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Estimating the Point-Spread-Function using 1 μm diameter microspheres for image restoration in biomedical multiphoton microscopy

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ABSTRACT

Multiphoton microscopy (MPM) is an advanced imaging technique used in biological and biomedical research to visualize and study living tissues and cells with high resolution. It is particularly well-suited for deep tissue imaging, as it minimizes photodamage and provides improved penetration compared to traditional microscopy techniques like confocal microscopy. The Point Spread Function (PSF) plays a crucial role in multiphoton microscopy and describes how a point source of light is imaged as a spatial distribution in the microscope. Understanding the 3D PSF is essential for deconvolution and other post-processing techniques used to reconstruct 3D images from a stack of 2D images. The imaging of fluorescent beads used as point sources is a solution to evaluate the PSF. Usually, this strategy involves a bead of dimensions smaller than resolutions, typically with diameter of about less than 200 nm. However, in this setting, it is often complex to detect correctly the beads and thus to estimate accurately the PSF. We develop a computational solution for the PSF estimation based on the imaging of micro objects bigger than the resolution limit. We use fluorescent microspheres with a diameter of 1 μm and estimate the PSF from the deformations observed in the image of these microspheres. A deconvolution strategy illustrates the performance of our method, where we successfully restore an unsliced whole striated skeletal muscle utilizing the PSF estimated with 1 μm diameter beads.

Keywords: Multiphoton microscopy, PSF estimation, 1 μm bead diameter, in situ characterization, image restoration

1. INTRODUCTION

Multiphoton microscopy is an advanced imaging technique used in biomedical sciences to visualize structures within living tissues [1]. The principle of multiphoton excitation is based on nonlinear optics, where two or more lower-energy photons interact simultaneously. The spatial distribution of light intensity resulting from the excitation of a point source within the sample is described by the Point-Spread-Function. The precise knowledge of the PSF in situ is crucial for characterizing the resolution and imaging capabilities of a microscope system [2]. Moreover, the mathematical estimation of the PSF allows to apply a computational process for image restoration while considering a PSF

representing the local distortions of the optical field [3]. A well-characterized PSF is essential for precise depth profiling and for determining the focal volume of the microscope. Indeed, the PSF estimation is dependent on the spatial localization of the recorded image. For example, the PSF blurring highly increases in the optical axis with the imaging depth but not in the optical plan [4]. The current strategy for the estimation of PSF consists in the creation of a point source produced by fluorescent beads with dimensions smaller than the resolution and which can emit light at a single point. Usually, microspheres with sub-resolution diameter of less than 200 nm are often chosen [5-7]. However, their detection is not really easy. The risk of their destruction is high with the high peak power of the laser excitation and the distinction between a single bead or a pair of taped beads is not evident. This can induce a miss interpretation and thus a false estimation of the PSF. This recording procedure requires an initial step for the selection of single microspheres. In the present work, we propose an alternative strategy for the in-situ estimation of the PSF of a microscope pipeline. The originality of this strategy rests on the use of microspheres which are about five times larger than the optical resolution of the microscope. The PSF is mathematically detected thanks to the evaluation of the distortion of the image of this relatively large and standardized object. The experimentations were tested with entire mouse muscle. The restoration procedure illustrates the robustness of the strategy.

2. EXPERIMENTAL SETUP AND COMPUTATIONAL STRATEGY

The new approach proposed in the current manuscript results in an end-to-end pipeline devoted to the 3D image restoration of living samples. This strategy is divided into three parts: the experimental set-up, the preparation of samples including 1 μm beads and the mathematical strategy for estimating PSFs and image processing.

2.1 Microscope device

The images recorded for this work were obtained from the commercial multiphoton microscope BX61WI from Olympus. A water immersion objective from Olympus (XLPLN25XWMP, 25 \times , NA 1.05) corrected for multiphoton acquisitions focalized the beam on the sample. The femtosecond infrared laser pulses were emitted by a tunable titanium-doped sapphire laser excitation (Ti: Sa) from Coherent. The central wavelength was fixed at 810 nm. The detection channels were composed by two photomultiplier tubes. The first one recorded the second harmonic generation (SHG) signal emitted from the myosin assembly of muscle. The second one recorded the fluorescence emission of microspheres with 1 μm of diameter. The recordings were built from a superposition of 2D stacks with a pixel dimension of 49 nm in the optical plan (XY) with a stack spacing of 100 nm between two distinct slices in the depth (YZ).

2.2 Sample preparation

By integrating the microspheres with 1 μm of diameter into the sample, we aimed to establish a reliable reference image for analyzing the spread of light in microscopy images. This approach provided a controlled and standardized method for experimentally determining the PSF characteristics of the microscope used in imaging the muscle sample. Three-month-old male FVB mice were used to isolate Extensor Digitorum Longus (EDL) muscles, which were dissected from tendon to tendon. This procedure ensured the extraction of intact EDL muscles from the mice for further experimentation or analysis. For the purpose of estimating the PSF, a water solution containing polystyrene microspheres with a diameter of 1 μm was used as a reference. These yellow-green fluorescent microspheres from Molecular Probes™ (FluoSpheres™, 1 μm , yellow-green fluorescent 505/515) were strategically positioned along the perimeter of a mouse muscle sample immersed in the solution.

2.3 Mathematical strategy

The mathematical strategy goes through the formulation and resolution of an original non-convex minimization problem. The detailed mathematical strategy can be found in reference [8] where a proximal alternating method along with convergence guarantees is detailed. The parameters of the chosen heteroscedastic noise model were estimated. A restoration procedure was combining an estimation of the PSF and noise with a constrained optimization problem with a minimal hyper-parameter tuning.

