All-optical, *in-situ* histology of neuronal tissue with femtosecond laser pulses

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Goal

We describe the application of femtosecond laser pulses to image and ablate neuronal tissue for the purpose of automated histology. The histology is accomplished in-situ by serial two-photon imaging of labeled tissue and removal of the imaged tissue with amplified, approximately 100 fs duration pulses, as illustrated schematically in figure 1A.

Area of application

The ablation of tissue with femtosecond, infrared laser pulses requires large optical fluences, in excess of 1 J/cm². In the past, such high fluences have been achieved with commercially available ~ 100 MHz laser oscillators with ~ 100 fs pulse duration, whose 1 to 10 nJ pulse energies can be focused to a less than 1 μ m² spot size with high numerical aperture (i.e., tight focusing) objectives. Such beams have been used for fine-scale ablation of subcellular structures (Tirlapur and Konig, 2002). More typically, femtosecond laser pulses have been amplified with an optical amplifier to obtain pulses with comparable duration and microjoule energies, albeit at repetition rates of only 1 to 10 kHz. These amplified pulses have been used to ablate a wide variety of tissues, including cornea (Loesel et al., 1996; Oraevsky et al. 1996; Juhasz et al. 1999; Lubatshowski et al., 2000; Maatz et al., 2000), dental tissue (Loesel et al., 1996; Neev et al., 1996), skin (Frederickson et al., 1993) and brain (Suhm et al., 1996; Loesel et al., 1999; Tsai et al., 2003). In this chapter, we describe the coupling of femtosecond ablation with two photon laser scanning microscopy (TPLSM)

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for the purpose of serial histology of neuronal tissue (Tsai et al., 2003). This extends the histological use of TPLSM to image tissue throughout arbitrarily thick samples.

Materials

The integration of the imaging and ablation optomechanical set-up is shown schematically in figure 1B. The individual components are as follows:

- Two photon laser scanning microscope. We used a custom-built system based on a commercial Ti:Sapphire laser oscillator (Mira-F900 pumped by a Verdi V-10, Coherent Inc., Santa Clara, CA). The microscope design is derived from the original *in vivo* system that was constructed by Denk (Swoboda et al., 1997) and has been previously described in detail (Tsai et al., 2002). Alternatively, turn-key TPLSM systems are available from various commercial vendors.
- 2. Optical amplifier. A subset of the nanojoule pulses from the Ti:Sapphire oscillator are amplified in a multi-pass optical amplifier based on the system of Kapteyn and Murnane (Backus et al., 1995). This results in a 1 kHz train of 400 μJ pulses. Alternatively, appropriate amplified sources are now commercially available that produce either a 1 kHz or faster train of millijoule pulses (e.g., Legend pumped by an Evolution-15, Positive Light, Los Gatos, CA or competitive systems from Quantronix, East Setauket, NY, or Spectra Physics, Mountain View, CA) or a 200 kHz trains of pulses with a few microjoules of energy. (RegA9000; Coherent Inc.). The beam of amplified pulses is integrated into the TPLSM with polarization optics to allow sequential two-photon imaging with 0.1 to 1 nJ pulses and ablation with 1 to 10 μJ pulses within the same apparatus. The amplified pulse energy is controlled with neutral density filters.
- 3. Fluorescently labeled tissue. A tissue preparation, typically fixed with 4 % (w/v) paraformaldehyde (PFA), is placed in the modified TPLSM and imaged using a water-immersion objective with high numerical aperture (NA), i.e., 0.5 to 1.0. The structures of interest are tagged, either by surface application of a low molecular weight fluorescent dye, or alternatively, with the use of transgenic animals selectively expressing a fluorescent protein.

4. Fast programmable translation stages. The tissue is mounted on a set of fast translation stages, capable of speeds up to 8 mm/s with micrometer accuracy (RCH22 series stages and 300 series controllers, New England Associated Technologies, Lawrence, MA). These stages raster the tissue across the focus of the objective lens so that centimeter-sized areas may be ablated, and allow for lateral displacement of the tissue so that multiple fields of view may be imaged using TPLSM.

Protocols and Procedures

Tissue Preparation and Laser Alignment

- Adhere the fixed tissue to a Petri dish by partially encasing the tissue with a solution of 2 % (w/v) agarose (Sigma, St. Louis, MO) in phosphate buffered saline (PBS), and allowing the agarose to cool and gel. Additionally, a thin layer of cyanoacrylate cement (Superbonder 49550; Loctite, Hartford, CT) may be used to secure the tissue to the bottom of the Petri dish.
- 2. Center and align the imaging and ablation beams. This can be accomplished with calibration samples that consist of a coverslip atop a block of 100 μM fluorescein in 2 % (w/v) agarose gel. First, the imaging beam is focused at the interface of the coverslip and the gel by locating the axial onset of fluorescence. The focus of the ablation beam is the set with the use of telescopes and adjustable mirror mounts in the amplified beam path to center the focus of the ablation beam ablation within that of the imaging beam. Cavitation bubbles, visualized as dark spots in a sea of fluorescence, are generated when the focus of the ablation beam lies within the gel, but not when it lies within the coverslip.

Iterative Imaging and Ablation

The iterative process of imaging and ablation are laid out as a flowchart in figure 2, and technical details pertaining to particular steps are provided in the figure caption. Examples of cutting parameters that have been used for sample ablation with a 1 kHz train of 100 fs pulses are provided in Table 1. Note that these parameters represent cutting speeds that are overly conservative by a factor of between 2 to 10.

