

# A Practical Guide: How to Build a Two-Photon Microscope Using a Confocal Scan Head

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This chapter provides practical guidelines for the conversion of an Olympus Fluoview confocal microscope into a two-photon microscope. This enables the investigator to have access to two-photon microscopy without the large budget necessary to purchase a commercial instrument.

Two-photon fluorescence microscopy allows deep-tissue imaging in highly scattering preparations and long-term imaging of live tissue without the photodamage that is caused by out-of-focus light (see Chapter 7). It is, therefore, an essential tool for imaging cells in physiologically relevant conditions such as acute or cultured brain slices or *in vivo*.

## MATERIALS

### Laser

The key component of the system is a femtosecond pulsed laser, which can generate reasonable power in the near-infrared (IR) spectral region needed for convenient practical imaging (average power >50 mW). Our current setup uses the Chameleon laser from Coherent. This is a fully automated turnkey laser which can be tuned to any wavelength between 715 and 955 nm.

### Optical Table

The laser beam is delivered to a modified Olympus Fluoview confocal laser-scanning system through the set of optical elements on the optical table (Fig. 1):

- Intermediate mirrors
- Spatial filter
- Retardation waveplate
- Pockels cell

BB1-E02 dielectric mirrors from Thorlabs are

used as intermediate mirrors. These reflect >99% of light between 700 and 1150 nm at a 45° angle of incidence for all polarizations and do not introduce additional group velocity dispersion of ultrafast pulses.

An optical spatial filter, which has two planoconvex lenses and a pinhole in the focus of the first lens, acts as a simple telescope. It is used to restore a smooth Gaussian profile to the intensity of the laser beam cross-section and also to modify beam size to ensure proper overfilling of the back aperture of the microscope objective (Tsai et al. 2002). A retardation waveplate ( $\lambda/2$  or  $\lambda/4$ ) is used for complex experiments in which the polarization of the scanning laser beam must be controlled.

### Pockels Cell

3030C from Quantum Technology, a nonlinear optical modulator, is included to allow the dynamic modulation of laser light intensity with

excellent contrast ratio (>200:1) and sub-microsecond temporal resolution. The temporal resolution of the Pockels cell is limited in practical terms only by the electronics of the high-voltage driver. The model in our current setup (302 from Quantum Technology) can work in DC-10 MHz modulation range (thus providing 100 nsec temporal resolution).

### Scanning Microscope

A BX50WI Olympus upright scanning microscope is coupled to a modified Fluoview scanning unit. The Olympus Fluoview platform relieves the need for a separate, custom-built scanning system and software package (Majewska et al. 2000).

The scanning box contains the only essential optical component: a set of two galvanometer mirrors, which steer the laser beam and scan the image. The scanning box is optically linked to the infinity-corrected BX50WI microscope through a pupil transfer lens, which is part of the original Fluoview system. This lens, together with the tube lens of the microscope (original part of microscope) forms a telescope, which provides collimated light for the infinity-corrected objective lens (see optical scheme in Fig. 1). This telescope also approximately images the scanning mirrors onto the back aperture of the objective. This minimizes the movement of the laser beam at the back aperture, thus minimizing variation of laser power at the sample. The laser beam is reflected downward by a short-pass dichroic mirror (650DCSP from Chroma Technology), placed inside the standard trinocular tube of the Olympus microscope.

### Fluorescence Detection

In the case of two-photon absorption, excitation of fluorescence is essentially limited to the diffraction-limited spot in the focal plane. This provides the 3D sectioning characteristic of two-photon microscopy. Since fluorescence from the excited region irradiates in all directions, it is important to use a high-numerical aperture objective in order to collect as many fluorescence photons as possible. Our system uses an external photomultiplier tube (PMT) as a detector. The tube is mounted on the camera port of the microscope's trinocular tube—along with a dichroic mirror, which transmits visible fluorescent light collected by the objective. An addition-

al IR-blocking filter (BG39 from Chroma Technology) is placed in front of the PMT to filter out residual infrared fluorescent light reflected from the excitation path. The external PMT could also be positioned right next to the objective (see schematic in Fig. 1). In this case, the PMT would have to be mounted via a custom-made adapter with a long-pass dichroic mirror, which transmits excitation infrared light and reflects visible fluorescence to the detector. Positioning the PMT on top of the objective improves light collection efficiency but compromises the convenient positioning of micromanipulators.

