A cost-effective approach to Super-resolution Optical Fluctuation (SOFI) microscopy using an industry-grade CMOS camera

Robin Van den Eynde[1,+], Alice Sandmeyer[2,+], Wim Vandenberg[1], Sam Duwé[1], Wolfgang Hübner[2], Peter Dedecker[1], Thomas Huser[2], and Marcel Müller[1,2]

[1]Lab for NanoBiology, Department of Chemistry, KU Leuven, 3001 Leuven, Belgium
[2]Biomolecular Photonics, Faculty of Physics, Bielefeld University, 33501 Bielefeld, Germany
[*]muellerphysics@gmail.com
[+]these authors contributed equally to this work

ABSTRACT

Super-resolution (SR) fluorescence microscopy, especially at high speeds, is typically carried out on high-performance research microscopes. The substantial cost of such equipment, combined with the limited distribution of such instruments in imaging facilities, have complicated access to super-resolution imaging for research groups in the biosciences. In this work we promote the accessibility of Super-resolution Optical Fluctuation Imaging (SOFI) to the scientific community by demonstrating its flexibility in terms of the minimal required performance of the imaging system. We show that SOFI data can be acquired using a very cost-efficient industrial-grade detector on both a standard research microscope and an entirely home-built wide-field system. This enables more scientists to enter the field of live-cell super-resolution imaging, as it provides access to a robust and fast SR imaging modality at comparatively modest cost.

Introduction

The broad availability of specific and quantitative fluorescent labelling makes fluorescence microscopy an important tool for the analysis of structure, activity and interactions within living cells. However, its spatial resolution is fundamentally limited in a conventional imaging system due to diffraction. To overcome this limitation, a range of super-resolution techniques have been developed and optimised over the last two decades[1]. These techniques offer a very high imaging performance, though at the cost of requiring high-end imaging systems. This has limited their utility for scientists who do not have access to extensive imaging facilities. Driven by this observation, the last years have seen the development of several initiatives that aim to ”democratise” microscopy, by aiming for a careful trade-off between performance and cost, instead of maximal system performance. This is made possible by a combination of open-source / open-access software and hardware blueprints, as well as repurposing existing commodity hardware[2–9].

Cameras often pose a substantial portion of a microscope’s cost (a high-end sCMOS camera will cost 10 k€ or more), which leads to a high threshold for purchasing a super-resolution imaging system. High performance and extensive signal processing are the main reasons for this cost: Unlike a conventional industry-grade camera, these systems provide a higher Signal-to-Noise-Ratio (SNR), more sensitivity, an increased acquisition speed and extensive manufacturer-provided calibration enabling the quantitation of photon counts. Nevertheless, recent work has shown that scientific-grade cameras can be replaced by less expensive industrial-grade equipment, while maintaining most of the performance of super-resolution microscopy in suitable samples. For example, an industrial-grade camera is sufficient for super-resolution techniques such as dSTORM and fPALM, which was confirmed by extensive analysis and characterization of the individual components[2,5]. A key reason for this success is the fact that most organic fluorophores are very bright, emitting many photons before photodestruction occurs, and thus reducing the overall sensitivity requirements of the camera.

Localisation microscopy provides a very high spatial resolution but a low temporal resolution, and accordingly it is typically not used for live-cell imaging. It is therefore natural to ask to what extent inexpensive detectors can be combined with live-cell superresolution imaging. Superresolution optical fluctuation imaging (SOFI)[10] is an established super-resolution technique that has been shown to work well in living samples, working not only with organic dyes[11] and quantum dots[10] but also with genetically-encoded fluorescent proteins[12]. The technique combines isotropic increases in spatial resolution with good temporal resolution, rendering it especially useful for live biological samples[12–15].

Similar to other diffraction-unlimited techniques, the key ingredient for SOFI microscopy is the use of dynamic fluorescent labels. SOFI relies on the statistical analysis of multiple fluorescence images acquired from the same sample, labelled with fluorophores that show continuous and independent on-off 'blinking', or transient non-emissive intermittencies. Spatial resolutions that are two- to three-fold improved over diffraction-limited resolutions can be readily achieved using photochromic fluorescent proteins[12,16]. In addition to the conventional visualisation of fluorophore distribution, SOFI has also been used to visualise biosensor activities with spatial resolutions that surpass than diffraction-limited[17] resolutions.

In this work we set out to establish whether live-cell SOFI imaging can be combined with industrial cameras in order to enhance its accessibility. We focus on the use of genetically-encoded fluorescent proteins, whose overall brightness is considerably lower than those of organic dyes. As we will show in the following, we find that such imaging indeed works well and can readily enhance the utility of this versatile imaging technique, both in using a commercial imaging system and a home-built instrument.

Results

We selected the *IDS µEye UI-3060CP-M-GL Rev.2* camera as a cost-effective CMOS-based detector. This camera is based on the *SONY IMX174LLJ-C* image sensor with 5.86 μm pixel size and up to 80% quantum efficiency. This system was previously shown to be well suited to dSTORM measurements[5].

