# Trouble-free 2-Photon Microscopy

## Two-Photon Microscopy with Fixed-Wavelength Femtosecond Lasers

Two-photon (2P) absorption spectra of fluorescent dyes are broader than single-photon ones. Simultaneous 2P excitation of several dyes with a single wavelength (WL) is feasible and can be seen as an advantage. We used femtosecond (fs) pulsed fixed-WL infrared lasers with center WLs at 780 nm and 1040 nm, respectively, for Two-photon microscopy (2PM) in biologically relevant samples. The 1040 nm laser proved to be very efficient in exciting fluorescence from YFP and red fluorescent dyes, but also in generating second harmonic generation (SHG) from muscle tissue and collagen. We demonstrate that economical, small-footprint fixed-WL lasers can present adequate replacement for commonly used Ti:Sapphire (Ti:Sa) lasers, the size, price and handling of which causes considerable pain for microscope users.

### Introduction & Methods

Two-photon microscopy has become a widespread tool for 3D imaging with subcellular resolution in live, intact tissues [1]. Most 2PM are equipped with a Ti:Sa laser. Their output WL is tunable to allow excitation of fluorescent proteins and dyes with various absorption maxima [2].

Ti:Sa laser systems add considerably to system cost and complexity. Their high power, size, noise, and the sensitive alignment of typical 2PM require installation in dedicated labs [3, 4].

Therefore, we strived to set-up a 2PM with alternative laser sources and investigated the suitability of two different fixed-WL laser designs:

- Toptica FemtoFErb 780, a glass fiber laser producing 780 nm pulses of <100 fs at 50 mW average power from a passively cooled, 1.7 l volume box.
- Spectra Physics / HighQLaser FemtoTrain 1040, a solid state laser that produces 1040 nm pulses of <250 fs at 2.5 W average power from a 7.3 l box.

A typical Ti:Sa-based model (Spectra-Physics MaiTai HP) produces any WL between 690 and 1040 nm with <150 fs pulses, average power between 400 mW and 3 W, from a 30.3 l box. The FemtoTrain costs a third of the MaiTai. The FemtoFErb costs less than a quarter.

Based on the fixed-WL lasers and a commercial FEI Intravital 2PM, we built a low-budget 2P system that fits on a mobile 750 x 900 mm floating table.

Both fixed-WL lasers were coupled simultaneously (fig. 1). 780 nm laser power attenuation to 10 mW was performed by changing pump power; 1040 nm laser power was attenuated by retarder plate and polarizer to 10 to 20 mW. The Nikon 25x objective lens aperture (Nikon CFi75 Apo Water LWD, N/A 1.1, w.d. 2 mm) was modestly over-illuminated. End-to-end Transmission of the system was 40% at 780 nm / 30% at 1040 nm.

The 2PM was equipped with two non-descanned GaAsP PMTs with large sensitive surface. Dichroic mirrors were used to split signals into color channels. Laser safety was achieved by boxing lasers and beam path.

Using typical samples taken from ongoing research we tested if fixed-WL lasers present a viable alternative to tunable laser systems.

### Results & Discussion

We started with a crayfish ventral nerve cord (VNC) preparation stained with two nuclear dyes - propidium iodide (PI) and Hoechst 33342 (Hoechst). PI stains dead or leaky cells, while Hoechst stains alive cells. The double-staining allows quantifying alive/dead ratio. Both dyes can be excited with UV light of ~360 nm, allowing simultaneous imaging in camera-based wide-field fluorescence systems. Inherent to such systems is limited imaging depth and inexistent optical sectioning, prohibiting analyses below the surface of the tissue. Our 2PM yielded good optical sectioning at depths where giant axons are located (30-40 µm from the surface), but also near the bottom of the preparation, at 250 µm. The result show that periaxonal glial cells show heightened survival compared to other glial cells in the crayfish nerve cord after oxidative stress, confirming the findings in [5].

Fluorescent proteins (FP) like GFP have redefined fluorescence microscopy with numerous powerful uses, among these specific labeling of cell lineage [6], gene expression monitoring, and combinatorial expression of several GFP variants for Brainbow experiments [7]. GFP and its derivatives are expressed by the living specimen, ridding staining procedures and avoiding toxic influences from synthetic dyes.

