Two-photon Fiber STED Microscope Using Polarization Maintaining Fiber

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Abstract: We demonstrate a two photon (2P) fiber STED microscope in which the excitation and STED light are delivered to the sample in polarization maintaining (PM) fiber.

Two photon (2P) microscopy is popular among biologists because of its ability to achieve optically sectioned images deep in scattering tissue [1]. This makes it particularly well-suited to studying complex organs such as the brain. However, many neural mechanisms of interest can only be studied *in vivo*, and so a goal of the neuroscience community has been to realize flexible microscopy platforms to enable such studies. Recently, two photon microscopes have been combined with fiber delivery systems and miniaturized, head-mounted objectives in order to study the brains of awake and freely moving subjects. These depth and time-resolved measurements provide an unprecedented window into the functioning of the brain [2]. However, these microscopes are limited in resolution, which leaves them unable to resolve many important processes, for example the dynamic reshaping of synapses during the learning process [3]. A solution to this challenge is to combine a 2P fiber system with the super-resolution technique of stimulated emission depletion (STED).

In the standard implementation of STED, a doughnut-shaped depletion beam is overlapped with the excitation beam. The depletion beam quenches fluorescence everywhere but its dark center, resulting in a fluorescing spot smaller than the illuminated area. Performing fiber-coupled STED involves co-propagating the doughnut shaped depletion beam and the excitation beam through fiber. To be practically useful, neither beam should degrade in quality from fiber perturbations. This stipulation presents a challenge: the doughnut-shaped (fully vector) eigenmodes of step-index fiber are nearly degenerate, often resulting in intermodal coupling from fiber bending and a variable output shape [4]. Furthermore, even perturbative bending of step-index fiber changes the polarization at the output, which degrades the quality of the doughnut beam shape at high NA and renders it unfeasible to use for a fiber-STED microscope. Suitably engineered custom fiber has been used with promising results but involves expensive manufacturing [YKR19]. We recently pioneered another approach, which leverages the higher order modes of polarization maintaining (PM) fiber to support the depletion beam, and results in bend-insensitive operation [6]. Here, we demonstrate this method with 2P excitation.

The 2P excitation source for our fiber STED microscope is a homebuilt Ti:Sapph laser oscillator with a rep rate of 81 MHz, mode locked at a wavelength of 910 nm. The excitation beam passes through a grating stretcher in order to compensate for dispersion in the fiber and microscope optics. Using a dichroic mirror, we couple the beam in to 1 m of PM fiber with a core diameter of 4.5 μm. A photodiode monitoring the Ti:Sapph pulse train triggers the STED laser (NKT Katana HP-06) so that the two lasers are synced. The STED beam (wavelength of 592 nm) is shaped into a Hermite-Gaussian (HG) beam with an SLM in order to couple to the higher order modes of the fiber. It passes through a half wave plate, and a polarizing cube beam splitter breaks the beam into two arms of a Mach-Zehnder interferometer. In one arm, a dove prism rotates the beam shape by 90^{o} so that the combined HG beams create the doughnut shape, as shown in Fig. 1. This also provides a delay so that at the end of the interferometer, the two halves of the doughnut no longer interfere. The composite STED beam is combined with the 2P excitation light using a dichroic mirror and then carefully coupled into the PM fiber: the STED doughnut is coupled into the higher-order fiber modes, while the 2P excitation light is coupled into the fundamental mode. An apochromatic 20x objective collimates the excitation and depletion beams at the output of the fiber. The beams then impinge on a MEMS mirror (Mirrorcle A7B2.1-3600AL-TINY20.4-A/TP) that steers them through a scan and tube lens. We use a 100x 1.4 NA oil immersion objective for imaging. The fluorescence is de-scanned through the system and split from the beam path using a low-pass dichroic mirror. It is coupled to a multimode fiber and detected with a PMT (Hamamatsu H7422P-40). The voltage signal from the PMT is discretized using a photon counting unit (Hamamatsu C9744). The microscope is controlled using a customized program written in MATLAB.

