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

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Abstract: We demonstrate the first use of femtosecond laser pulses for serial histology. Successive iterations of multiphoton imaging and ablation provide diffraction-limited volumetric data that is used to reconstruct the architectonics of labeled cells or microvasculature.
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1. Introduction

Current techniques in histology involve the manual slicing of frozen or embedded tissue, which is both labor intensive and may affect tissue morphology [1]. Advances in molecular labeling and the introduction of transgenic animals have brought about a need for high throughput analysis of architectonics and patterns of gene expression.
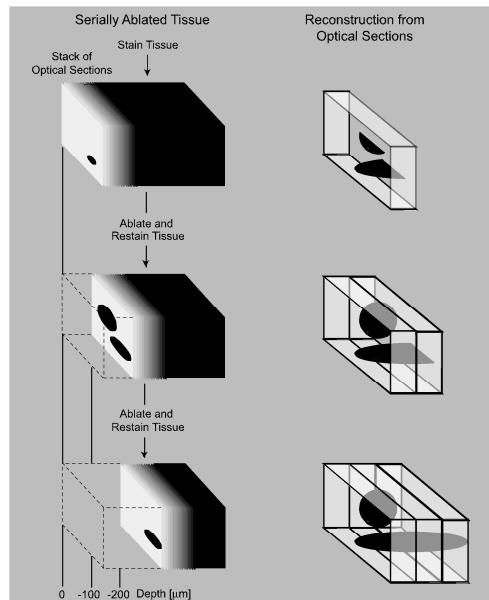

Figure 1. The iterative process by which tissue is imaged and cut. A sample (left) containing two fluorescently labeled structures is imaged by two-photon microscopy to collect optical sections through the ablated surface until scattering of the incident light reduces the signal-to-noise ratio below a useful value; typically ~ 150 μm in fixed tissue. Labeled features in the stack of optical sections are digitally reconstructed (right). The top of the now-imaged region of the tissue is cut away with femtosecond pulses to expose a new surface. The sample is again imaged down to a maximal depth, and the new sections are added to the previously stored stack. The process of ablation and imaging is repeated until no tissue remains.

We used femtosecond pulses to both image and ablate tissue for the purpose of serial sectioning. The tissue sample to be processed is positioned on an automated x-y translation table and is rastered across the focus of the laser beam. The top layers of the tissue are stained and then imaged using two-photon laser scanning microscopy [2,3]. The region of the tissue that has been imaged is subsequently removed by laser ablation with amplified femtosecond laser pulses [4]. The newly exposed surface is then stained, imaged, and ablated. This sequence repeats serially until the desired volume of tissue has been analyzed and leads to a digitized block of optical sections of the labeled tissue. Lastly, as an alternative to labeling with exogenous dyes, one can use tissue in which specific cells are labeled via the transgenic expression of endogenous fluorescent proteins [5].

2. Results

We demonstrated all optical histology under a variety of conditions and tissue types, i.e.,

>   Fixed, in 4 % (w/v) paraformaldehyde, as well as perfused but fresh neuronal tissue.

>   Rat and mouse, including transgenic mice.

>   Adult and embryonic; as early as E15.

Neuronal tissue was stained with either a lipid analog, 5-hexadecanoylamino-fluorescein, which labeled membranes down to 100 μm below the ablated surface, or 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 that comprised the cortical vasculature were intrinsically labeled by their expression of cameleon. The latter construct is the linear fusion of 4 constituents: a cyan fluorescent protein, calmodulin, a calmodulin-binding peptide, and a yellow fluorescent protein [5].

The localization and precision of the ablation was evaluated by three complementary methods.

>   Surface roughness was assayed by staining the cut tissue with a lipophilic dye and imaging the ablated surface with two-photon laser scanning microscopy. We observed a root-mean square roughness of 1.0 μm.

>   Immunoreactivity of the tissue adjacent to the ablated surface was assayed by conventional immunohistochemical staining for the axonal label tyrosine hydroxylase. We found that staining occurred within as little as 2 μm of the laser cut surface (Fig. 2).

>   Protein viability in the tissue adjacent to the ablated surface was further evaluated in transgenic animals expressing cameleons. Fluorescence was maintained within 10 μm of the laser cut surface.