Preliminary experiments showed that the 780 nm laser evokes only weak signals marginally brighter than background auto-fluorescence (data not shown).

Our hope was that the 1040 nm fs laser would be more suitable for excitation

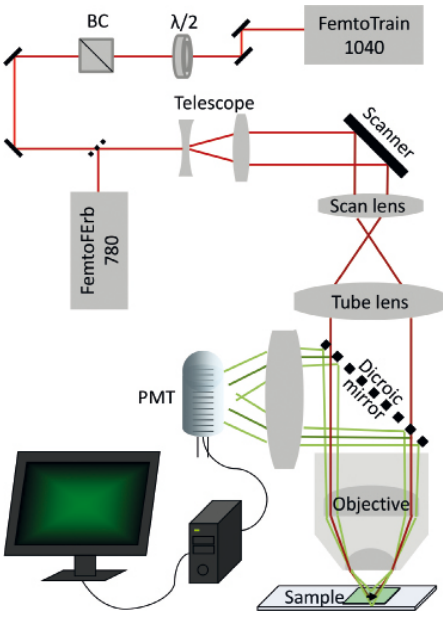



Fig. 1: 2PM with two fixed-WL lasers. λ/2 = Lambda-half plate, BC = polarising beam splitter cube, PMT = photo multiplier tube.

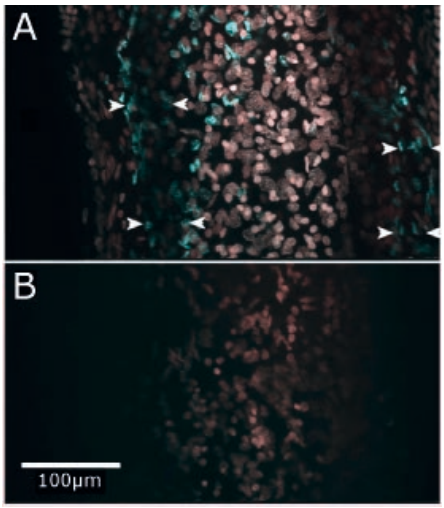



Fig. 2: Glial cells in connective of crayfish VNC after oxidative stress. A: Plane where giant axons lie (30-40 µm deep), B: Optical section 250 µm below surface. Cyan: living glial cells; Pink: necrotic glial cells. Imaged with 780 nm laser at 10 mW. Emission Split at 562 nm. Arrowheads: edges of giant axons. Sample courtesy of Mikhail Kolosov; recording by Tilman Franke.

of FPs. We tested this with a mouse brain sample in which a sub-population of neurons expressed YFP (fig. 3).

The upper image in figure 3 shows a 415x415 µm field of view in the Hippocampus area of the mouse brain. The magnification, recorded with 150 nm pixel resolution, reveals fine details of the YFP-expressing neurons. Low image noise indicates efficient excitation of YFP by the 1040 nm laser at 10 mW power.

To test if fixed-WLs lasers are suitable for imaging in organs that are still embedded into their tissue context, we imaged arteries of living mice that were injected with Alexa594-stained white blood cells (WBCs) in an acute preparation. Figure 4 shows Alexa-stained WBCs in the microvasculature of the muscle tissue surrounding the carotid artery (vasa vasorum) [9].

Such imaging is performed with the aim to observe the interaction of WBCs with the vessel wall *in vivo* during the inflammation process that goes along with atherosclerosis. While we could not image the time-course of WBC movements in the vasa vasorum of atherosclerosis-prone old ApoE-/- mice, we could detect WBCs attached to blood vessels (arrows) and streaks of moving WBSs (red lines). Intriguingly, muscle tissue (striped, cyan) was visualized without prior dye labeling as SHG.

