volume rendering of labeled vasculature (13).

Figure 2. Schematic of the microscope for *in vivo* two-photon imaging and multiphoton ablation.

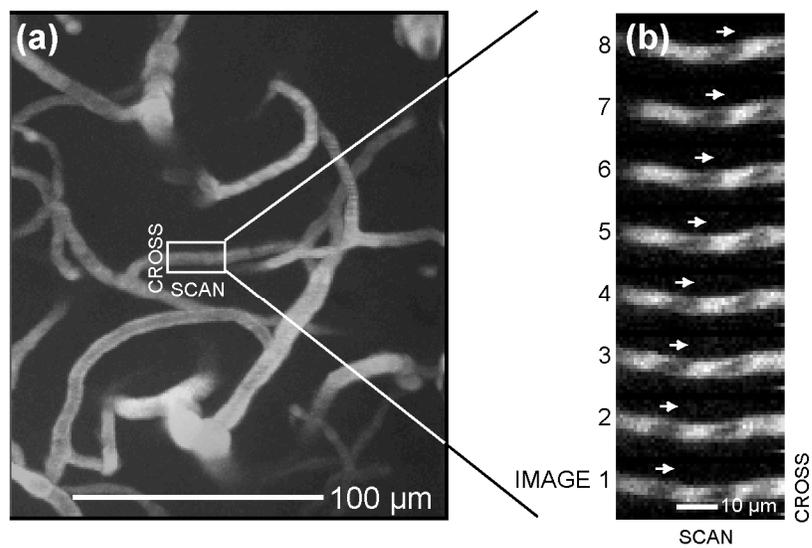
Figure 3. Two-photon *in vivo* imaging of cortical blood flow. (A) Submicron resolution images of blood flow. **(B)** Time series depicting the transit of a red blood cell against the fluorescently labeled plasma (9).

Figure 4. Schematic and two-photon images of vascular disruption induced by ultrashort laser pulses. Photodisruption is initiated by focusing amplified ultrashort pulses into the target vessel. Subsequent dynamics result in the formation of three types of vascular injury (10).





Clay, Nishimura, Schaffer, Tsai and Kleinfeld - Figure 2



Clay, Nishimura, Schaffer, Tsai and Kleinfeld - Figure 3