By way of example, we used our procedure to iteratively image and ablate cortex in a mouse in which the vascular epithelial cells express cameleon. The optical sections were taken at axial steps of 0.5 μm and were processed to form the volumetric reconstruction shown in figure 3.
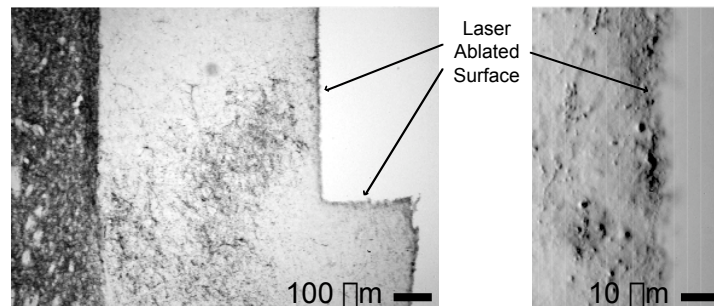

Figure 2. Preservation of protein integrity as evaluated by immunohistology near an optically cut surface in fixed neuronal tissue. The laser was focused onto the cut face of the tissue with a 20X magnification, 0.5 NA water objective. Single passes, at a line-scan rate of 4.0 μm/s, were made to ablate successive 10 μm thick planes and form a channel with a final depth of 370 μm. The energy per pulse was 2.2 μJ. After completion of the optical cutting, the tissue was frozen, sectioned at 25 μm on a sliding microtome, immunostained with anti-tyrosine hydroxylase, visualized with DAB, and photographed under brightfield (left) or Nomarski (right) optics. The dark regions correspond to immunostained axons.
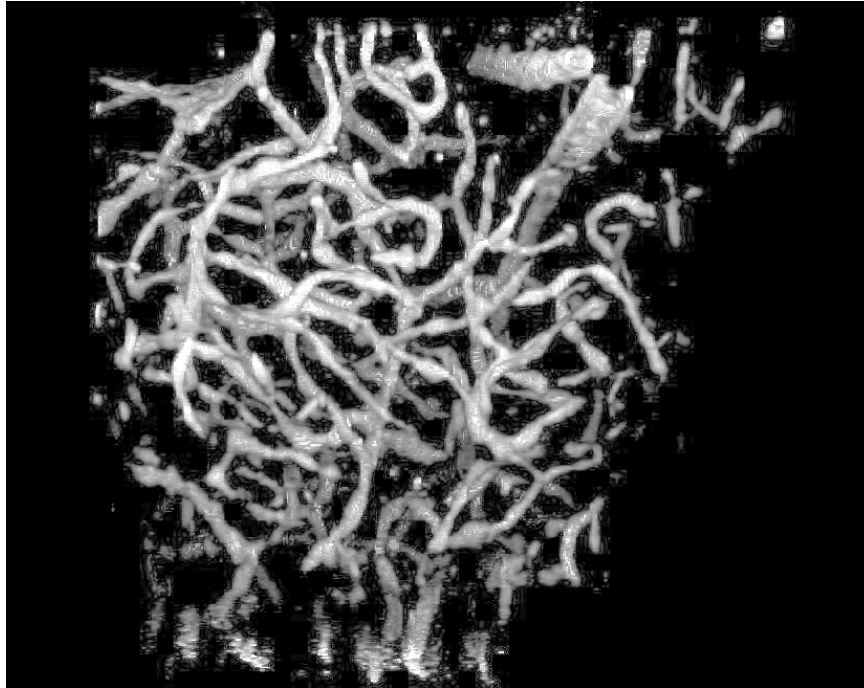
Figure 3. Volume tissue reconstruction of the vasculature in a 250 μm by 375 μm by 400 μm region of neocortex. We performed optical sectioning, two photon imaging, and volumetric reconstruction in neocortex of a transgenic mouse that expressed cameleon in the vascular endothelia. Four successive cutting and imaging cycles were performed. The laser was focused onto the cut face with a 20X magnification, 0.5 NA water objective and single passes, at a scan rates of 5 mm/s, were made to optically ablate successive planes at a depth of 10 μm each with total thicknesses of 70 μm per cutting cycle. The energy per pulse varied from 0.4 to 1.7 μJ. Optical sections were obtained with two-photon laser scanning microscopy at λ = 850 nm to excite the cyan fluorescent protein in the cameleons. The data was band pass filtered between spatial wavelengths of 2.4 and 39 μm, passed through a threshold nonlinearity to remove noise, and median filtered with a square block that was 2.4 μm on edge to remove isolated fragments and fill defects in the vessel walls. Shown is a single view from a volume renderings, constructed with ray casting, of the processed images.

3. 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 5 terapixels of data that reports the distribution of a label at the diffraction limit of spatial resolution.

4. References

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5. Acknowledgements