We first measured cos-7 cells transfected with ffDronpa (see methods) using a commercial Olympus IX-71 microscope body. The camera was coupled into the side port through a 0.5x demagnifying C-mount adaptor, which together with a 100× objective lens brought its effective pixel size to 117 nm. The intensity of the excitation was adjusted by tuning the SOFI relaxation time in multi-τ analysis[18] to reach $\tau \approx 1$. Representative SOFI images are shown in fig. 1a–b. For each SOFI image 350 images were acquired at an exposure time of 30 ms per image, for a total measurement duration of approx. 11 s. All experiments were continued on up to 500 frames and beyond, to allow for an analysis of the ideal number of frames in the SOFI reconstruction, balancing SNR against motion blur (see methods). Overall these findings demonstrated that the IDS camera can indeed be used to obtain sub-diffraction imaging on samples labeled with photochromic fluorescent proteins.

Home-built systems offer an attractive combination of flexibility and lower cost compared to commercial systems, and therefore present attractive avenues for reducing the overall cost of super-resolution imaging. We tested the viability of such a system, build from free-standing optics, for SOFI measurements. Again, 350 images were acquired for a SOFI reconstruction (see fig. 1c-d), here at an exposure time of 10 ms and thus a total experiment duration of less than 4 s. We similarly found that this resulted in usable sub-diffraction images of the labelled cells.

Afterwards, the flexibility of this system was used to straightforwardly introduce an additional camera via a 50/50 beamsplitter for biplane imaging. Both cameras can be made to visualise a different depth within the sample by positioning them at different distances from the tube lens (see Suppl. fig. 1). Calibration measurements and analysis show that these two planes are 543 ± 15 nm apart (see Suppl. fig. 2). Since we split the emission light equally between the two cameras, each separate plane only has half of the photons available for SOFI analysis compared to single plane acquisition. The laser power used was similar to the power for the single plane data. However, due to splitting the signal between both cameras, the loss over time due to photobleaching of the fluorophores had a greater impact on the SOFI image reconstruction of the biplane data. Nonetheless, this biplane data still proved adequate in order to achieve clear SOFI reconstructions, using 350 raw frames, acquired at 20 ms per frame and thus less then 8 s total experiment time (see fig. 3).

For a quantitative evaluation of all datasets, we analysed the signal-to-noise ratio (SNR) in all of our SOFI calculations. We found SNR = 10.3 for the CMOS dataset obtained on the commercial microscope system, SNR = 2.6 for the single plane measurements of the freestanding microscope system, as well as $\mathrm{SNR}_1 = 5.6$ and $\mathrm{SNR}_2 = 4.5$ for the first and second plane of the biplane dataset. All datasets thus reach SNRs that are suitable for further analysis. It is quite obvious that the industry-grade camera allows for comparable performance with regard to SOFI image reconstructions compared to high performance sCMOS or EM-CCD systems[19], given that imaging conditions are adjusted accordingly. It is apparent that high variations in SNR are caused by sample-to-sample variations, so for a full, quantitative comparison between systems, a large set of samples would have to be statistically analysed.

Discussion and Conclusion

In this paper, we evaluated the use of an industry-grade CMOS camera for SOFI imaging, using both a commercial and freestanding microscope. Driven by the low cost of this camera, we also investigated how easily two cameras could be combined for biplane imaging.

We showed that the industry-grade CMOS camera is clearly capable of obtaining data suitable for SOFI, even using cells labeled with genetically-encoded fluorophores, using commercial microscope optics implemented in a mechanically rigid frame. An added benefit of the industry-grade CMOS camera, is that it can be used to record data with a higher frame rate compared to

much more expensive EM-CCD detectors at larger fields of view. This increase in speed is important for live cell imaging, since it enables scientists to study dynamic biological and medical problems. For samples with fewer detectable photons, on the other hand, it is more advisable to use an EM-CCD camera, as read-noise, dark current and uniformity surpass the CMOS detector, which becomes most important at low signal levels. In addition, the CMOS detector strongly reduces system cost compared to an EM-CCD since it more than 20× less expensive (20 k€ for a scientific EM-CCD compared to less than 1 k€ for the industry-grade CMOS camera).

Even though using an industry-grade detector is already a significant cost-saving factor, we also verified the camera for SOFI imaging on home-build system, constructed from free-standing optics. This system follows the design ideas proposed for cost-effective localization microscopy[2,3], which allows it to be constructed from readily available opto-mechanical components. We visualised our samples on this fully home-built microscope with industry-grade CMOS camera attached, which resulted in super-resolved images with good SNR.

Additionally, we challenged ourselves to go even further and show that even biplane imaging is readily possible with this home-build imaging system. Due to the low price point of the industry-grade CMOS cameras, a second or even multiple detector(s)[9], can easily be coupled to this system while preserving the low-cost aspect. Due to the free-standing optics, the opto-mechanical changes are minimal, as only a beam-splitter and the second detector have to be added. We were able to obtain usable biplane data with good SNR since the sensitivity of the industry-grade camera was well suited for capturing a sufficient number of photons.

