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# A custom two-photon and second-harmonic microscope

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## Abstract

**The introduction of two-photon microscopy has revolutionized life sciences by enabling long-term imaging of living preparations in highly scattering tissue while minimizing photodamage. At the same time, commercial two-photon microscopes are expensive and this has prevented the widespread distribution of this technique to the biological community. As an alternative to commercial systems, we provide an update of our efforts designing custom-built two-photon instruments by modifying the Olympus Fluoview laser scanning confocal microscope. With the newer version of our instrument we modulate the intensity of the laser beam using a Pockel's cell in arbitrary spatiotemporal patterns, perform simultaneous optical imaging and optical stimulation experiments and also can combine them with second harmonic generation measurements.**

Keywords: GFP, Imaging, Pockels, Uncaging

**Introduction**

The introduction of two-photon excitation [1] to life sciences has opened novel experimental territories [2]. Two-photon excitation occurs when two low-energy photons are simultaneously absorbed by a molecule in the ground state, resulting in a similar excitation to that produced by a single high-energy photon [3]. This process has important consequences for microscopy because it enables fluorescence with infrared excitation light, which can penetrate without major scatter through living tissue [4]. In addition, the non-linear reaction confines the excitation essentially to the focal point [1], thus effectively solving a major problem in optical microscopy, that of out of focus excitation. These improvements of two-photon excitation over conventional fluorescence microscopy have proven to be of great practical advantages: two-photon microscopy have enable, among other things, physiological analysis of dendritic spines [5, 6] and direct functional mapping of synaptic receptors [7] or channels [8] on living neurons in brain slices.

The spread of two-photon microscopy, however, has been hampered by the high costs associated with commercially available two-photon systems. As a solution to this, over the last seven years our laboratory has designed and built two-photon microscopes based on modifying relatively low-cost confocal systems. We find that this strikes a good compromise between cost, flexibility and ease of engineering the system. In a previous publication we describe in detail our initial design of a custom-build two-photon microscope based on the Olympus FLUOVIEW scanning system [9]. The large interest generated by this first publication in the neuroscience community has stimulated us to provide an update to the modifications and further improvements of our system, so that other investigators can also profit from them. In this manuscript, we specifically describe

how we have implemented the control of the beam positioning and intensity which is essential for fast imaging or photostimulation experiments, as well as the modifications of the microscope to enable the simultaneous measurement of second-harmonic generation (SHG) [10, 11], a novel non-linear microscopy technique which could have major implications for life sciences.

**Description of the system-**

Our current instrument consists of two commercial lasers, an external light path, a scanning head and an optical microscope (Figure 1). We proceed to describe in detail each part of it and the rationale behind each of our choices in design.

**Laser and laser diagnostics**

The lasers we are currently using are the Mira 900 basic – tunable Ti: Sapphire lasers from Coherent pumped by 5-W solid-state pump lasers (Verdi, Coherent) as described in previous publication [9]. As beam diagnostic devices we use a spectrum analyzer (Rees) and a power meter (Powermax 500A from Molectron, Portland OR; or FieldMaster with LM-1 detector head from Coherent). In previous publication [9] it was described that a coverslip reflects a small portion of the beam (<2 mW) at its exit from the cavity and sends it into the Rees for analysis. We found that it is not an optimal solution because the glass coverslip distorts the beam. Instead, we placed the detector of Rees spectrum analyzer inside the Mira module, replacing the internal fast photodiode originally designed by Coherent for detection of mode-locking. A high-quality semitransparent mirror deflects small portion of the beam to Rees.

These two instruments (power meter and spectrum analyzer) proved to be sufficient for proper alignment of laser cavity, maintaining mode locking, tuning the wavelength and determining approximate pulse durations.

**Modifications of optical pathway before scanning unit**

In our new system, it became necessary to modify the laser excitation pathway since we found that the laser beam, in reaching the back aperture of the objective, was not collimated (the Olympus BX50WI microscope is designed for infinity corrected objective lens). We diagnosed this problem by monitoring the beam profile at different points in the light path with a WM100 Omega Meter from Thorlabs (Newton, NJ). The lack of collimation is probably caused by incompatibility of our BX50WI upright microscope with the modified FLUOVIEW scanning head. This problem theoretically can be solved by moving the pupil transfer lens (a complex lens right after the scanning mirrors – see beam path on Figure 2) but we found that the available range of movement was not large enough to correct the problem.