By choosing appropriate dichroic mirrors, it is possible to use two channels of fluorescence imaging. Placing additional band-pass filters in front of detectors can also efficiently separate the emissions of two different fluorescent dyes. The Olympus Fluoview data acquisition board comes ready equipped with two independent input channels (see Section 8 in this manual for example applications and for advice on choosing filters and dichroic mirrors).

The PMT used in our instrument is the HC125-02 assembly from Hamamatsu, which comprises a bi-alkali, head-on PMT, a high-voltage power supply, and a wide bandwidth (8 MHz) signal preamplifier. This PMT provides a voltage signal that is proportional to the light intensity. The signal is fed directly into the signal input of the Olympus Fluoview data acquisition board. The standard Olympus Fluoview software is used in scanning mode for signal acquisition and reconstruction of a digital image.

To improve the signal-to-noise ratio, an additional intermediate amplifier is connected between the external PMT and the Fluoview signal input. Currently, our system uses a PE 5113 amplifier (AMETEK Advanced Measurement Technology; former EG&G and Perkin Elmer Instruments). This instrument has a variable gain and adjustable high/low-pass filters, and can be controlled through a user-friendly computer interface. The amplifier is very convenient for proper signal conditioning, since it allows proper filling of the dynamic range of the Fluoview signal analog-to-digital converter, something crucially important for imaging weak signals and quantitative measurements. For calibration curves of the available dynamic range of Olympus Fluoview signal inputs, see Nikolenko et al. (2003).

**Caution:** See Appendix 3 for appropriate handling of materials marked with <!>.



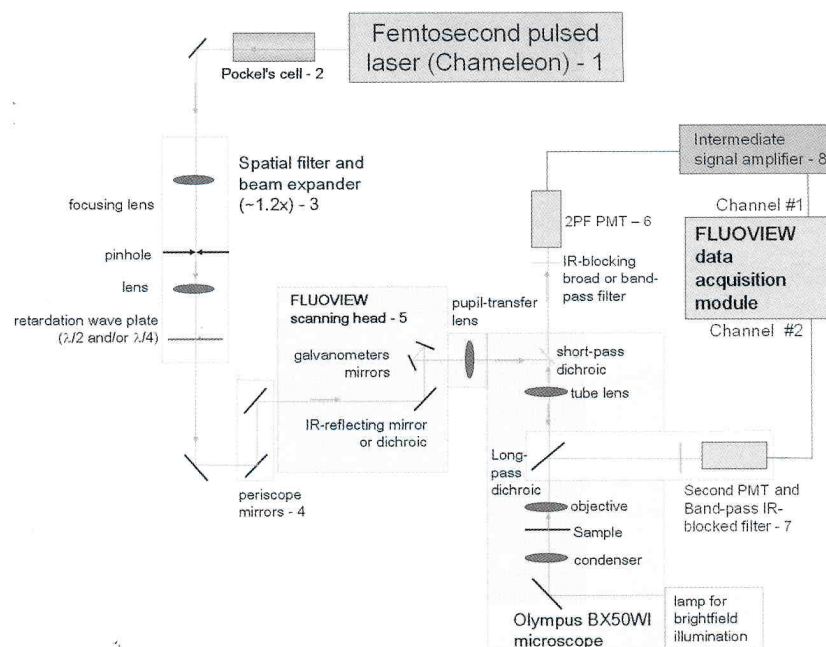


FIGURE 1. Optical design of the instrument. Key components: (1) NIR femtosecond pulsed laser; (2) Pockels cell modulator; (3) system of lenses, which works as spatial filter and beam expander ( $\sim 1.2\times$  in our case); (4) periscope mirrors (deliver laser beam from optical table level to scanning box of Fluoview, which is raised for upright microscope); (5) modified Olympus Fluoview scanning unit; (6) external PMT detector for 2P-fluorescence signal, attached to camera port of the microscope; (7) second external PMT attached to the microscope through custom-made adapter; (8) intermediate signal amplifier for matching dynamic range of signal source (PMT) and Fluoview data acquisition module.

