

Development of three photon large field of view microscope for mouse brain imaging

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ABSTRACT

A large field of view (LFOV) microscope is a type of multi-photon microscope that is capable of imaging a greater number of neurons at the same time than a regular multi-photon microscope. This microscope consists of many different components, and this paper is focused on power control. The LFOV microscope is mostly used to image brains of mice; the mice are prepared for imaging using craniotomy, a type of surgery which involves replacing a section of the skull with a glass window to allow deeper imaging.

INTRODUCTION

One of the main objectives of microscope development is to find and expand the fundamental physical limits of imaging. It is a perpetual search for new ways to image deeper, clearer, wider, and faster. This goal has lead to the development of multi-photon microscopes, which are a type of fluorescence microscopes.

A fluorescence microscope focuses laser light onto a fluorescent-dye treated sample, which excites the dye molecules and then causes them to emit photons.^{2,5} These photons then advance through the microscope to the photomultiplier-detector.^{2,5} To form an image, there are two scanners inside the microscope which aim the laser light across the x and y directions, a process called raster scanning [Fig. 1].^{2,5} To obtain a 3D image, one can raster scan multiple layers at different

depths and then assemble these layers together using software.

In the resulting image, light areas represent parts of the sample that were treated with dye, like cell nuclei for instance, while dark areas represent the space in between. The higher the contrast of the image, the better it's possible see the desired structures and therefore the better the quality.^{2,4}

There are several types of fluorescent microscopy which are useful for different purposes. In traditional single-photon microscopy, the electrons in the dye are excited using one photon whose energy is equal to the energy gap between the ground and excited states [Fig. 2(A)].^{2,5} In multi-photon microscopy, the electrons are excited using the combined energies of multiple photons that arrive at the molecule almost simultaneously, within about 0.5 femtoseconds.² In this case, the energy of the photon is equal to one-half [Fig. 2(B)] or even one-third [Fig. 2(C)] of

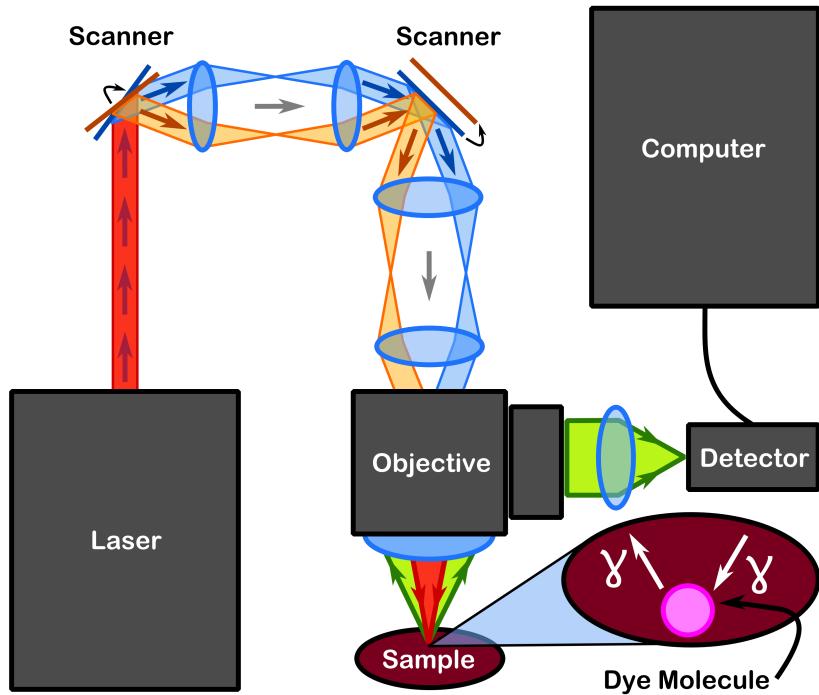


Figure 1: Schematic diagram of a typical fluorescence microscope. Large arrows represent the direction in which the light travels. Light (red) originates from the laser and travels through two scanners, which rotate along perpendicular axes. From there, the light advances into the objective, where it's focused onto the sample. The sample is pre-treated with fluorescence dye (type of dye), and if we “zoom” into the sample, we'll observe photon excitation. The emitted photons (green) travel through the objective to the detector, which consists of two photomultiplier tubes. The photomultiplier tubes convert light into electric current, which travels into the computer through the wire. Computer software called ScanImage converts the electric current into a tif image.