As an alternative solution, we used a system of additional lenses – a simple telescope of two plano-convex lenses - to make the beam divergent before the scanning mirrors and thus compensate for the strong convergence in the microscope between the tube lens and objective lens (Figure 2). In practice this is achieved with our external telescope by adjusting it to make the beam slightly convergent such that it comes to a focus before the scanning mirrors, and then starts to diverge. Special attention must be paid to ensure that the beam is not focused on the surface of one of the intermediate optical elements, like dielectric mirrors and filters, since intense focused laser light can damage their coating.

Our external lens optical system was designed to easily allow the adjustment of laser beam before objective. We not only ensure its collimation but also its appropriate size by choosing the magnification of the telescope to slightly overfill the back aperture of the objective lens. This is necessary for carefully designed optical system for laser scanning microscopy: to ensure that the system uses all the possible numerical aperture of the objective lens, and thus achieving diffraction limited spot size at the object plane, which is important for the highest optical resolution. This also minimizes the power loss of laser light and provides minimal variations in the power of the excitation light across the image during scanning [12].

Our system of correction of laser beam before scanning head also performs the function of a spatial filter: it is a system of two lenses and pinhole. The pinhole is placed at the focal plane of the first lens. This spatial filter is a convenient way to remove random spatial deviations from the intensity profile of a Gaussian laser beam, which are picked up by scattering from optical defects and particles in air [13]; See 'spatial filter' section in Mells Griot or Newport catalog for practical choice of components - http://www.newport.com/store/xq/ASP/lone.Optics/ltwo.Technical+Reference/lthree.Spatial+Filters/lfour./id.3873/lang.1/qx/product.htm). The beam, after passing the pinhole, has a smooth intensity profile and, in addition, any pointing fluctuations in its direction are removed. This comes at the expense of reduced laser power (~10% loss), which is not a major problem since for most practical cases of two-photon microscopy we do not use the laser at full power but rather use a neutral density filter to reduce power. It therefore provides the best beam conditions for laser scanning microscopy and improves the image quality. In addition, the spatial coherence of the beam (the phase distribution along the

beam cross section) is restored, which is important for second harmonic generation microscopy (see below).

**The scanning head and beam pathway**

We used a modified FLUOVIEW confocal unit, which we find ideal for conversion to two-photon microscopy [9]. It has a simple beam path, is easy to align and the unit is fully accessible, making it easy to modify without disturbing essential components. A full description of our scanning head modifications is found in [9]. Briefly, the minimal modifications for its conversion to two-photon microscopy are:

1. The side panel in the laser input port has to be removed and a hole drilled into the casing to accommodate the beam from Ti-Sapphire laser without clipping.
2. The entrance dichroic should be removed completely in order to allow the beam to enter the body of the confocal box.
3. In the case of using an external photomultiplier tube (PMT) in whole-area detection configuration (see below) the second dichroic has to be changed to a regular mirror of appropriate size or another dichroic with good reflectivity in near IR to allow the beam to be reflected onto the galvanometers.

A description of the light path follows (Figure 2). After entering the confocal box through the drilled hole, the laser beam encounters a shutter, under control from the FLUOVIEW software, which prevents the laser from entering the microscope when the unit is not scanning. The galvanometers scan the beam into the upright microscope (BX50WI, Olympus) through a pupil transfer lens. In order to increase the power throughput of the unit, the pupil transfer lens was substituted with a lens that has high transmission in the IR (available from Olympus). Another dichroic (Chroma Inc.;

650DCSP which reflects IR and transmits 95% between 425 nm and 640 nm) inside the trinocular head then directs the beam downward towards the sample through the microscope tube lens and objective. The visible fluorescent light returns back from the sample through the objective and microscope tube lens and then is transmitted to an external PMT by the same dichroic in the trinocular head. Additional IR blocking filters (BG39 from Chroma Inc. or similar – see [9]) are placed in front of the external detector (PMT).

