

Real-time two-photon fluorescence microscopy of blood flow dynamics following photothrombotic stroke in rat neocortex

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Abstract: We use two-photon microscopy to observe changes in blood flow after localized photothrombotic clotting of individual blood vessels in rat neocortex. We find that flow reverses direction downstream from clotted arterioles, thereby maintaining tissue perfusion.

Stroke affects approximately 700,000 people per year in the United States alone. Options for the treatment of stroke, however, remain few and of limited efficacy despite years of basic and clinical research. Future progress depends critically on animal models that allow ischemic stroke to be studied at various stages, from initial changes in blood flow, to the physiological reaction to ischemia, to neuronal death, to behavioral impairment and recovery [1]. Most stroke models developed to date focus on events that occur from hours to days after ischemic injury. Here we describe a photothrombotic stroke model in near-surface vessels in rat neocortex that allows direct observation of events occurring in the vasculature on the timescale of seconds to hours. Using this model, we demonstrate that downstream flow in arterioles does not stop after clot formation. Rather, flow is reestablished at the first downstream branch, albeit with reduced speed, with the flow direction reversed in one of the branches.

Craniotomies over parietal cortex were performed on 200 – 300-g rats anesthetized using urethane (1.5 mg/kg animal weight). The exposed cortex was covered with a thin glass window, allowing optical access to the cortical vasculature [2]. We use two-photon laser-scanning microscopy (TPLSM) of intravenously injected fluorescein-dextran to visualize blood flow [2] during and after clot formation. The setup is shown schematically in Fig. 1. The fluorescein labels only the blood plasma, so red blood cells and other solid blood constituents appear as dark objects in the fluorescence image. Red blood cell motion between the successive line scans that form the image leads to streaks that can be used to quantify the blood flow velocity.

We induce clot formation by optical excitation of an intravenously injected photosensitizer, the fluorescein derivative rose bengal (4 mg/injection). Photoexcited rose bengal interacts with molecular oxygen to form singlet oxygen, which is known to damage fatty acids and proteins in the vessel endothelium, thereby triggering the natural clotting cascade and leading to vessel obstruction [3]. In this study, we excite rose bengal using 1-s duration, 0.5-Hz repetition-rate pulses of 100- μ W, 532-nm laser light, focused to a 10- μ m diameter on the inner wall of the vessel. TPLSM images are taken between pulses of 532-nm irradiation. Clots are characterized by a non-fluorescent region of densely packed cells, frequently accompanied by a brightly fluorescent region of stalled flow upstream from the clot.

Figure 2 shows changes in blood flow during and after clot formation in an arteriole. The arrows in the figure indicate the direction of flow and the circle in Fig. 2a indicates the region irradiated with 532-nm laser light, beginning 30 s after the start of observation. At 300 s a clot begins to form (vessel region near * in Fig. 2b), and at 455 s the clot is complete, obstructing both the targeted vessel and the downstream branches (Xs in Fig. 2c). Later, and after the 532-nm irradiation is turned off, flow is restored in the downstream branches, with a reversed flow direction in the branch on the right (Fig. 2d). This flow pattern remained stable for the entire four hour duration of observation. In all cases studied (18 clots in 10 rats, vessel diameters 20 – 60 μ m), downstream flow continues due to flow reversal at the first downstream branch as shown in Fig. 2d. We find that the speed of red blood cells in the branches is roughly 45% (n = 12) of the speed of the original, unperturbed flow.

In conclusion, we have demonstrated a powerful model for the study of real-time changes in blood flow following the formation of a localized photothrombotic clot. Data obtained with this model has revealed the previously unobserved, persistent nature of downstream tissue perfusion following clot formation in an arteriole.

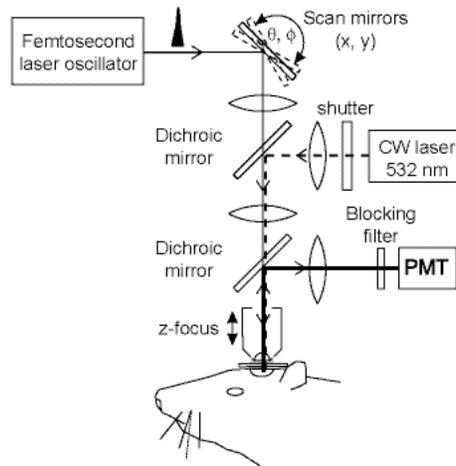


Fig. 1. Setup for real-time observation of blood flow and localized photothrombotic clotting. A commercially-available femtosecond laser (Mira, Coherent, Inc.) is used with a TPLSM of local design [4]. The femtosecond beam (thin line) is raster scanned in the focal plane of the objective using galvanometric scan mirrors and a telescope system. The position of the focal plane in the sample is controlled by moving the microscope objective along the beam axis. The fluorescence (bold line) is collected by the microscope objective, separated by a dichroic mirror, spectrally filtered, and detected on a photomultiplier tube. Continuous wave, 532-nm laser light (dashed line) is brought into the microscope using a dichroic mirror. The focus of the 532-nm light is aligned with the center of the field imaged by the TPLSM. The 532-nm laser is mechanically shuttered, allowing TPLSM fluorescence images to be taken between pulses of 532-nm light.

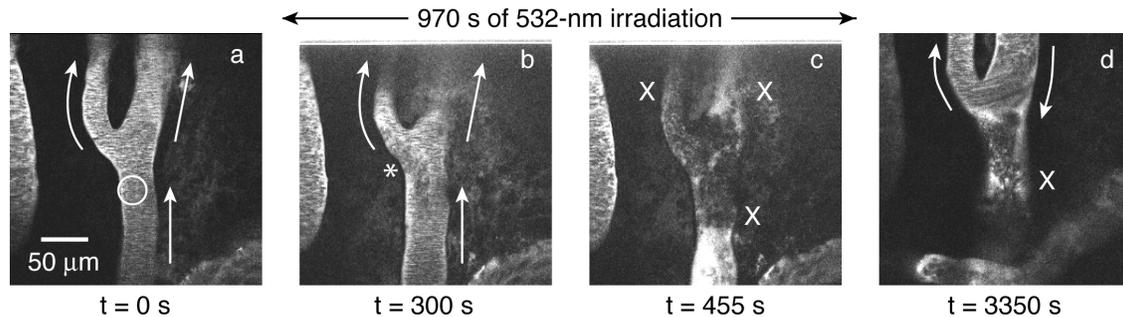


Fig. 2. Photothrombotic clotting dynamics in an arteriole, as observed by TPLSM. **a)** Image prior to photoexcitation of rose bengal. The circle indicates the region of the vessel that will be irradiated with 532-nm laser light and arrows indicate the direction of blood flow. **b)** After the start of rose bengal excitation, a clot begins to form near the region indicated by *. **c)** Later, the vessel is completely occluded (stalled flow indicated by X). **d)** Flow spontaneously restarts in branches downstream from the clot. Flow direction has reversed in the branch on the right.

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