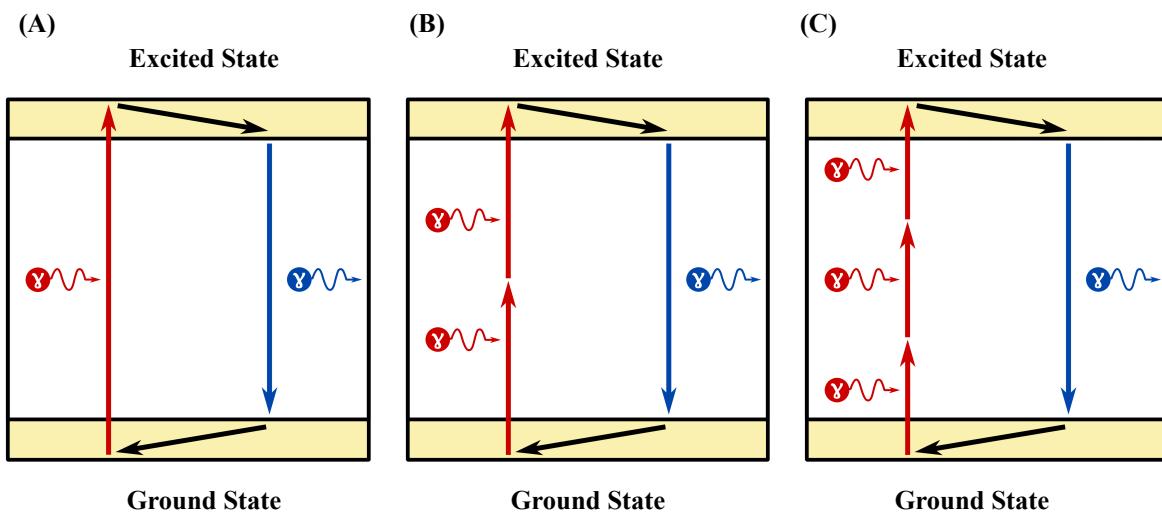


Figure 2: (A) One-photon absorption. (B) Two-photon absorption. (C) Three-photon absorption.

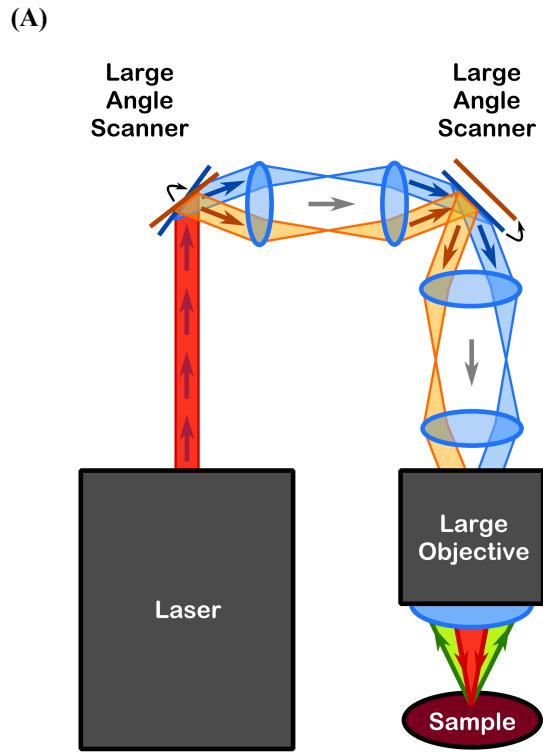
the energy gap.^{2,5}

The lower the energy of a photon, the longer its wavelength, and so the kind of microscope that one uses boils down to what wavelength would work best for what we want to achieve. Specifically, it depends on the depth that we want to image. This is because as one images deeper into a sample, there will inevitably be attenuation, or power loss, due to absorption and scattering.⁴ It so happens that there is less attenuation when we use longer wavelength, specifically near-infrared light.⁴ This is why two photon microscopy allows us to image deeper than single-photon microscopy, and three-photon microscopy allows us to image even deeper than that.⁴ Unfortunately one trade-off is that lower energy light results in much dimmer images, so single-photon microscopy is still the best method to use for shallower regions.^{2,4}

LARGE FIELD OF VIEW

Depth isn't the only limit that researchers want to stretch. The field of view, or the "area" that could be imaged at once is also very important. In particular, this matters a lot for imaging the brain. This is because with a larger field of view it's possible to capture more neurons simultaneously. Seeing more neurons at once allows us to observe biological processes occur over time. To get an image of the same dimensions using a microscope with a smaller field of view, we would have to image several volumes and combine them together. Because these volumes would have to be imaged at different times, one after another, the resulting image would not show us how our sample changes with time.