**PMTs**

In our previous study [9] we report the major improvement in the signal using external PMTs, which we use routinely. For the common applications of two-photon fluorescence imaging we use this external PMT in whole-area detection configuration, by mounting it to the camera port of the trinocular head of our Olympus BX50WI upright microscope. The signal to noise ratio can be additionally increased by placing detector right after objective with special holder [9] but this configuration is not very convenient for everyday work because of its position. We use it only in special cases that require very high sensitivity or simultaneous PMT/camera imaging with a CCD camera attached to camera port usually occupied by external PMT. The PMT that we prefer is the HC125-02 (Hamamatsu). It is a self-contained assembly of a head-on bi-alkali PMT with wideband amplifier (bandwidth 8MHz) and a high voltage power supply. Although this is useful as it avoids the necessity for the user to deal with high voltages, it requires a custom-made low voltage power supply. Our custom-made power supply allows the regulation of the bias voltage of the PMT by changing the position of knob of the variable resistor.

We found that the uncorrelated dark noise of the HC125-02 PMT (primarily of thermal origin) has a strong dependence on the applied bias voltage (see Figure 3C). It is therefore important to correctly choose the bias voltage in order to balance the resulting gain of the PMT versus noise. In most cases, the normal charge of PMT is ~750V, but it is possible to increase bias voltage of PMT if active methods of averaging are used, such as Kalman filtering available in standard FLUOVIEW software package.

Care must be taken to ensure that the external PMT is compatible with the FLUOVIEW hardware and software as it was described earlier [9] by introducing an additional custom made signal amplifier, which is absolutely necessary for the correct detection of low light intensity signals (see Figure 3A and 3B for available dynamic range of FLUOVIEW hardware input signals). It is worth mentioning that this intermediate amplifier requires a battery power supply, because available power supplies working with AC power usually introduce additional noise.

**Direct software control over Olympus FLUOVIEW**

To gain flexibility in the scanning, we have created a program that interfaces with the FLUOVIEW software, by taken advantage of the Olympus application note "Restricted-Area Laser Scanning" which describes how to control the FLUOVIEW software in order to expose small selected regions of a specimen to laser light. This application note is the first phase of direct programmatic control of FLUOVIEW functions and initially was designed to provide an example of how to scan an image, target an area, expose it to light, and then scan again to measure visible results. But in practice this note explains how to obtain control and gain direct program access to the FLUOVIEW functions. This note and its accompanying sample software described how

to initialize the FLUOVIEW hardware, control the laser shutter, control the z stage motor, change the bias voltage of internal PMTs and move the galvanometers mirrors in order to direct the laser beam to any desirable position in the field of view. The FLUOVIEW acquisition ActiveX control DLL (gbx.dll file in FLUOVIEW software version 2.1.22) exports a number of "C" callable functions. But for our version of FLUOVIEW software (2.1.22), the control is really a hybrid. COM technology can be used to access and set a number of parameters, but no COM methods were implemented (COM – "component object model", see more for example on Microsoft website http://www.microsoft.com/com). Instead, "methods" are made available via direct "C" calls (see Figure 5A).

It should be noted that the method shown in this application note is not supposed to be used to make extreme movements of galvanometers mirrors – since large, non-smooth waveforms can cause the two mirrors to collide.

An internal function in FLUOVIEW hardware library (in gbx.dll file), which actually moves the galvanometers, accepts command values in internal units and sends these values as command signals to the galvanometers. We implemented the earlier regime "park mode" [9] for point measurements, which actually can be considered as a special case of this direct control over galvanometers mirrors when command values in internal units are equal to (0; 0). In order to calibrate the internal units we moved the beam in a regular fashion in the horizontal and vertical directions and then let the beam stay at this position until it produced a visible spot of photobleaching (Figure 5C). Then we analyzed the images and found a unique transfer function between the coordinates in internal values and the real pixels coordinates of digital image. It is important to notice that the center position in the galvanometers mirrors internal coordinates is not at the

geometrical center of digital image: for 800x600 scanning mode, the coordinates (0; 0) in internal units translates to coordinates ~(350,300) in the digital image, mainly because the sampling period for each line (region of linear movement) starts not far from the left edge.

The simplest forms of direct control of the FLUOVIEW hardware by the manual modification file gbscasn.ini (regime "Park Mode") and direct calls of hardware functions from gbx.dll by sample software has been used extensively for fluorescence measurements with microsecond time resolution ([14, 15]; Mansvelder and Yuste, unpublished observations). However, to reach a higher degree of control, we created our custom software for direct access to FLUOVIEW hardware functions by using LabView™ graphical programming interface (National Instruments, Austin, TX), which combines low-level programming tools with simplicity of development application with convenient user interface (Figures 5B and 5D). We should mention that this kind of custom software can be created by using any modern programming language which supports direct "C"-calls or COM technology in general such as C++, Visual Basic, etc.

