Towards widefield multiphoton mesoscopy with the Mesolens

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ABSTRACT

We report progress on the development of multiphoton excitation methods for the Mesolens, with the aim of increasing the field of view of multiphoton imaging without stitching and tiling or stochastic sampling methods. We give details on our method of upscaling widefield two-photon microscopy to the mesoscale, where we have imaged specimens exceeding 2.5 mm x 2.5 mm in size at a frame speed of 0.2 Hz using a Ti:Sapphire laser for fluorescence excitation and a sensor-shifting camera for fluorescence detection and imaging. We give details on the opportunities and limitation of this technology for multiphoton imaging at the mesoscale, and we conclude with details on our progress to develop a new mesoscale imaging technology that is chromatically corrected from 400-1080nm for both single-photon and multiphoton excitation mesoscopy.

Keywords: Mesoscope, Mesolens, multiphoton, fluorescence, optical instrumentation

1. INTRODUCTION

Multiphoton excitation has proven indispensable in the study of both preserved and living organisms, and there is a great drive within the imaging community to extend the field of view of study to enable study of larger specimens. However, multiphoton excitation necessitates the use of an objective lens with a high numerical aperture: given the historical relationship between numerical aperture and magnification, the field of view of a multiphoton microscope is usually limited to less than 0.5 mm in diameter.

We previously reported a method for two-photon excitation of fluorescence without scanning using a narrow parallel beam of ultrashort-pulsed laser light delivered via a widefield microscope [1]. We used a commercial Ti:Sapphire laser with a pulse duration of around 140 fs at a repetition rate of 80 MHz and we coupled this into a widefield microscope that was modified to include a dichroic reflector to direct the near-infrared laser emission to the specimen and transmit the visible wavelength fluorescence. Up to 1 Watt of average power from this near-infrared laser was delivered to the specimen plane. With this setup we performed imaging of a 90 μm diameter field at speeds of up to 100 Hz using a 60x objective lens and a sCMOS camera. Importantly, we observed a considerable reduction in the rate of photobleaching in multiphoton excitation when compared with single-photon excitation.

Although the high speed of imaging proved useful for imaging of cell signalling processes including calcium transients in hippocampal neurones, the 90 μm diameter field of view was capable of imaging only a few cells in a single image dataset. As such, extensive imaging studies were required to achieve statistical significance.

Here we report our progress in widening the field of view of multiphoton excitation to study large cell populations. We have extended our multiphoton excitation method without scanning to allow use of the Mesolens, a giant custom objective with the unusual combination of low magnification and high numerical aperture (4x/0.47). The use of the Mesolens for widefield multiphoton excitation of fluorescence has led to an optical illumination and detection methodology that is considerably different to that reported in previous work.

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2. METHODOLOGY

Insstead of using a low power Ti:Sapphire laser as in our previous work [1] we have used a laser oscillator capable of delivering up to 4.5 W to the specimen plane. This presents the advantage of being able to spread the beam more widely while preserving the high intensity needed for multiphoton excitation of fluorescence.

In addition, instead of delivering a collimated 90 µm laser beam to the specimen through the objective lens, we deliver a much wider (multi-millimetre) diameter laser spot to the specimen.

We also excite fluorescence from the specimen in transmission mode through a Mesolens-specific high numerical aperture condenser lens, rather than via epi-illumination. In the current Mesolens objective there is over 15 cm of glass which causes significant pulse broadening that is intolerable for multiphoton excitation of fluorescence. By contrast, the condenser lens glass [2] is less than 1 cm thick and it has a numerical aperture of 0.5. We note that although the average (and peak) power of the illumination system may seem high, when spread across a multi-millimetre diameter spot, the power per unit area is around two orders of magnitude lower than the beams used in point-scanning two-photon excitation microscopy [1].

Instead of an off-the-shelf objective lens we use the Mesolens for detection of fluorescence. We choose the Mesolens not only because it can image up to a 4.4 mm x 3.0 mm field of view when used with a camera while preserving sub-micron lateral resolution [3], but because it has an optical throughput more than 20x that of a commercial objective with comparable magnification [4]. We therefore use the high collection efficiency of the Mesolens to increase the probability of capturing multiphoton excited fluorescence.

In our latest work we use a totally different photodetector not normally used in microscopy or mesoscopy to capture images. Images were acquired with a thermoelectric Peltier cooled camera (VNP-29MC, Vieworks) with a chip-shifting mechanism. The chip-shifting mechanism is needed to benefit from the large diameter field and high spatial resolution (700 nm lateral) provided by the Mesolens. The camera port on the Mesolens system was adapted to house a focusing lens providing an additional magnification of 2x, bringing the total system magnification to 8x. The camera could be operated without chip-shift at a resolution of 6576×4384 pixels (28.8 Megapixel), with 4x chip-shift at 13152×8768 pixels (115.3 Megapixel) and with 9x chip-shift at 19728×13152 pixels (259.5 Megapixel). For our widefield multiphoton imaging the Mesolens, the chosen mode was always 9x chip-shift. In this mode, the sampling rate was 4.46 px/µm, corresponding to a 224 nm pixel size, satisfying Nyquist sampling [2]. With a frame exposure time of 100 ms the overall time taken to capture an image was 5 seconds. This is due to the large file size of the data (approximately 0.5 GB per image) and the time taken to transfer the data from camera to the computer.

As such, the camera is presently the primary limiting factor in the slow speed of widefield multiphoton mesoscopy with the Mesolens, but new chip-shifting camera technologies are emerging. Cameras that use the sensor-shifting methodology are now available with frame speeds of up to 6.2 Hz with effectively 604 Megapixel images after the shift operation. The pixel size, quantum efficiency, and noise levels of these devices are commensurate with multiphoton mesoscale imaging at high speeds.

3. CONCLUSIONS

Using this wide parallel beam of ultrashort-pulsed laser light for excitation of fluorescence together with the uniquely high optical collection efficiency of the Mesolens, we have performed multiphoton excitation over a field of view exceeding 2.5 mm x 2.5 mm in diameter at a rate of 0.2 Hz, which is limited only by the chip-shifting camera needed to image the field of view of the Mesolens at full resolution.

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