Design and building of modern two-photon laser scanning microscope for using in neuroscience

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Abstract. One of the main visualization method in neuroscience is two-photon microscopy. However, scientists need to upgrade their microscopy system so regular because they are interested to get more specific data. Also this is due to the fact that this technique allows to obtain 3D images of tissues due to laser focus change, that is possible due to substantially greater penetration depth on the main wavelength into biological tissues. Self-developed microscopy system allow to modify the construction of microscope in not-complicated manner depending on specialized experimental models and scientific tasks. This work is small attempt to help researchers who are interested to build their own two-photon laser scanning microscope for using in neuroscience. The work was performed with financial support of the government represented by the Ministry of Education and Science of the Russian Federation, the unique identifier of the project is RFMEFI57814X0079, the agreement on granting a subsidy №14.578.21.0079 dated 28.11.2014.

1 Introduction

Application of modern techniques offer high resolution properties for living systems research is significant key for neurobiology progress. there are certain peculiarities arising from the specifics of experiments using this technique. In one case, for example, the analysis of the morphological cell data of hippocampal slices of the brain of rodents, other experiments *in vivo* depending on the design using the full-sphere of the virtual reality or, for example, in the form of a cylindrical hover platform. Working with selected methods require both of high-technology equipment availability and knowledge and skills to applying non-trivial mathematical algorithms for control two-photon microscopy system [1] and for well-formed data analysis [2], [3]. Two-photon microscopy offers several advantages compared with other forms of microscopy. Creation of two-photon system taking into account its specific use in neuroscience is probably the best solution. This article may be regarded as a quick reference to laboratory staff who are wishing to develop their own microscopy system for self-service and modernization of the system and in order to save the lab budget.

2 Optical part

Suggested type of microscope is module-based (Figure 1), where main module of it is body represented as metal base to which other modules are mounted. It could be

stereotaxic part to fix the animals (chamber in case of in vitro experiments), scanners, shifting platform with fixed objectives nosepiece, optical elements using for work of differential interference contrast (DIC) [4] and multi-photon mode. Technically, the operation of DIC is provided with installation of a visible light source and an IR filter, an iris-shutter, a diffuser, a mirror to reflect the beam in the vertical direction and lens. After passing through the lower block and being reflected the light beam reaches the polarizer, and then passes onto a condenser with Nomarski prism. Then a light beam achieve the sample, which is fixed on the chamber. Passing through the sample, the beam achieve the optical components on a shifting platform (fixed to the frame of the microscope), and passes the second Nomarski prism and the analyzer. Here the interference of previously separated rays is performed. After passing the mentioned optical path the light beam falls onto the so-called illuminator of reflected light, that does not contain any filters or mirrors in the DIC mode operation, and then the beam passes onto IR camera that displays the image on the monitor. Galvo [5] and resonant [6] scanners are presented on Figure 1. The first one may be used for rectangular area (line-by-line) scanning, line scanning or point scanning and for uncaging some drug (e.g. glutamate, GABA), the second one – for fast frame rectangular area scanning. Maximal speed for galvo scanner is up to 2 fps (for 512x512 pixels frame), and for resonant scanner is up to 30 fps (for 512x512 pixels frame). IR Ti:Sa femtosecond pulsed lasers (tuning

wavelength range is 690–1080 nm) are using for fluorophore activation. Non-descanned detectors (NDDs) are using for detecting of fluorescence. Each NDD based on pair of bandpass filter to extract dye absorption wavelength and photomultiplier to detect fluorescent signal. Photodetectors are installed in close proximity to the aperture of lenses, besides, the fluorescence beam doesn't pass the way back through the scanning module, but directly gets onto the detector. The use of several dyes simultaneously is often the necessary requirement in multiphoton microscopy. Therefore the design of the microscope detector allows to make fast change of emission filters and dichroic mirror. The standard cube for filters mounted on the slider like "swalow-tail" that is easily inserted and taken out from the detector is used for this purpose and significantly facilitates the work of the researcher. The multiphoton detector must also be equipped with means of electronic protection and a shutter preventing the damage of the photo multiplier and increase in its performance term. Modern microscopy systems allow to mount up to 4–6 detectors depending on researcher goals.
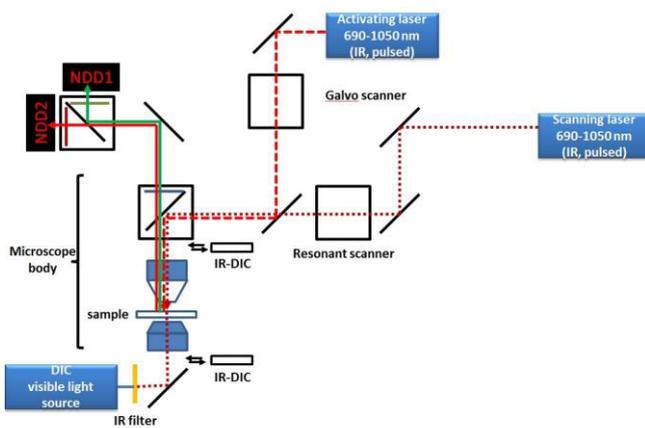

