

All optical histology : Serial ablation and multiphoton imaging of neuronal tissue with femtosecond laser pulses

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Abstract : We use femtosecond laser pulses to serially image and ablate neuronal tissue. This allows histological imaging throughout the entire depth of the brain; a procedure that is unattainable by multiphoton imaging alone.

Current techniques in histology involve the manual slicing of frozen or embedded tissue, which is labor intensive and may adversely affect tissue morphology¹. In addition, these slide-based histological techniques do not readily lend themselves to automation. Meanwhile, recent advances in molecular labeling, and the introduction of transgenic animals have spurred a need for high throughput analysis of architectonics and patterns of gene expression. To address this need, we demonstrate an all-optical histological technique², which involves successive iterations of multiphoton imaging with nanojoule, femtosecond pulses^{3,4} and ablation with microjoule, femtosecond pulses⁵, as shown schematically in Figure 1. The histological imaging is carried out in-situ; therefore image registration is automatic, and the technique is conducive to both automation and 3-dimensional volumetric reconstruction of the image data.

To demonstrate the feasibility of all-optical histology, we focus on the compatibility of femtosecond laser ablation in combination with multiphoton microscopy for imaging structures within the brain. We obtained tissues from a variety of preparations, including adult rat, and adult and embryonic mice that were genetically engineered to express fluorescent protein associated transgenes. In the case of the embryonic mice, the ablation and imaging also included developing extracranial structures such as the skin and the skull. Tissues were either perfusion fixed with buffered paraformaldehyde or saline perfused and left un-fixed.

Neuronal tissue was surface stained either with a lipid analog, 5-hexadecanoylamino-fluorescein, which labeled membranes down to 100 μm below the ablated surface, or with the water soluble nucleic dye, acridine orange, which labeled nuclei down to 150 μm below the surface. Alternately, we used transgenic animals in which either neurons or endothelial cells comprising the cortical vasculature were intrinsically labeled by their expression of fluorescent proteins.

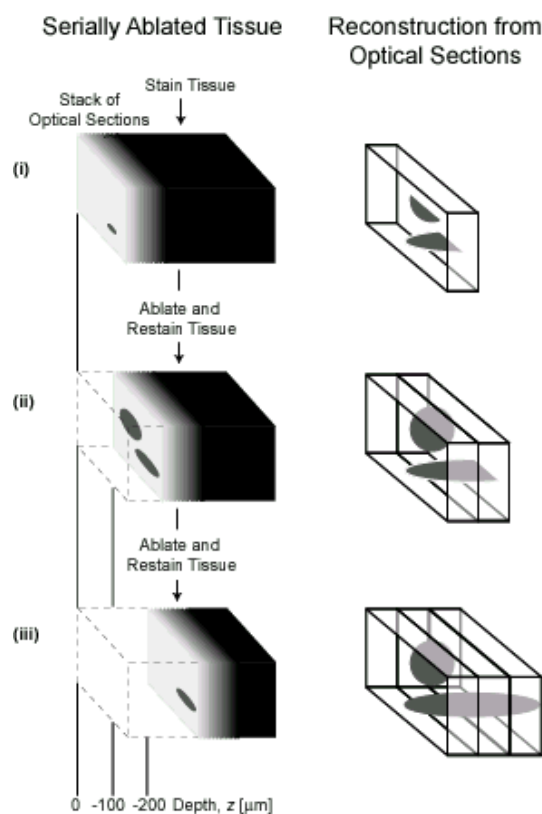


Figure 1. Schematic illustration of the iterative process of all-optical histology. (i) The tissue sample (left column) containing two fluorescently labeled structures is imaged by conventional two-photon laser scanning microscopy to collect optical sections. Sections are collected until scattering of the incident light reduces the signal-to-noise ratio below a useful value; typically this occurs at $\sim 150 \mu\text{m}$ in fixed tissue. Labeled features in the resulting stack of optical sections are digitally reconstructed (right column). (ii) The top of the now-imaged region of tissue is cut away with amplified femtosecond laser pulses to expose a new surface for imaging. The sample is again imaged down to a maximal depth, and the new optical sections are added to the previously stored stack. (iii) The process of ablation and imaging is again repeated so that the structures of interest can be fully sectioned and reconstructed.

Protein integrity after ablation was assayed both by conventional immunohistochemical staining and by the retention of fluorescence by endogenous fluorescent proteins. We found that immunostaining occurred within as little as 2 μm of the laser cut surface, while fluorescence was maintained to within 10 μm of the ablated surface.