For this reason, a large field of view (LFOV) microscope has been built. This



(B)

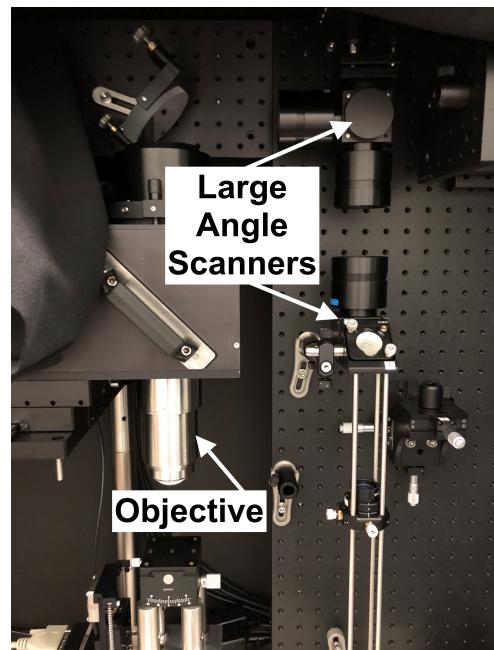
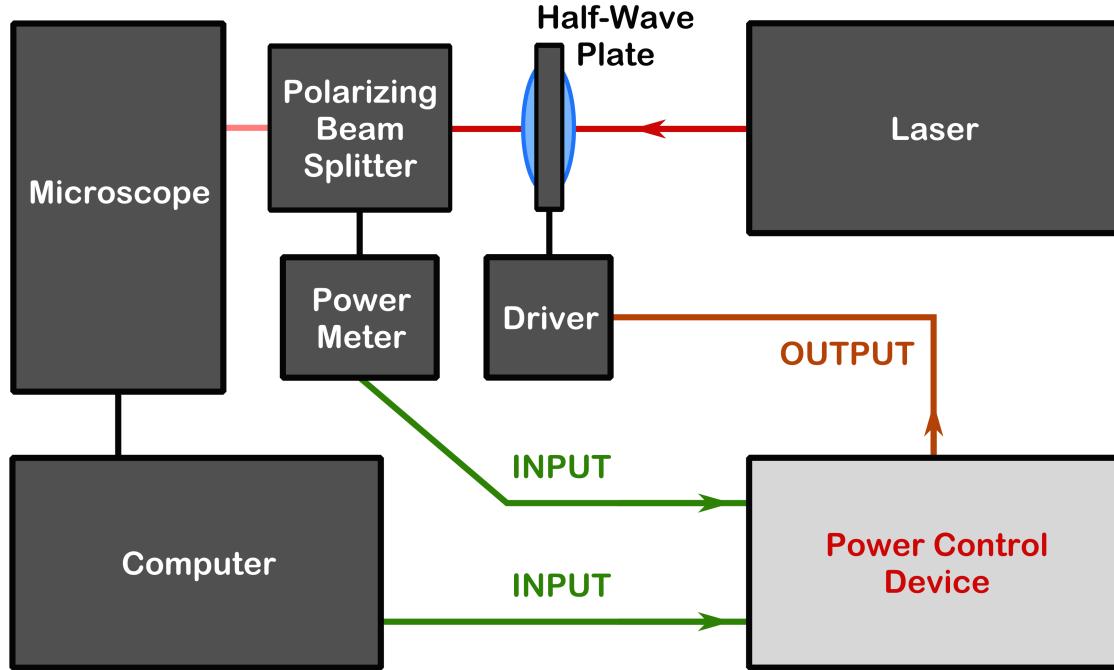


Figure 3: (A) Schematic diagram of the LFOV microscope, which highlights the large angle scanners and the large objective lens. (B) A photograph of the actual LFOV microscope which labels these components.