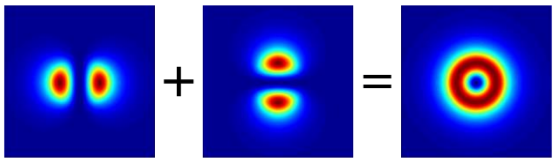

Fig. 1. Simulated intensity distributions of the two higher-order modes of PM fiber that are combined to create the doughnut-shaped depletion beam. Prior to being coupled into fiber, the depletion beam is split into two arms of a Mach-Zehnder interferometer. The path length difference between the two arms is much greater than the coherence length of the STED laser, so there is no interference between the two modes during fiber propagation. This makes our fiber STED microscope robust against fiber bending [6].

Images from our microscope are presented in Fig. 2. The imaged specimen is a 500 μm coronal slice of the brain of a Plp1-eGFP mouse, where the myelin is labeled with eGFP [7]. While there is clearly an improvement in the sharpness of the image when acquired with STED (Fig. 2 b), a more quantitative measure is made by taking column averaged line cuts (Fig. 2 c and d). To extract a column averaged line cut, a rectangular region of interest is found (shown in Fig. 2 a and b) and pixels spanning the short distance of the rectangle are averaged as columns. These line cuts confirm that features in our sample are resolved more clearly when using STED. A 180 nm structure is shown in Fig. 2c, which is roughly half of its apparent size without using STED. However, it is not possible to extract the resolution of the system from these images alone, as the sizes of the features themselves are not known.

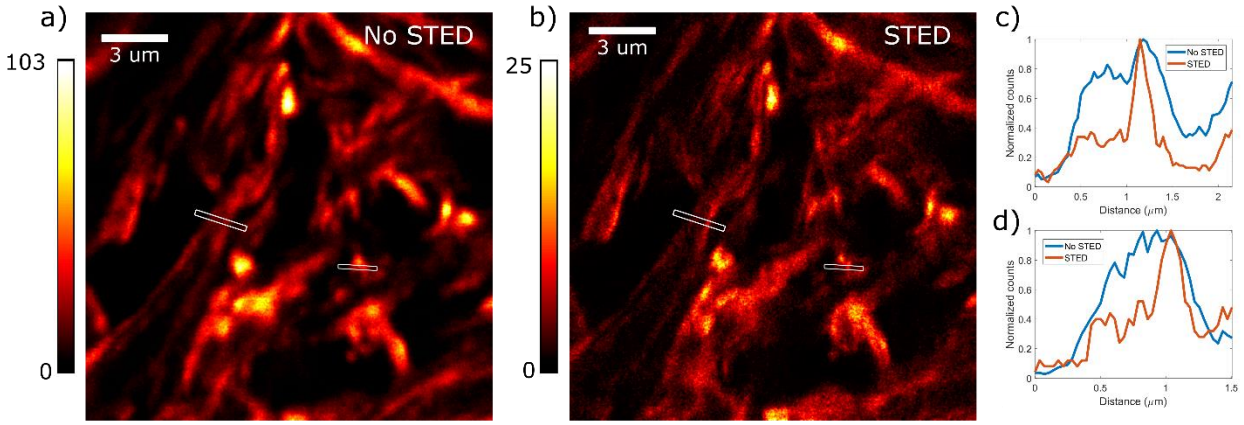

Fig. 2. Images of a Plp1-eGFP mouse brain with eGFP labeled myelin [7] taken on our 2P fiber microscope a) without STED light and b) with 40 mW of STED light, measured before the objective. Images have a pixel size of 35 nm and the dwell time for each pixel is 50 microseconds. A Gaussian blur filter with a sub-pixel waist of 25 nm is applied to both images to smooth pixel shot noise. The STED image appears sharper, with features that are not evident in a). As a more quantitative measure, column averaged line cuts are taken at two locations, with the leftmost presented in c) and the rightmost in d). Both the STED and no STED have been normalized by their largest count to facilitate comparison. A description of the column averaged line cuts is available in the text.

These results represent the first demonstration of a 2P fiber STED system in which both the excitation and STED light are delivered by PM fiber. Our microscope design provides the advantage of being robust against fiber perturbation, as shown previously for single-photon STED [6]. We expect our microscope will be useful for studying dynamic structures in the brains of awake behaving animals.

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