**Basic and Advanced versions of custom software and Windows scripting**

Our initial version of this software ("basic version", Figure 5B) gives full access to the following hardware functions of our FLUOVIEW system: move z-motor, lock/unlock fine focus manipulator, open/close shutter, move galvanometers mirrors with maximum available accuracy to direct laser beam to any desirable point on the pre-scanned image pointed by cursor or even to track beam cursor movement over the image in real time. This version is fully functional for point measurements of fluorescence or/and SHG signals.

The more recent version of our software ("advanced version") allows laser irradiation of an array of selected targets by sequentially pointing the laser beam to the individual targets in "vector mode"– see Figure 5D. This program also allows easy regulation of the intensity of laser irradiation for each target by changing irradiation time or intensity for individual target (see Pockel's cells below).

In addition, another way to control the Olympus FLUOVIEW system is to use a high-level control over the native Fluoview software by the Windows Script Host (full documentation can be found on Microsoft Development Network website: http://msdn.microsoft.com/scripting). This high-level tool provided by the OS practically allows creating a "virtual operator" on the computer which can launch any applications and switch between them ("Run" and "AppActivate" methods), and, more importantly, can send sequence of keyboard commands to selected application ("SendKeys" method) – see Figure 5A. The main drawback of this type of control over any software is the fact that SendKeys method needs some delay related with the productivity of available computer system: for our current FLUOVIEW PC system (PII 400 MHz, 512 MB RAM, Microsoft Windows NT 4.0 ws) this delay is on the order of hundreds of milliseconds. The necessity of this delay limits the use this type of high level control to relatively "slow" actions of Olympus FLUOVIEW software: start/stop scanning, saving files etc. Also, the appearance of a macro language in latest versions of FLUOVIEW software (FV300 and FV500) probably will make unnecessary extensive use of this type of control over software. At the same time, Windows Script Host can be considered as universal macro-language which gives more flexibility in types of available commands, not limited to one application and in principle allows to organize data flows between different applications running simultaneously (data acquisition software such as FLUOVIEW +

data processing software such as ImageJ (http://rsb.info.nih.gov/ij/) or Matlab (MathWorks, Inc., Natick, MA) – software packages widely used for off-line data processing in our group).

**Pockel's cell**

We extensively use a Pockel's cell – an electro-optical modulator [9], as a fast neutral density filter for the dynamic regulation of laser light intensity. The original model (Pockel's cell model 350-50 and high-voltage driver model 302 from Conoptics Inc., Danbury CT) gave us limited flexibility in wavelength and bandwidth. Recently, we obtained a newer model of the Pockel's cell (model 327) and high-voltage driver (model 3030C) from Quantum Technology, Inc. (Lake Mary, FL) which allows modulation laser light intensity with 0.1 μs time resolution and maximum contrast ratio as good as 600:1. A Pockel's cell is probably the best choice for modulation of pulsed femtosecond lasers used in non-linear optical microscopy (multi-photon fluorescence (MPF), second-harmonic generation (SHG), coherent anti-stokes Raman microscopy (CARS), etc. Another solution, which is the one implemented by Olympus in the latest versions of FLUOVIEW confocal laser scanning systems, is to use an acousto-optical tunable filter (AOTF) [16]. Nevertheless, an AOTF has limited use as fast modulator/deflector for near-infrared femtosecond pulsed lasers because of significant pulse broadening, although it can be compensated at least partially by introducing of additional optics in laser pathway, albeit at the expense of laser power [17].

In simplest case, we use a Pockel's cell in everyday work, when our system works in a regular scanning regime, for blocking the laser beam in "flyback movement" – because the system does not collect data but the sample is irradiated [9]. This flyback

time accounts for ~40% of the scanning time, so blocking the laser during flyback allows to increase the average irradiation power (and thus obtain a better fluorescence signal to noise ratio) without increasing the average level of photodamage and photobleaching. For conventional, single photon laser scanning microscopes, this flyback problem can be corrected with AOTFs but again, this is not practical for pulsed femtosecond lasers, necessary for two-photon excitation. Another solution is the bi-directional scanning mode, in which data is also collected during flyback. Bidirectional scanning, available in the latest versions of Olympus FLUOVIEW, leads to a two-fold improvement in the time resolution, but also produces a degraded spatial resolution and is generally not recommended for imaging of fine structures (Yiwei Jia, personal communication). Also, most custom-made laser scanning microscopes [12], use unidirectional X-scanning for simplification of adjusting parameters of moving in Y-axis.