(A)



(B)

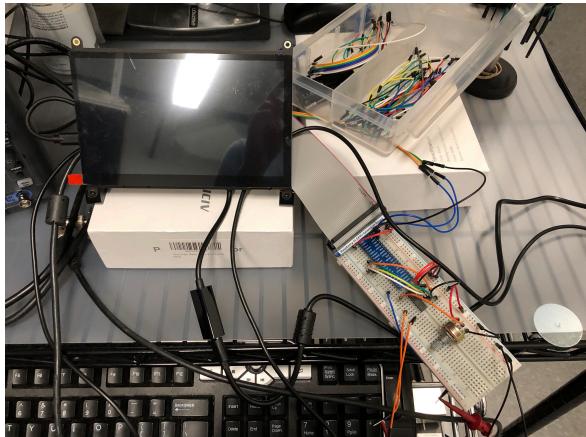


Figure 4: (A) Schematic diagram of LFOV microscope with the power control device. Laser light travels from the source to a half-wave plate, which is rotated by a driver to change the polarization of the light. Next, the light advances into the polarizing beam splitter which, as the name suggests, splits the beam into components, only allowing one to pass on. The power of the exiting beam depends on how much the half-wave plate changed the polarization of the light, which is determined by how much the driver rotated the half-wave plate. To adjust the power to a desired value, a power control device can be used. This device has two inputs - the desired value from the computer and the current value from the power meter, which is attached to the beam splitter - and one output into the driver. The power control device works by telling the driver to turn the half-wave plate while comparing the current power to the desired power until the two values are equal. (B) A photograph of the power control device, which consists of Raspberry Pi attached to the back of a touch screen, a keyboard, a mouse, and a breadboard.

microscope can image an area of $3000 \mu\text{m}^2$ whereas a standard one can cover an area of $600 \mu\text{m}^2$. A LFOV microscope is distinguishable by its large objective lens and large angle scanner.¹ These features allow the light to scan through a larger angle during a raster scan, which is how this microscope can image such a large region.¹

POWER CONTROL

A large device like the LFOV microscope has many different components that control its various aspects. One such aspect is power control. To image a sample at different depths, one would need to adjust the power of the laser - as one goes deeper, the laser power needs to increase exponentially.^{2,4} Before, one would have to manually adjust the laser power for each depth until the desired power for that depth is reached, which was not efficient. This problem was solved by building a power control device that would do this automatically and make imaging faster and easier.

The setup is similar to one in a previous paper.³ To be brief, laser power is adjusted using three devices: a half-wave plate, a driver, and a polarizing beam splitter. A half-wave plate is an optical component that can change the polarization of light when rotated by the driver. The beam splitter controls the power by selecting one polarization state. The power is measured by a power meter.

The power control device works by taking in two inputs: desired power from the computer and current power from the power meter. Using this information, it sends a signal to the driver telling it to rotate the half-wave plate until the current power is the same as the desired power.

(A)



(B)

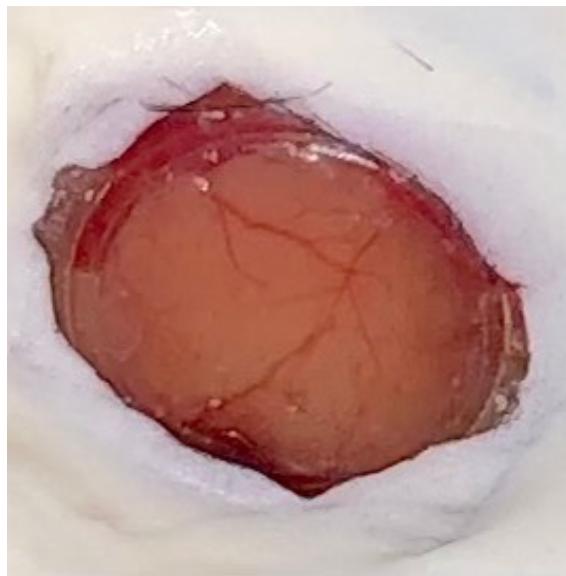


Figure 5: (A) Photograph of a mouse that had craniotomy performed on it. This same mouse was used for three-photon structural imaging. (B) Two-photon excitation (C) Enlarged photograph of the glass window, through which the brain and blood vessels are visible.