Example of Application

We visualized layer V pyramidal neurons from mouse neocortex via the iterative, optical histology described above (Figs. 1C and 1D). The tissue was obtained from the transgenic mouse strain B6.Cg-TgN(thy1-YFPH)2Jrs (Feng et al., 2000), available through Jackson Labs (Bar Harbor, ME). The animal was perfused with PBS followed by 4 % (w/v) PFA in PBS. The perfusion volume was 5 ml per gram animal and the flow rate was 20 ml/min. The tissue was stored in 4 % (w/v) PFA in PBS for postfixation before mounting in agarose.

Ablation was performed using a 20-times magnification, 0.5 NA water-immersion objective. A beam of 8 μ J pulses, arriving at a rate of 1 kHz, was focused through the objective onto the sample. The sample was translated in a raster pattern at a speed of 4 mm/s with a 10 μ m lateral step between linear passes. The axial step between ablation planes was chosen to be 10 μ m. Accumulated debris and hydrolysis bubbles under the water-immersion objective were removed by washing the tissue with buffer between successive ablation planes.

Imaging was performed with a 40-times magnification, 0.8 NA, water-immersion objective, which achieves 0.4 μ m lateral resolution and 2 μ m axial resolution (Williams et al., 1994). The field size was 200 μ m x 200 μ m and was stored as an 800 pixel x 800 pixel image. The axial step between images was 1 μ m.

The result of 24 iterations of imaging and ablation along a radial axis of neocortex are shown in figure 1C. The imaging in each iteration progressed from the dorsal to ventral direction (left to right in the figure), over a single 200 μ m x 200 μ m field of view through at least 100 μ m of tissue depth. The ablation also progressed from the dorsal to ventral direction, removing an average of 60 μ m per iteration. Each image stack was then projected along the coronal direction and overlaid to produce the composite in figure 1C. The 24 individual stacks were then smoothly merged and contrast-inverted to produce the image in figure 1D.

Advantages and Limits

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The primary advantage of this all-optical histological technique is the ability to image *in-situ* large volumes of unfrozen and unembedded soft tissue. Tissue distortion and misalignment due to the generation of thin physical sections is eliminated, allowing for straightforward image registration and generation of 3-dimensional maps and reconstructions. The technique is also conducive to automation for high-throughput applications.

The technique is limited to fluorescently labeled tissues whose optical properties are amenable to two-photon microscopy, i.e., the scattering depth must be large compared to the axial extent of the focal volume. The speed of TPLSM imaging is currently the time-limiting aspect of this technique (Tsai et al, 2003), by a factor of as much as 100, although faster imaging can be achieved with the use of resonant scanners.

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CAPTIONS

Figure 1. Overview of all-optical histology.

(A) 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 ~ 150 μ 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.

(B) Schematic of laser and imaging systems. A train of 100 fs pulses from an optically pumped Ti:Sapphire oscillator are directed with scanning mirrors to image a preparation placed on a programmable translation stage. A portion of the imaging beam is picked off by an electrooptic pulse picker, e.g., a Pockels cell, for amplification in an optical amplifier before being directed to the sample for ablation. The tissue is moved laterally by the programmable translation stages to allow ablation over large areas as well as imaging over multiple fields of view.

(C) 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 laser was focused onto the cut face with a 20-times magnification, 0.5 NA water objective and single passes, at a scan rate of 4 mm/s, were made to optically ablate successive planes at a depth of 10 μ m each with total thicknesses between 40 and 70 μ m per cut. The energy per pulse was maintained at 8 μ J. Each stack of images represents a maximal side projection of all accumulated optical sections obtained using TPLSM at λ = 920 nm. The sharp breaks in the images shown in successive panels demarcate the cut boundaries.

(**D**) A maximal projection through the complete stack with the breaks removed by smoothly merging overlapped regions. The contrast in 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).

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Figure 2. Flowchart of the all-optical histology. The following notes apply.

1. For example, to fluorescently label nucleic acids, apply 100 μ M acridine orange (Molecular Probes, Eugene, OR) to the tissue surface for 3 minutes, followed by 5 washes with PBS for 1 minute each.

2. Laser power should be increased approximately exponentially with depth to compensate for scattering and absorption losses.

3. Use the programmable translation stages to move to an adjoining lateral field of view for imaging. It is useful to allow roughly 10 % spatial overlap with previously imaged fields of view to verify proper registration

4. Program the translation stages to perform a raster scan across the entire lateral extent of the tissue. Parameters for ablation can be varied to trade off between speed and precision. Higher pulse energies can allow faster translation speed and larger step sizes, but may increase the surface roughness of the tissue, thereby potentially reducing the quality of subsequent images.

5. Debris and hydrolysis bubbles resulting from the ablation process can be removed either with a continuous buffer flow across the tissue during the ablation or by manually washing the tissue with buffer between successive ablation planes.

6. The total depth of tissue to be removed per ablation round will vary with the tissue scattering properties and the strength of the fluorescent signal. It is important to ablate less depth than was imaged in the previous imaging round, as a means to verify registration.

7. To maximize spatial overlap, so as to minimize surface roughness, the lines of ablation in successive planes can be interlaced by offsetting the start position in each plane by one half the lateral step size between lines.









Objective NA	Energy [microjoules]	Translation Speed [microns/sec]	Lateral Step Size [microns]	Axial Step Size [microns]	Volume Rate [mm ³ /sec]
0.5 Water	2	4000	2.5	10	1 x 10 ⁻⁴
0.5 Water	8	4000	10	10	4 x 10 ⁻⁴
0.3 Water	20	4000	10	50	2 x 10 ⁻³
0.2 Air	30	1500	10	20	3 x 10 ⁻³

 Table 1. Ablation parameters that were successively used with a 1 KHz train of amplified 100 fs pulses.