Laser light was blocked during flyback by using a Master 8 stimulator (A.M.P.I., Jerusalem, Israel) – a computer-independent, RS232 programmable externally triggerable square pulse generator, which was synchronized with scanning by FLUOVIEW generated TTL pulses ("line active") corresponding to regions of sampling in each line [9]. We find that these TTL synchronization pulses cannot be used directly for modulating of Pockel's cell because of the incorrect length of the pulses in case of 2-channel data acquisition and because of the impedance mismatch between the TTL output of the FLUOVIEW hardware and the signal input of the Pockel's cell driver. Also, the use of external square-pulses generator of adjustable amplitude provides a lot of flexibility in the use of the Pockel's cell.

**Selective excitation of regions of interest (ROIs)**

We find that the Master 8 stimulator is ideal for function generation of stereotypic square pulses and thus creating regions of interest (ROIs) in the image, where higher intensity illumination is enabled for space-selective photo-stimulation [18], photobleaching [14] and uncaging [9, 15]. The simplest form of space-selective excitation was implemented earlier [9], but we have now developed more complicated modes of space selective excitation (Figure 4A). For example by programming the Master 8 stimulator to generate trains of square pulses with an interval equal to the time interval of the line scans, we can generate a "bright" box – an approximately rectangular region of increased laser light intensity in the image during scanning. In this case, the generation of this train by Master 8 is triggered by the FLUOVIEW TTL synch-pulse ("frame active"). The start of this pulse corresponds to the upper left-corner of the current frame and the delay in its generation actually defines the relative position of the "bright box" in current image. This train sets a high voltage for the Pockel's cell at the defined region of image and maintains constant "background" level of laser light intensity for the rest of the image. This is done by connecting the Master 8 summed output of 2 channels – one used in a "DC mode" (constant voltage), and another which actually generates the train of pulses.

Unfortunately, this approach for space-selective excitation has intrinsic problems caused by the limited flexibility of the Master 8 generator. The main drawback is that it is not possible to create many regions of excitation – the number of such regions is limited by the number of independent Master 8 channels (8 channels). Practically it is not convenient to arrange more then one box since other channels of Master 8 stimulator are usually used at the same time for other tasks and trigger the electrophysiological

protocols. Also, the numerical parameters of "boxes" such as size and position cannot be precisely adjusted because the Master 8 has only 4-digits precision for the numerical parameters of pulses. This is not enough precision to define the interval in pulses train to generate a perfectly rectangular box, or to create a box at the arbitrary part of the image. Although the last problem can be solved by using additional independent channel of Master 8 stimulator, in practice this limits the freedom to arrange "bright boxes" at any desirable part of image. Finally, the Master 8 stimulator cannot be reprogrammed very fast, because it uses a serial RS232 interface and sending command takes some time limited by the bandwidth of the RS232 standard.

To solve these problems, a generic data acquisition board with buffer memory and externally triggered output can be used for the purpose of creating any appropriate waveform synchronized with FLUOVIEW "scan active" signal. We have used PCI-6052E data acquisition board from National Instruments (Austin, TX). By programming appropriate waveform as command for the electro-optical modulator it is possible to create any arbitrary distribution of light intensity at the image. Custom written software ("advanced version") in simplest case allows defining the set of arbitrary placed "boxes" of increased light intensity at the image for space-selective excitation (Figure 4B) – "raster mode" of selective excitation.