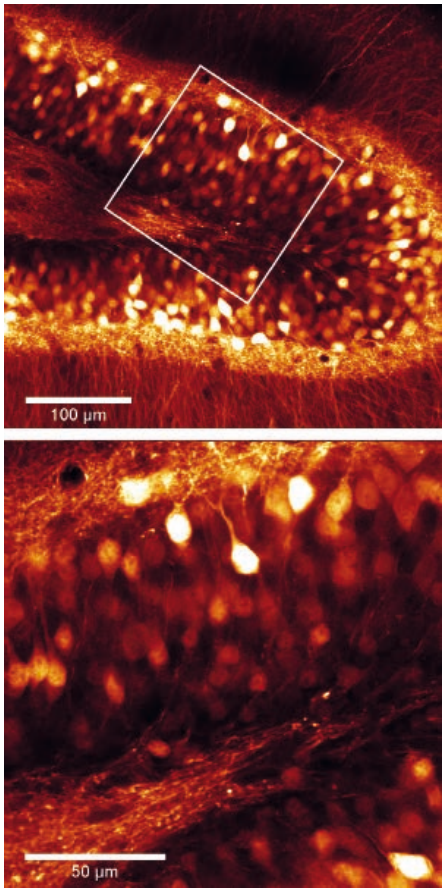



**Fig. 3: Mouse brain sagittal section, Hippocampus. Optical section from 40 µm below the surface of the slice. Excitation with 1040 nm laser at 10 mW. A sub-set of neurons express YFP. Lower image: magnification of marked area in upper image. False-colour code to enhance visibility of faint structures. Sample courtesy of Sabine Scheibe, LMU Munich, Germany; recording by Tilman Franke.**

## 780 nm Laser

The VNC experiment and preliminary tests in a variety of fixed samples demonstrate that exciting several synthetic dyes simultaneously is possible with the 780 nm laser.

The observation is backed up by cross section data acquired at Cornell [10], which shows that at 780 nm, more than half of all synthetic dyes tested can be excited with more than 50% efficiency.

## Power

Ti:Sa lasers have a lot more power at 780 nm than the fiber laser used here. However, given the transmission of typical 2PMs and the damage threshold of biological tissues, we conclude that laser power is never a limiting factor at 780 nm.

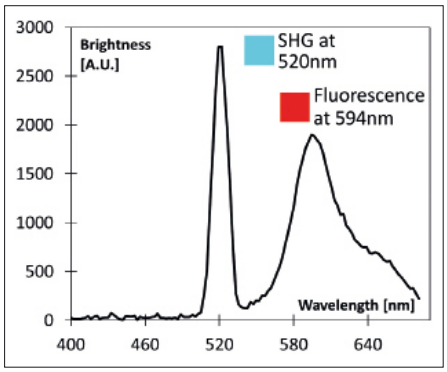
## 1040 nm Laser

For intravital and fresh tissue imaging, depth penetration and tissue damage are decisive of usability of a 2PM system. Using a longer excitation WL improves both parameters [11].

We showed that YFP can be excited efficiently with 1040 nm, in agreement with [2]. Furthermore, we got label-free SHG signals. SHG images were brighter and clearer than what we observed with Ti:Sa laser set to 880 nm in similar preparations. There are a number of explanations for exceptional SHG performance of the Spectra Physics / High Q laser:

- Long WL excitation light scatters less than short WL excitation light.
- Longer WL SHG (520 nm) scatters less than short WL (440 nm) SHG.
- The spectral sensitivity function of GaAsP detectors peaks at 520 nm, corresponding to the SHG signal.

We conclude that the 1040 nm laser is well suited for intravital imaging with red dyes, FPs, and in unstained samples. The 780 nm laser proves to be interesting for imaging with synthetic dyes.



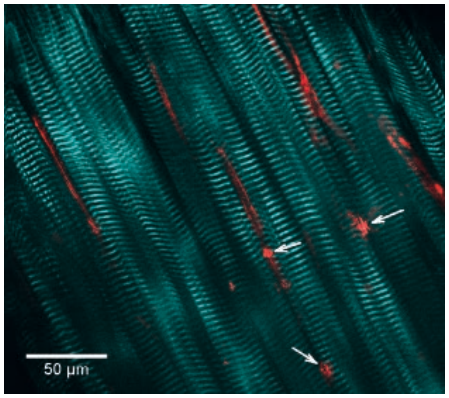




**Fig. 4: Striped muscle tissue and microvasculature imaged in acute preparation of mouse neck. Cyan: SHG signal; Red: Alexa 594 staining in WBCs. Arrows: WBCs attached to vessel wall. Excitation with 1040 nm laser at 20 mW. Recorded at the lab of Marc A.M.J. van Zandvoort in Maastricht, NL, by Tilman Franke.**

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