## PROCEDURE

### Building a Custom-made Two-Photon Microscope

The full description of practical changes in the standard Olympus Fluoview confocal system can be found in Majewska et al. (2000) and Nikolenko et al. (2003). A brief summary of the necessary modification is presented here.

1. Install the pulsed femtosecond near-IR laser.
2. Build the external optical pathway from laser source to laser-scanning microscope with the optional spatial filter and Pockels cell.
3. Modify the scanning unit of a confocal laser-scanning microscope for scanning by the IR beam from external laser source. This essentially requires drilling a hole in the back of the scanning unit and replacing the internal dichroic mirror with an IR reflecting mirror (see full list of Olympus Fluoview modification in Majewska et al. 2000 and Nikolenko et al. 2003).
4. Place a short-pass dichroic mirror into the trinocular tube of the optical microscope and install the external detector (PMT) on the camera-imaging port of the trinocular tube.
5. Connect the detector signal output to the data acquisition input of the confocal system through the intermediate signal amplifier.

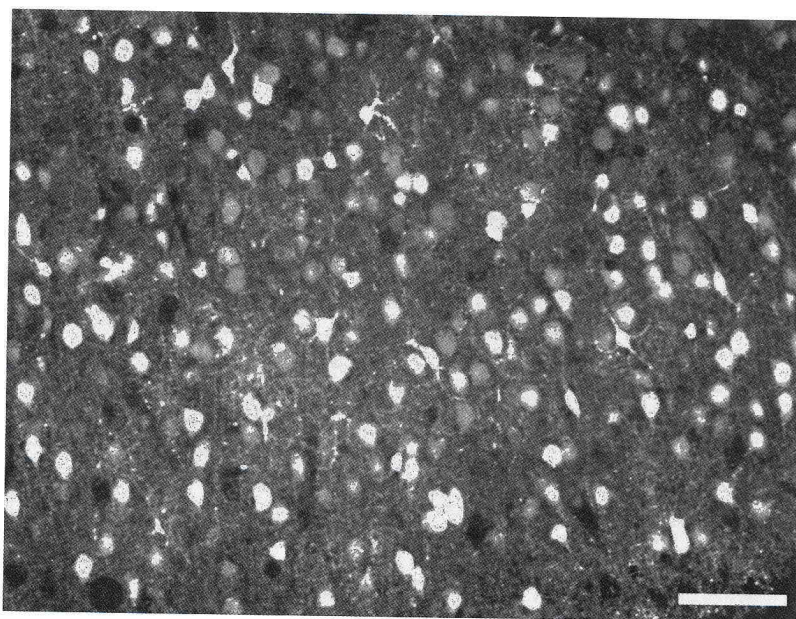


FIGURE 2. Example of imaging. A neocortical slice from a postnatal day-13 mouse, loaded with the  $\text{Ca}^{2+}$  fluorescence indicator indo-1AM. Two-photon fluorescence image acquired with  $\sim 730$  nm excitation wavelength. Bar, 50  $\mu\text{m}$ .

### SHORT EXAMPLE OF APPLICATION

The custom-made two-photon microscope described here has been used successfully for long-term imaging of action potential activity in large ( $>1000$ ) populations of neocortical neurons in acute brain slices, AM-loaded by  $\text{Ca}^{2+}$  fluorescent indicators such as fura-2 (Cossart et al. 2003) or indo-1 (Fig. 2).

### ADVANTAGES AND LIMITATIONS

The two-photon system described here, based on the Olympus Fluoview confocal system, successfully combines a customized home-made system with a reliable commercial instrument. Although tailor-made for the chosen application, the individual elements of a home-made system can be difficult to maintain in proper working condition. However, recent advances are easing this problem. For example, the appearance on the market of turnkey femtosecond-pulsed tunable lasers, such as the Chameleon, eliminated the need for realignment of the optical path. However, potential users should be aware of the correct procedures for cleaning optical surfaces that are exposed to dust, changes in humidity, etc., and follow proper laser safety guidelines (see Appendix 3).

### REFERENCES

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