**Second harmonic generation (SHG) microscopy**

The Olympus Fluoview/BX50WI microscope can also be modified with a minimum amount of effort to have the capability to acquire images of SHG, either from special chromophores [11, 19] [20] et al 2001, Kobayashi et al 2002) or from endogenous structures in biological tissue such as oriented collagen fibers [21]. SHG, which like two-

photon fluorescence is a nonlinear optical effect, is gaining recognition as an important mode of microscopy that allows researchers to probe biological cell's trans-membrane potential [10] and monitor the electrical activity of nerve cells [22, 23]. Unlike fluorescence, in which emitted photons are best detected with epi-illumination, SHG photons, which result from coherent scattering, are best detected in the transmission path of the microscope. One might think of the process in a simplistic picture as the partial conversion of the stimulating light (the IR beam) into an electromagnetic wave at twice the incident frequency (half the wavelength) with a similar bandwidth (actually times $\sqrt{2}$). The SHG photons, generated at the focal spot of the laser in the sample, are collected by the condenser lens which has to be of equal or greater numerical aperture (NA) than the objective lens NA in order to collect the whole cone of light. This is important since the SHG radiation in the forward direction is restricted to certain off-axis angles [20]. We used the Olympus Aplanat Achromat oil immersion condenser with a variable NA of up to 1.4 (one does not have to use the oil for NA values of less than 1). A PMT (Hamamatsu HC125-05) with the appropriate (blue) filter was placed in the auxiliary port (see schematic Figures 1b and 2) instead of the diffuser and fiber bundle, originally designed for imaging DIC in transmission mode in the FLUOVIEW confocal scanner. By moving a handle connected to a mirror (part of the original microscope) the user can choose to engage the path either for bright field illumination (with the white light lamp) as is usually done when viewing the sample through the eye-piece, or for SHG (in which case the PMT is switched on and the lamp should be switched off or at least turned down in order not to saturate the PMT that is placed just beyond the mirror).

The SHG filter is chosen according to the operating wavelength. For instance, for an operating wavelength of 840nm an interference filter centered at 420nm with a band

of 20nm appears a good choice. One needs to ensure that the filter is fully blocked in the IR. The narrower the bandwidth of this filter, the less susceptible it is to noise from ambient light or residual two-photon fluorescence. However, since the light is directed to the PMT by the condenser lens, any room light which contains blue light will be detected and hinder the experiment by adding noise. Therefore one must darken the room lighting when performing SHG microscopy with this type of detection, or alternatively optically isolate the microscope by other means. Finally, since two channels can be detected simultaneously by the FLUOVIEW hardware, two-photon and SHG images can be acquired and displayed as a composite image by the FLUOVIEW native software. Figure 6 is an example of such a composite image, which shows two live *C. elegans* nematodes expressing a YFP (yellow fluorescent protein) tagged mec4 protein (mec4::YFP), where the SHG signal comes presumably from the muscle (blue) and the two-photon fluorescence (green) is an auto-fluorescence from endogenous granules in the intestines of the worm as well as the YFP signal - a central green spot in the upper nematode, emitted from a neuron expressing the mec4 protein (the anterior part of the upper worm is towards the right and of the lower one is towards the left. The worms were paralyzed by Levamisole prior to taking the image. The blue and green colors are pseudo-colors to distinguish the two separate channels). Note how, although both SHG and TPEF signals come from the same location of the laser spot at the same time during the scanning, they do not overlap and show different structures –for example the YFP does not produce SHG signal. The laser power needed in this case to show this intrinsic SHG signal was quite high compared to the power used with bright fluorescent dyes - 270mw before the scanning head at 870nm with 1x digital zoom and a 40x water immersion objective lens 0.8 NA. Finally, the SHG signal is strongly dependent on the state of polarization of the

laser light and the orientation of the dipole moment in the molecules that interact with that light. It is therefore advantageous to be able to control the laser state of polarization, for example in order to determine molecular orientation, or to maximize SHG. We therefore chose to add a retardation plate (half and/or quarter wave plates depending on the type of experiment one does) at the position 11c shown in figure 2 where the laser beam is fairly collimated.

**Summary**

In this work we provide an update on the modification of our two-photon microscope since the Majewska et al. publication [9]. The major improvements are spatial filtering and refocusing of the incident laser beam, direct control of the scanners via custom-made software to enable imaging or photostimulation of any arbitrary number of ROIs and, finally, implementation of SHG imaging. This system is flexible and can be easily used for many different type of experiments and does not require large expenses, other than those associated with the laser system.

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## Figures Legends:

**Figure 1: Diagram of the instrument and part list**

A: Recent photograph of our setup, showing the basic features of beam pathway. Part numbers of elements used in the beam path between laser and confocal unit. (*Neat* New England Affiliated Technologies, *NF* New Focus, *NP* Newport, *S&H* Spindler and Hoyer, *Th* Thorlabs, *Ch* Coherent Inc., *QT* Quantum Technology, Inc.