By way of example, we used our procedure to iteratively image and ablate neocortex in two strains of transgenic mice. To visualize neurons, a transgenic mouse⁶ expressing the yellow-emitting variant of green fluorescent protein (YFP) was processed in 24 iterations, starting from the dorsal surface. Subgranular pyramidal neurons were visualized by maximal projection of the data, as shown in Figures 2a and 2b. To visualize cortical vasculature, we processed the cortex from a transgenic mouse in which the vascular epithelial cells express cameleon⁷, a fusion protein that contains the cyan-emitting variant of green-fluorescent protein (CFP) as one of its constituents. Vascular data

was obtained with four iterations of the all-optical histological process. The data was then filtered and projected with a ray casting algorithm to produce the image in Figure 2c.

In conclusion, we have developed a new histological method that renders the process entirely optical. The technique has significant advantages over traditional techniques for sectioning and visualizing neuronal tissue. In particular, the present technique does not require that the tissue be either frozen or embedded, does not require realignment of the imaged sections, and can be completely automated. Further, it is ideally suited for the analysis of transgenic animals with intrinsic fluorescent markers and thus is a timely addition to a burgeoning direction of molecular medicine. In particular, an entire mouse brain can be transformed into approximately 2 terapixels of data that reports the distribution of a label at the diffraction limit of spatial resolution.

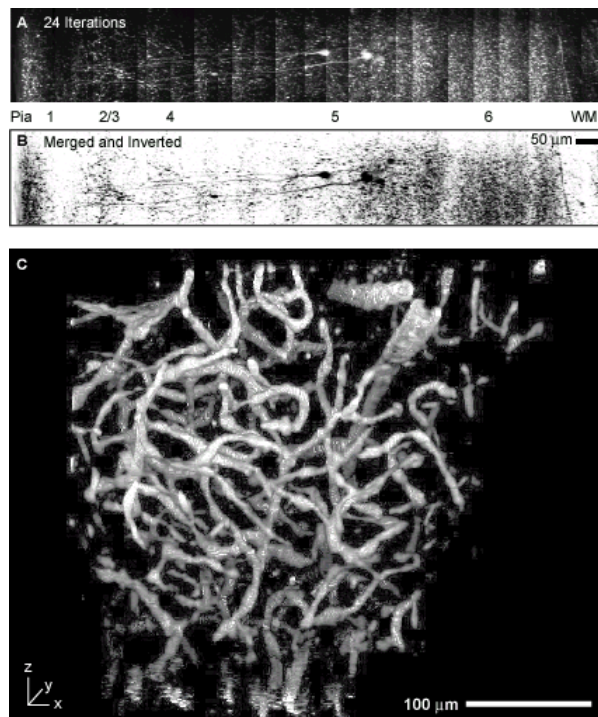


Figure 2. All-optical histology of transgenically labeled neuronal tissue.

(A) Iterative processing of a block of neocortex of a transgenic mouse with neurons labeled by the yellow-emitting variant of green fluorescent protein (YFP). Twenty-four successive cutting and imaging cycles are shown. The ablation beam was focused onto the cut face with a 20-times magnification, 0.5 NA water immersion objective and single passes, at a scan rate of 4 mm/s, were made to optically ablate successive planes at a depth of 10 μm each with total thicknesses between 40 and 70 μm per cut. The energy per pulse was maintained at 8 μJ . Each stack of images represents a maximal side projection of all accumulated optical sections obtained using TPLSM at $\lambda = 920 \text{ nm}$. The sharp breaks in the images shown in successive panels demarcate the cut boundaries.

(B) A maximal projection through the complete stack with the breaks removed by smoothly merging overlapped regions. The contrast is inverted to emphasize the fine labeling. Anatomical regions are labeled above the figure, including the pia mater (Pia), the white matter (WM), and the cortical layers (1 to 6)

(C) Volume rendering of cyan fluorescent protein (CFP) labeled vasculature in a block of neocortex of a transgenic mouse. The data was obtained with four successive cutting and imaging cycles, filtered, and reconstructed with a ray casting algorithm. The ablation beam was focused onto the cut face with a 20X magnification, 0.5 NA water immersion objective and single passes, at a scan rate of 0.5 mm/s, were made to optically ablate successive planes at a depth of 10 μm each with total thicknesses of 70 μm per cut. The energy per pulse varied from 0.4 to 1.7 μJ .

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