A two-photon and second-harmonic microscope

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Abstract

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 application 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 in arbitrary spatiotemporal patterns using a Pockels cell and software control over the scanning. We can also perform simultaneous optical imaging and optical stimulation experiments and combine them with second harmonic generation measurements.

Keywords: Imaging; Pockels; Uncaging

1. 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 an excitation similar 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 nonlinear 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 advantage: two-photon microscopy has enabled, among other things, physiological analysis of dendritic spines [5,6] as well as direct functional mapping of synaptic receptors [7] and channels [8] on living neurons in brain slices or in vivo.

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 years our laboratory has designed and built two-photon microscopes based on modification of relatively low-cost confocal systems. In a previous publication we describe in detail our initial design of a custom-built two-photon microscope based on the Olympus FluoView scanning system [9]. We find that this strikes a good compromise between cost, flexibility, and ease of engineering the system.

The large interest generated by the first publication in the neuroscience community has stimulated us to provide a detailed update to the modifications and further improvements of our system, so that other investigators can also profit from them. In this report, we describe how we have implemented the control of the beam positioning and intensity, essential for fast imaging or photostimulation experiments, as well modifications of the microscope to enable the simultaneous measurement of second harmonic generation (SHG) [10,11], a novel nonlinear microscopy technique that could have major implications for life sciences and that can be implemented with the same lasers as two-photon excitation.

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2. Description of the system

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

2.1. Laser and laser diagnostics

The lasers we are currently using are the Mira 900 basic tunable Ti:sapphire laser (Coherent) pumped by 5-W solid-state pump lasers (Verdi, Coherent) as described in the previous publication [9]. For beam diagnostic devices, we use a spectrum analyzer (IST-Rees model E201 from Imaging and Sensing Technology Corporation, Horseheads, NY) and a power meter (Powermax 500A from Molectron, Portland OR; or FieldMaster with LM-1 detector head from Coherent). In the 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 spectrum analyzer for analysis. We found that this is not an optimal solution because the glass coverslip distorts the beam. Instead, we placed the detector of the spectrum analyzer inside the Mira, replacing the internal fast photodiode originally designed by Coherent for detection of mode locking. A high-quality semitransparent mirror deflects a small portion of the beam to the spectrum analyzer.

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

3. Modifications of optical pathway before the 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 an 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 Fig. 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 (Fig. 2). In practice, this is achieved with our ex-

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Fig. 2. Light path. Part numbering same as in Fig. 1. Some nonessential elements are omitted. The fluorescence beam pathway to internal detector of FluoView is not shown (as it is rarely 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 infrared 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 the objective and, in the case of collimated laser light, at the input of scanning head. Pupil transfer and tube lenses work as telescope forming an image of scanning mirrors approximately at the back aperture of the objective [12].
ternal 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 adjustment of the laser beam before the 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. These steps are necessary to configure the optical system for laser scanning microscopy, and they also ensure that the system uses fully the numerical aperture of the objective lens, thus achieving a diffraction-limited spot size at the object plane and obtaining the highest optical resolution. A properly configured light path 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 method of correcting the laser beam before the 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 generated by scattering from optical defects and particles in air [13]; also see the Spatial Filter section in the Mells Griot or Newport product catalogs for a practical choice of components. 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). The loss 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).

3.1. 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 and 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]. Indeed, after the purchase of a femtosecond laser system, the basic conversion of a FluoView confocal microscope to a two-photon microscope requires little additional equipment (<$5000 of optical components) and effort (a few hours of work). 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 to allow the beam to enter the body of the confocal box.
3. When 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 (Fig. 2). After entering the confocal box through the drilled hole, the laser beam encounters a shutter that is controlled by the FluoView software, preventing 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. 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 and 640 nm) inside the trinocular head then directs the beam downward toward 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).

3.2. Photomultiplier tubes

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 the detector right after the objective with a special holder [9]. However, this configuration is not very convenient for everyday work because of its position. Consequently, we use it only in special cases that require very high sensitivity or simultaneous PMT/camera imaging with a CCD camera attached to the camera port (normally occupied by the 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 8 MHz) 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 us to regulate 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 Fig. 3C). It is therefore important to correctly choose the bias voltage to balance the resulting gain of the PMT versus noise. In most cases, the normal charge for the PMT is ~750 V, but it is possible to increase the PMT bias voltage if active methods of averaging are used, such as Kalman filtering available in the 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. This is absolutely necessary for the correct detection of low light intensity signals (see Figs. 3A and B 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.

3.3. Direct software control over Olympus Fluoview

To gain flexibility in the scanning, we have created a program that interfaces with the Fluoview software, by taking advantage of the Olympus application note “Restricted-Area Laser Scanning,” which describes how to control the Fluoview software 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 observe 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 describe 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 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 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, described on the Microsoft Corporation website http://www.microsoft.com/com). Instead, “methods” were made available via direct “C” calls (see Fig. 4A). 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—command large, nonsmooth sending waveforms can cause the two mirrors to collide.