1. "Verdi" pumping laser, *Ch*
2. Mira 900-F Ti:Sapphire femtosecond pulsed laser, *Ch*
3. Dielectric mirrors (10B20UF.25 ultrafast broadband 45 degrees mirrors, *NP*) mounted on combination of following components: mirror mounts: *Th* KM1 or *NF* 9891 (*NF* mirror mounts can be flipped); post: *Th* TR-2; post holder: *Th* PH2-ST; base: *Th* BA1 (more flexible bases are available but more expensive: *NF* 9910+*NF* 9909).
4. Polarizer (05FC16PB.5 polarizing cube beam splitter, *NP*) mounted on (KM-PM, *Th*) prism platform with (PM1 *Th*) mounting hardware; post: *Th* TR-2; post holder: *Th* PH2-ST; base: *Th* BA1.
5. Pockel's cell (Model 327 with 19GLE polarizer installed at end of modulator, *QT*) mounted on holder (MTM-1000 *QT*)
6. Pockel's cell high-voltage driver (Model 3030C *QT*)
7. Custom made block for fine adjustment of Pockel's cell driver input signal
8. Spectrum analyzer and oscilloscope of spectrum analyzer (Rees optical spectrum analyzer). Detector of spectrum analyzer is not visible – it is placed inside the Mira unit. Instead of the fast photodiode provided by Coherent, we use a semi-transparent

mirror which deflects small portion of output laser light to this detector without distortion of beam.

9. Laser light power meter (FieldMaster with LM-1 detector head *Ch*)
10. Fast photodiode (DET110 Si PIN detector, *Th*) mounted to MB175 magnetic base (*Th*) – used for diagnostics
11. Beam expander/contractor and spatial filter and (all components are from Thorlabs):
    a. Two coated plano-convex lenses (LA1131-B AR and LA1134-B AR) mounted to Z-translators (SM1Z) by SM1L05 lens tube and SM1 retaining rings
    b. Pinhole (P50S) mounted to XY translator with micrometer drives (ST1XY-S) and pinhole mounting cell (SM1L03)
    c. Half-wave plate (WPH05M-830, zero order) mounted to rotational mount (CRM1) – for certain experiments we also used quarter-wave plate (WP05-830 zero order) on rotation mount (CT-104)
    d. Iris diaphragms (SM1D12 used in SM1 series) mounted to threaded caged plates (CP02);
    e. Other components used in spatial filter: extension rods (ER6, ER8); posts: *Th* TR-2; post holders: *Th* PH2-ST; bases: *Th* BA1. Center hole alignment tool (CPA1) was also helpful in alignment of spatial filter.
12. Neutral density gradient filter for coarse adjustment of excitation power (Edmund Industrial Optics, Barrington NJ)
13. Periscope:
    a. Stand: S&H 02 6106; stand cover plate: S&H 02 6212;
    b. mounting plate (mounts stand to air table): S&H 02 4330;

c. carrier (attaches to stand and linear stage): S&H 02 6421; linear stage (attaches to circular plate): Neat 1122075C (bottom one for horizontal movement; top one for vertical movement); circular plate (attaches to mirror mount): S&H 02 4972; mirror mount: S&H 08 5811; mirror: 10B20UF.25 ultrafast broadband 45 degrees mirrors, *NP*

14. Olympus FLUOVIEW confocal scanning block.
15. Trinocular tube of Olympus upright BX50WI microscope
16. External PMT attached to camera port of trinocular tube via custom made adaptor (custom made parts, threaded caged plates (CP02 *Th*), retaining rings (SM1 *Th*), rods (ER2 *Th*)). IR blocking filter [9] is placed in front of photocathode of PMT. PMT usually additionally protected from light with black rubberized fabric (BK5 *Th*) and black masking tape (T137-1.0)
17. Epi-fluorescent illuminator of BX50WI.

**Figure 1B:** SHG detection block, view from the back of Olympus upright BX50WI microscope Epi-fluorescent illuminator of BX50WI (17) is also shown.