Figure 1. Optical part of two-photon microscopy system including galvo and resonant scanners.

To improve the quality of the resulting images it is necessary to use not only good program support but also to employ the advantages of the technical (physical) characteristics of the microscope, on the one hand, helping to move the lens to the sample as quickly and often as possible, and on the other hand, providing the most accurate movement when scanning a sample. The design must take into account all these features. In the first case (rapid movement to the sample) it is advisable to use a mechanical movement using a larger number of gear, and in the second case - the minimal number of the gear and electronic movement.

3 Design

Researchers should develop and design high-technology platform for realisation module-based microscope (the three-dimensional model of future microscope could be developed using software, e.g. Compass). Microscope consists of high-technology alloy allowed both to follow

requirements of structure rigidity and minimal mass of microscope. All the components and elements of microscope should be developed in such a way as to have possibilities for receiving all the performance goals on the one hand and provide to evolution and addition techniques which could be used in that unique microscopy system on the other. For example if researchers are planning to have an in vivo experiments than working space (Figure 2) and, thus, the body of microscope should be higher than systems without in vivo approaches. Moving platform located above DIC elements and working space is using for mounting of objective nosepiece, filter cube(s) and NDDs. The electric drive control system for practicing a given angle or motion uses feedback sensors providing data concerning the angle or position of the output shaft of the operating engine. If the actuator motor employs a synchronous stepping motor, it is possible to dispense with the feedback sensor (DR) and to simplify the engine control system (CS).
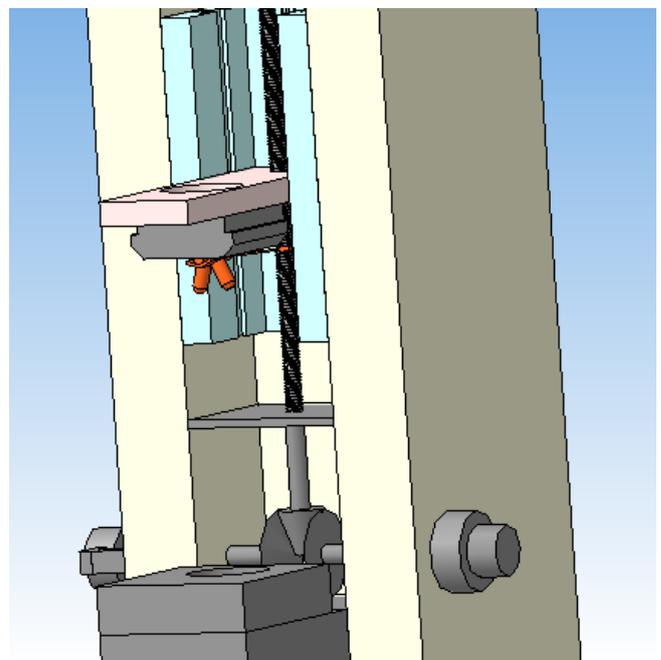

Figure 2. Working space of the microscope model developed using Kompas software.

Moving platform should be constructed as handy element in the context of ergonomics and functional. Moreover, developer should turn attention to minimal approach increment (stepper motor with at list 500 steps per rev should be used) of moving platform on the one hand, and to high-speed displacement and no slop (free motion) on the other. Other important aspect is accurate positioning of chamber for brain tissue (or alive animals) as well as micromanipulators for using electrophysiological and other (e.g., bolus loading) methods. U-shaped moving table can be adapted for specified type of microscope. It is possible to to set up this table independently from the microscope. For maintenance purposes it is possible to remove this table without disassembling the microscope. Motorized axes could be controlled by independent controller (control

box and joystick or keyboard) or PC using especial software which could be integrated in mail control software for microscopy system.