An internal function idaSetDACInitialValue() in the Fluoview hardware library (gbx.dll file) actually moves...
Fig. 4. Software design, interface, and calibration of scanning head. (A) Schematic 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 that controls hardware resources. Using C programming, the user has low-level access to the FluoView hardware via calls to the functions in the FluoView hardware dynamic-linked library (the “gbx.dll” file 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 our basic version of custom-made software illustrating direct control of FluoView hardware. Our software 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 galvanometer mirrors. Note that the center of the scanning (0,0) does not coincide with the geometrical center of the scanned digital image. The grid pattern corresponds to systematically bleached regions. The bright circles are fluorescent particles in the sample. (D) Example of user interface in custom-written software that allows selection of “targets” (“vector mode”—see text) for space selective point photostimulation, uncaging, or multipoint measurements. The user can define the number of targets and the duration of the excitation for each target.
the galvanometers. It accepts command values in internal units and sends these values as command signals to the galvanometers. Previously, we implemented the “park mode” routine [9] for point measurements. Park mode can be considered a special case of direct control over the galvanometer mirrors as command values in internal units are equal to (0; 0). 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 (Fig. 4C). 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 note that the center position in the galvanometer mirrors’ internal coordinates is not at the geometrical center of digital image: for 800 × 600 pixels scanning mode, the coordinates (0; 0) in internal units translate 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 methods of direct control of the FluoView hardware are made by the manual modification of gbscan.ini file (e.g., “Park Mode” routine) and direct calls of hardware functions from the gbx.dll library by sample software. This has been used extensively for fluorescence measurements with microsecond time resolution ([14,15]; Mansvelder and Yuste, unpublished observations). However, to reach a greater 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, easy development of application, and a convenient user interface (Fig. 4B and D). We should mention that this kind of custom software can be created by using any modern programming language that supports direct “C” calls or COM technology in general such as C++ and Visual Basic.

3.4. Basic and advanced versions of custom software and windows scripting

Our initial version of this software (“basic version,” Fig. 4B) 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 prescanned image pointed by cursor, and even to track beam cursor movement over the image in real time. This version is fully functional for point measurements of fluorescence and/or 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 Fig. 4D). This program also allows easy regulation of the intensity of laser irradiation for each target by changing irradiation time or intensity for individual targets (see Pockels cells below).1

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 creation of a “virtual operator” on the computer that can launch any applications and switch between them (“Run” and “AppActivate” methods). More importantly, it can send a sequence of keyboard commands to selected applications (“SendKeys” method) (see Fig. 4A). The main drawback of this type of control over any software is the fact that the SendKeys method needs some delay related to the productivity of the 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 the FluoView system (FV300 and FV500) probably will make extensive use of this type of control over software unnecessary. At the same time, Windows Script Host can be considered as a universal macro language that gives more flexibility in the types of available commands, and is not limited to one application. In principle, this allows the organization of data flow between different applications running simultaneously [data acquisition software such as FluoView and 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].

3.5. Pockels cell

We extensively use a Pockels cell, an electro-optical modulator [9], as a fast neutral density filter for the dynamic regulation of laser light intensity. The original model (Pockels 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 newer models of the Pockels cell (Model 327) and high-voltage driver (model 3030C) from Quantum Technology, Inc. (Lake Mary, FL) which allow modulation laser light intensity with 0.1-μs

1 Send E-mail requests for the software components to vn59@columbia.edu.
Fig. 5. Photostimulation of regions of interest (ROIs). (A) ROI excitation created via the temporal modulation of laser light intensity by gating the Pockels cell during scanning. The Master 8 square pulse generator was used as a source of pulse trains to modulate the Pockels cell (see text). The bright box at the center of the image is not perfectly rectangular because of hardware limitations of Master 8 stimulator (see text). The sample is from a cortical slice from a Postnatal Day 12 mouse loaded with the Ca\textsuperscript{2+} fluorescence indicator fura-2 AM [24]. Two-photon fluorescence image acquired with 800-nm excitation wavelength. Bar = 20 μm. (B) Regions of space-selective excitation created using the “raster mode” routine in the 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.
time resolution and maximum contrast ratio as good as 600:1. A Pockels cell is probably the best choice for modulation of pulsed femtosecond lasers used in non-linear optical microscopy [e.g., multiphoton fluorescence (MPF), second harmonic generation (SHG) and coherent anti-stokes Raman microscopy (CARS)]. Another solution, which is the one implemented by Olympus in the latest versions of the 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 partially compensated, at the expense of laser power, by introducing additional optics in the laser pathway [17].