18. Olympus FVX-TD-BX transmission detection module (cover removed for illustration) allows switching between halogen lamp for bright field illumination and additional detector port. Originally Olympus placed a diffuser and fiber-optics bundle in order to simultaneously perform bright-field and DIC images with FLUOVIEW confocal scanner. These parts were removed in our system.
19. Hamamatsu HC125-05 PMT was placed to transmission detection port instead of fiber bundle via custom-made adaptor.

20. Custom made adaptor (custom made parts, threaded caged plates (CP02 *Th*), retaining rings (SM1 *Th*), rods (ER2 *Th*)). D425/50M fully blocked filter from Chroma Technology Corp. is placed before photocathode of PMT inside custom made adaptor to protect it from fundamental IR beam and remove residual two-photon fluorescence signal.
21. PMT battery-powered custom made power supply.
22. Halogen lamp of BX50WI for conventional bright-field imaging and DIC.

**Figure 2: Drawing of the light path**

Part numbering same as on Figure 1. Some non-essential elements are omitted. The fluorescence beam pathway to internal detector of FLUOVIEW is not shown (it is practically not used). Additional lenses in transmission detection pathway also are not shown for simplicity (these lenses are not serviceable in BX50WI and we did not change them). Red arrows show propagation directions of excitation near infra-red beam. Green arrows show pathway of two-photon fluorescence emission to external detector. Blue arrows show pathway for SHG light. For an ideal system, an infinity corrected set of lenses should provide a collimated excitation beam at the back aperture of objective, and in case of collimated laser light at the input of scanning head, pupil transfer and tube lenses work as telescope which forms image of scanning mirrors approximately at the back aperture of objective [12].

**Figure 3: Measurements of PMT dark noise and linearity**.

**A & B**: Response of FLUOVIEW data acquisition module to a simulated signal input. In order to check reliability of using FLUOVIEW data acquisition system for quantitative

image analysis and verify linearity of analog circuits before analog/digital converter, signal input of FLUOVIEW data acquisition block was supplied with series of voltages and output digital image was analyzed. Our system shows good linearity in respect to analog gain and offset. The variable gain allows accommodating wide range of amplitudes from external signal sources (custom attached external PMTs, PMTs + additional amplifier, etc.).

**C***:* Dependence of not-correlated dark noise of external PMT (Hamamatsu HC125-02) versus bias voltage.

**Figure 4: Photostimulation of ROIs**.

A: ROI excitation created via the temporal modulation of laser light intensity by gating the Pockel's cell during scanning. The Master 8 square pulse generator has been used as source of train of pulses (see text). The "bright box at the center of scanned field is not perfectly rectangular because of hardware limitation of Master 8 stimulator (see text). The sample is a postnatal day 12 mouse cortical slice loaded with the $Ca^{2+}$ fluorescent indicator fura-2 AM [24]. Two-photon fluorescence image acquired with 800 nm excitation wavelength. The scale bar is 20μm.

B: Illustration of regions of space-selective excitation regime in "raster mode" of custom software ("advanced version" – see text for details). The current version of our custom software allows users to manually define (or read coordinates of origins from file) a set of rectangular regions of excitation – the regions of increased laser light intensity.

**Figure 5: Software design, interface and calibration of scanning head**

**A:** General scheme of the software control over the FLUOVIEW software/hardware by our custom-made software. Basically, the FLUOVIEW software can be represented as a container with a graphical user interface module and a module which controls hardware recourses. The user can have low level access to the FLUOVIEW hardware via "C-calls" of functions in the FLUOVIEW hardware dynamic-linked library (file "gbx.dll" for version 2.1). The Windows Scripting Host works as a universal macro language at the higher level and allows access to FLUOVIEW via its standard user interface.

**B:** User interface of the basic version of custom-made software which illustrates the direct control of FLUOVIEW hardware: it initializes the hardware and provides direct access to basic hardware resources: galvanometers mirror positioning, shutter and z-axis motor.

**C:** Calibration of internal coordinates of galvanometers mirrors. Note that the center of the scanning does not coincide with the geometrical center of the scanned digital image.

**D:** Example of user interface in custom-written software which allows to select "targets" ("vector mode" – see text) for space selective point photostimulation, uncaging or multi-points measurements. User can define the number of targets and the duration of the excitation for each target.

**Figure 6: Second harmonic Generation imaging of c. elegans.**

Two live nematodes imaged with SHG (blue) and two-photon excitated fluorescence (green). The scale bar is 20μm. See text for more details.