CRANIOTOMY

For LFOV microscope imaging we use mice because the mouse is a common animal to use in a lab setting. To prepare mice for imaging we perform craniotomy, a kind of surgery that involves removing a part of a mouse's skull and replacing it with a glass window [Fig. 5A]. That way, the light doesn't have to go through the opaque skull and it's possible to image deeper. A more detailed step-by-step description of the procedure was outlined in a previous paper.³

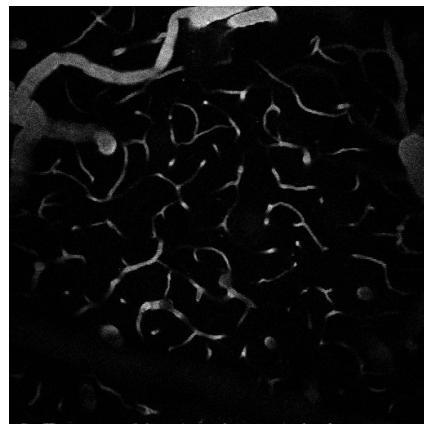
Good images require good surgery, one after which the mouse can recover without complications or brain damage, and where the glass window is clean. A clean glass window is one where the brain and the blood vessels are clearly visible without blood spillage under the glass [Fig. 5B]. The quality of the surgery is assessed based on how deep the mouse can be imaged: the deeper, the better.

Also, if the surgery is done well, the same mouse could continue to be used for imaging for at least a month post-surgery without degradation in image quality due to healing; if the surgery isn't done properly, the glass window could get blocked or displaced during the healing process instead of permanently adhering to the mouse in the desired position. After a craniotomy the implant should merge with the mouse's skull and the animal should continue living a normal life.

EXPERIMENT

To demonstrate the effectiveness of craniotomy, we conducted an experiment which demonstrates an example of what kind of images a good craniotomy could yield immediately following the procedure. The imaging was not performed on a LFOV

(A)



(B)



(C)

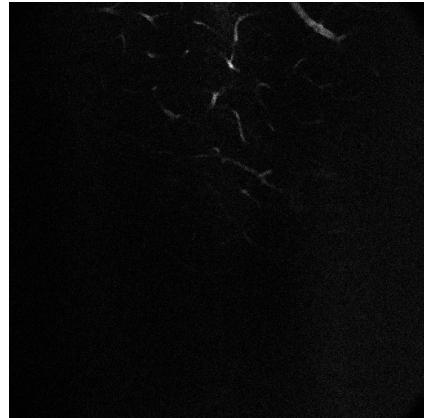


Figure 6: (A) Single frame from a stack imaged at a depth of 0 μm , which is the surface of the brain. (B) Single frame from a stack imaged at a depth of 500 μm from the surface, which was the middle stack. (B) Single frame from the deepest stack, at a depth of 1000 μm .

microscope but rather another three-photon microscope as the purpose of the experiment was not related to field of view. The subject of this experiment was the same mouse as pictured in Figure 5.

To image the blood vessels, fluorescein dye was injected into the mouse's blood stream prior to imaging. A total depth of 0 to 1270 μm was imaged. This paper includes three frames from three different depths; specifically at 0 μm [Fig. 7A], 500 μm [Fig. 7B], and 1000 μm [Fig. 7C] depths.

Depth	Power
0 μm	1.20 mW
100 μm	1.69 mW
200 μm	2.26 mW
300 μm	3.53 mW
400 μm	7.05 mW
500 μm	13.37 mW
600 μm	21.23 mW
700 μm	42.07 mW
800 μm	76.46 mW
900 μm	108.39 mW
1000 μm	108.39 mW

Figure 7: Table that shows the laser power used (in mW) at each depth (in μm).

As stated in the previous section, sample quality is evaluated based on the maximum depth that's possible to image within the sample, where "deep" means approximately 1000 μm and more. Because we were able to obtain images at that depth and beyond, this particular sample is quite good, at least for imaging shortly after

craniotomy. Because this sample was only once, the day after craniotomy, it is unknown whether this kind of imaging quality would hold up in the long term. This is something that can be investigated in future experiments.

The brightness is similar in all three images despite the fact that they were taken at three different depths. This is because as imaging depth was increased, the power of the microscope was also increased [Fig. 7]. Changing the power allows us to maintain consistency in image brightness, which emphasizes the importance of power control.^{2,4}

CONCLUSION

This paper gave a brief overview of the large field of view (LFOV) three-photon microscope and how it works, some of its main components, and the kind of experiments that it could be used for. It is a relatively new, innovative technology that has a lot of potential, and even though it already works quite well, there is still room for improvement. And so the next step is to look for new ways to further push the limits and break the boundaries of fluorescence microscopy.

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