In the simplest case, we use a Pockels cell when our system works in a normal scanning mode to block the laser beam in “flyback movement” (i.e., during repositioning of the laser after the scanning of a line or of a frame), because the system does not collect data but the sample is irradiated [9]. This flyback time accounts for up to ~40% of the scanning time, so blocking the laser during flyback allows an increase in the average irradiation power (and, thus, attainment of better 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 bidirectional scanning mode, in which data are also collected during flyback. Bidirectional scanning, available in the latest versions of Olympus FluoView, leads to a twofold improvement in the time resolution but also produces a degraded spatial resolution. Thus, it 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 moving in the 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. The stimulator was synchronized with the laser 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 the Pockels cell because of the incorrect length of the pulses in case of two-channel data acquisition and because of the impedance mismatch between the TTL output of the FluoView hardware and the signal input of the Pockels cell driver. Also, the use of an external square pulse generator of adjustable amplitude provides a lot of flexibility in the use of the Pockels cell.

3.6. Selective excitation of regions of interest

We find that the Master 8 stimulator is ideal for generation of stereotypic square pulses and thus creation of regions of interest (ROIs) in the image, where higher-intensity illumination is used for space-selective photostimulation [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 (Fig. 5A). 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 train of pulse generation actually defines the relative position of the “bright box” in the current image. This train sets a high voltage for the Pockels cell at the defined region in the image and maintains a constant “background” level of laser light intensity for the rest of the image. This is done by connecting two channels from the Master 8 and summing their output: one used in a “dc mode” (constant voltage), and another 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 (eight channels). Practically, it is not convenient to arrange more than one box since other channels of the 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 four-digit precision for the numerical parameters of pulses. This is not enough precision with which to define the pulse train interval and generate a perfectly rectangular box, nor to create a box at an arbitrary part of the image. Although the last problem can be solved by using an 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 quickly reprogrammed, because it uses a serial RS232 interface that is limited by the narrow 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 ap-
appropriate waveform synchronized with FluoView “scan active” signal. We have used a PCI-6052E data acquisition board from National Instruments (Austin, TX). By programming appropriate waveforms as commands for the electro-optical modulator it is possible to create any arbitrary distribution of light intensities at the image. Custom-written software (“advanced version”) allows the user to define a set of arbitrarily placed “boxes” of increased light intensity on the sample for space-selective excitation (Fig. 5B): “raster mode” of selective excitation.

3.7. Second harmonic generation 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] 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 biological researchers to probe a cell’s transmembrane potential [10] and thus 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 simple terms, as the partial conversion of 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

Fig. 6. Second harmonic imaging of C. elegans. Two live nematodes imaged with SHG (blue) and two-photon excited fluorescence (green). The SHG signal is believed to arise from the muscle (blue) and the two-photon fluorescence (green) from the autofluorescence from endogenous granules in the intestines of the worm as well as from the expression of a mec4:YFP construct. A central green spot in the upper nematode is emitted from a touch-sensitive neuron expressing the mec4 protein. Note how, although both SHG and two-photon fluorescence signals arise from the same location and at the same time during laser scanning, they do not overlap and show different structures. For example, YFP does not produce a SHG signal. Worms were paralyzed by levamisole prior to taking the image. Bar = 20 μm.
condenser lens, which has to be of equal or greater numerical aperture (NA) than the objective lens NA to collect the whole cone of light. This is important since the SHG radiation in the forward direction (toward the condenser) 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 oil for NA values less than 1). A PMT (Hamamatsu HC125-05) with the appropriate (blue) filter replaced the diffuser and fiber bundle in the auxiliary port (see schematic Figs. 1(B) and 2). This port was originally designed for DIC imaging in transmission mode in the FluoView confocal scanner. As part of the original microscope, a lever-operated mirror allows the user to choose to engage either the path for bright-field illumination (with the incandescent lamp) for viewing the sample through the eyepiece or the PMT path for SHG. When engaged for SHG, the incandescent lamp should be switched off or turned down not to saturate the PMT that is placed just beyond the mirror.

The SHG filter, in line with the PMT, is chosen according to half the operating wavelength. For instance, an operating wavelength of 840 nm would require an interference filter centered at 420 nm with a band of 20 nm. One needs to ensure that the filter fully blocks IR wavelengths. The narrower the bandwidth of the 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 that 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. Fig. 6 is an example of such a composite image, and shows two live Caenorhabditis elegans nematodes expressing a yellow fluorescent protein (YFP)-tagged mec4 protein (mec4::YFP). The laser power needed to show the intrinsic SHG signal was quite high compared with the power used with bright fluorescent dyes—270 mW before the scanning was quite high compared with the power used with the laser power needed to show the intrinsic SHG signal.

4. Summary

In this work, we provide a substantial update to our original modification of the Olympus FluoView system for two-photon microscopy [9]. The major improvements are spatial filtering and refocusing of the incident laser beam, direct control of the galvanometers via custom software enabling imaging or photostimulation of any arbitrary number of ROIs, and, finally, implementation of SHG imaging. The system we describe is flexible and can be easily used for a variety of experiments. Importantly, this system does not require a large expenditure beyond that associated with the FluoView and the laser system.

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