4 Control

To improve the quality of the resulting images it is necessary to use not only advantages of the technical (physical) characteristics of the microscope, but also good program support. Control of system is realized either exactly by using digital-to-analog (DAC) and analog-to-digital (ADC) converter(s) (e.g. National Instruments boards and others) or by using control element of microscope components (e.g. USB, COM connection). However development of cross functional and convenient software to control the system is very important object for researchers. For achieving these goals developers could use commercially available software (ScanImage, Micro-Manager, YouScope or others) or create their own software using Matlab, LabView, Python or others. In case of software development researcher could create few sub-programs for each of elements controlled by software (e.g. manipulators, focus, lasers, scanners, acousto-optic modulator (AOM), detectors and others). After this step sub-programs could be combine to one main program which will control all of them and synchronize their integrated work. Having practical experience researchers could develop design of their software more friendly for end user than commercially available software because of their sector-specific goals. Software also should implement algorithms to scan region(s) of interest. These algorithms will depend on scanner type. Galvo scanner could be used for rectangular area (line-by-line, few options are presented in Figure 3) scanning, line scanning or point scanning and for uncaging some caged drug (e.g. glutamate, GABA) in sequence of points. At the same time resonant scanner could be used for fast frame rectangular area scanning in relation to feature of resonant scanner construction.
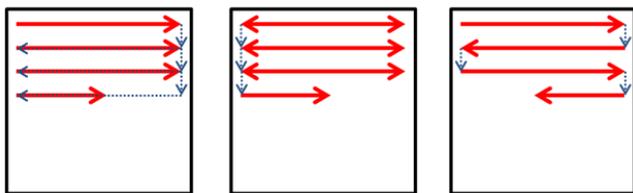
Figure 3. Few options of laser beam movement in case of rectangular area (line-by-line) scanning using by galvo scanner.

Working cooperatively, these scanners could fast scan rectangular area (resonant scanner) and activate caged drugs (galvo scanner) using different wavelength from different IR Ti:Sa femtosecond pulsed laser sources. Important aspect during any type of scanning is synchronizing between movement of laser beam by scanner and input signal from detector(s). Input signal received in one moment of time is detected fluorescent signal from the point where was directed laser beam by scanner at the same moment of time. Thus, software could construct fluorescent signal frames and simply

analyse them (overlapping to each other, scale bar, construct 3D images and others). Moreover, researchers could realize more complicated data analysis allow represent data correctly. Additionally all the experimental data (include parameters of hardware such as laser intensity and wavelength, photomultipliers sensitivity and others) could be saved in especial file which could be opened both in that software to repeat experimental parameters and specially developed software to represent data and analyze it (e.g. using Matlab and others).

5 Conclusions

As a result of development of the own two-photon system taking into account all special characteristics allows to lower the expenses and to increase the quality of methods helping to improve the quality of the obtained data, enables to conduct researches on live samples with the minimum damage of fabric, makes possible to carry out researches of the data of morphological slices of mice or rats hippocampus, as well as researches on live samples, that is carrying out experiments in vivo. Due to the optimal location of photomultipliers the fluorescence from the activated objects is caught optimally, that allows qualitatively to visualize the object, and does not result in an overheat and burning down of the sample. Comparative characteristics of the self-building microscope and commercially available microscopes (Nikon AIR MP, Olympus FV1000 MPE, Carl Zeiss LSM 510 NLO) are presented in the Table 1.

Table 1. Comparative characteristics of the self-building microscope and commercially available microscopes.

Parameter	Self-building microscope	Nikon (AIR MP)	Olympus (FV 1000 MPE)	Carl Zeiss (LSM 510 NLO)
Scanning velocity	Seconds (512x512 pixels)	Seconds (512x512 pixels)	Seconds (256x256 pixels)	Seconds (256x256 pixels)
Flexibility of construction (availability *in vivo* experiments)	Yes	No	No	No
Resolution (depends on objective), µm	0.4–0.8	0.4–0.8	0.4–0.8	0.4–0.8
Light source	Ti:Sa IR femtosecond pulsed laser	Ti:Sa IR femtosecond pulsed laser	Ti:Sa IR femtosecond pulsed laser	Ti:Sa IR femtosecond pulsed laser
Detector	Photomultiplier	Photomultiplier	Photomultiplier	Photomultiplier

Detecting wavelength renge, nm	300–920	400–750	400–750	400–750

Acknowledgments

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