3. VISUAL RESULTS

Figure 1 illustrates the images recorded in the optical plan (XY) before (Figure 1A) and after (figure 1B) the image restoration. The observed signal corresponds to the muscle organization of myosin at the submicrometric scale. The expected shape of myosin is revealed thanks to the detection of the SHG signal.

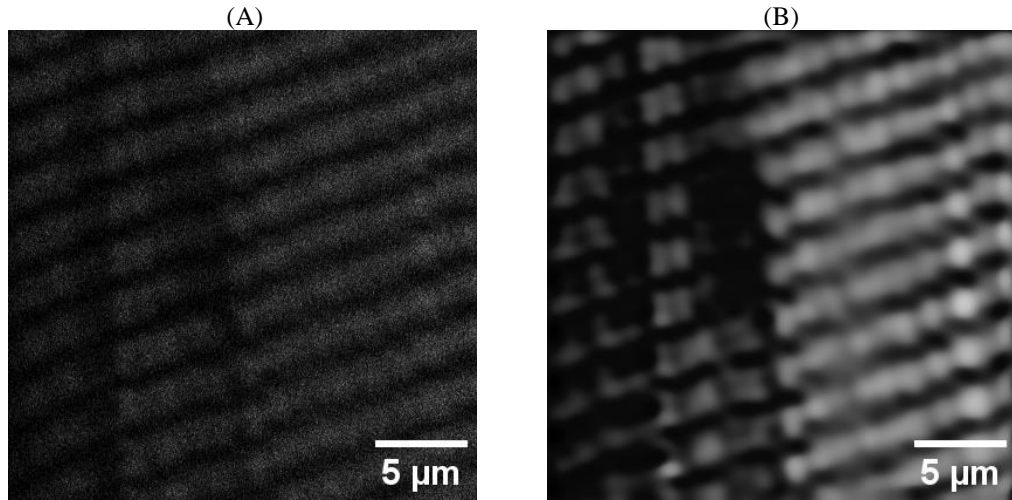


Figure 1. Raw (A) and restored (B) images of the myosin organization of EDL with the estimation of the PSF from 1 μm diameter microspheres.

Figure 2 illustrates the quantification of the optimization of the image quality. The SHG intensity profile is drawn for the line identified in Figure 2A for raw (left) and restored (right) images. Figure 2B gathers the raw and restored intensity profiles for the same regions.

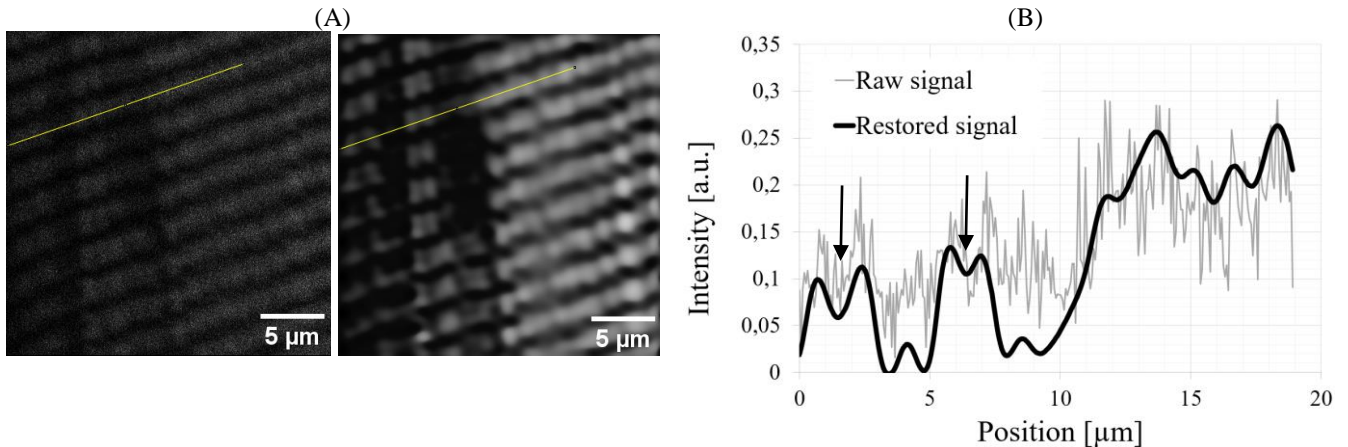


Figure 2. A. identification of the measured intensity profile of SHG. B. Intensity profiles of SHG for the raw and the restored image. The two arrows locate the Z-line which is clearly identified thanks to the image restoration process.

Figure 2B shows the interest of the restoration process which allows to identify the presence of the Z-lines of myosin organization. Two arrows are presenting the identified Z-lines which cannot be clearly seen from the raw data. Thanks to these observations, we can state that the PSF estimation with microspheres having a diameter of 1 μm allows to reconstruct images satisfactorily.

4. CONCLUSION

The estimation of the PSF is a standard procedure in optical microscopy for the calibration of the device and also for the image restoration of living samples. However, the estimation of the PSF is a tricky procedure which requires an adapted biological procedure for the inclusion of microspheres and a mathematical solution considering the noise and blur conditions inherent to multiphoton microscopy. The use of standardized object with dimension larger than the resolution for the image restoration of living samples is demonstrated here. Thanks to this strategy, images of spheres with a diameter of 1 μm are easier with clearly visible contours more easily accessible for a mathematical treatment and estimation of the distortion.

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