In conclusion, we showed that successful live-cell SOFI imaging can be performed with an extremely cost-effective detector. This allow other researchers to implement SOFI imaging rather easily in Biology and Bio-Medical departments, by either attaching such a camera to an existing research microscope or by building a simple and robust system from readily available parts. Certainly this becomes more or more a viable research tool option with the amount of open-source / open-access software and hardware blueprints made available to the scientific community.

Materials and Methods

Acquisition Hardware and Software

The microscope systems and corresponding hardware that we used for our measurements are described below. For the CMOS data acquisition we used the open-source and freely available Micro-Manager software[20,21] and a custom device adapter providing full camera speed[5]. For data reconstruction we made use of the Localizer[22] package.

Commercial microscope with industry-grade CMOS

The commercial Olympus IX71 system is equipped with a 491 nm, 50 mW diode laser for excitation. The excitation beam is focused into the objective (UPLFHLN 100x, 1.30 NA oil objective, Olympus). The fluorescence signal is collected with the same objective and the emission is separated from the excitation by a dichroic mirror. The emission is than focused on the industry-grade CMOS (IDS uEye) camera with a 180 nm tube lens combined with a 0.5× c-mount demagnifier, giving an overall magnification of 50× and thus a projected pixel size of 117 nm.

Cost-efficient home-build microscope with industry-grade CMOS

The home-built system was equipped with a 473 nm, 50 mW diode laser for excitation. An acousto-optic tunable filter (AOTF) was used to enable fast switching rates of the laser, and therefore, only illuminating the sample during data acquisition. Thus, photobleaching effects are reduced to a minimum. After passing through the AOTF, the excitation beam is coupled into a single mode fiber and then focused into the objective (UPLSAPO60XO 60x, 1.35 NA oil objective, Olympus). The fluorescence signal was collected with the same objective and the emission was separated from the excitation source by a dichroic mirror. The emission is then focused on the industry-grade CMOS (IDS uEye) camera using a 250 mm tube lens and after filtering with an emission filter, resulting in an overall projected pixel size of 70 nm.

Biplane calibration

We used a 200 nm diameter TetraSpeck bead slide (Life Technologies, Ref T7280, Lot 1718181) to calibrate the biplane data. The beads were diluted in doubly destilled H_2O with a 1 to 1000 concentration and then mounted in glycerol on a #1.5 glass cover slip. First, one of the CMOS cameras was adjusted, so that it was focusing into the sample. Then, the objective was moved with a piezo-electric positioner (PIFOC P-721 with digital amplifier E-709) by 500 nm and the second CMOS camera was focused to this plane. For the correct analysis of the distance between the planes, a z-stack extending over 10 μm with 50 nm stepsize was recorded and analysed (see fig. 2).

Optical design software was used to verify that the chosen realization of biplane imaging (beam-splitting in a non-coherent path) does not introduce significant aberrations.

Transfection and sample preparation

Cos-7 cells were cultured in DMEM supplemented with FBS, glutamax and gentamycin at 37 °C with 5% CO_2. Cells were washed and detached from the growth flask using a 0.05% Trypsin solution. The cell suspension was then seeded on 35 mm glass bottom culture dishes (#1.5 thickness, MatTek) to ensure a confluency of 50% to 80% for transfection. Cells were then transfected with pcDNA::MAP4-ffDronpa[18] using FuGENE6 (Promega) according to manufacturers instruction, and cells were incubated for a maximum of 24 hours before imaging.

Set-up of the experiment and SOFI analysis

The laser power and acquisition that were used in our experiments were chosen to achieve a τ-value (numbers of frames that a single blink lasts) around 1. To our experience this has given us the best results, in terms of blinking behaviour of ffDronpa for reconstruction.

We additionally analysed all data sets using 200 and 500 raw data frames for a 2nd order SOFI reconstruction (Suppl. figs. 2 to 4). This allows to set 350 frames at an ideal number for the final reconstruction, as 200 frames resulted in a SOFI signal that was too low, while 500 frames picked up increased blurring due to cell movement.

All measurements where obtained at room temperature. Samples were maximally used for 24 hours after transfection and incubation. Samples were embedded in PBS for imaging.

Acknowledgements and author contributions

R.V. performed measurements and SOFI reconstructions, and wrote parts of the manuscript. A.S. performed measurements, built the freestanding microscope system, and wrote parts of the manuscript. W.V. performed SOFI reconstructions and analysis. S.D. provided the ffDronpa constructs and helped with the measurements. W.H. performed the cell transfections. P.D. and T.H. conceived of and supervised the project, and helped writing the manuscript. M.M. supervised the projects, performed data analysis, and helped writing the manuscript.
All authors participated in the reading and editing of the final manuscript.

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There are no competing interests. Raw image data is made available upon request.

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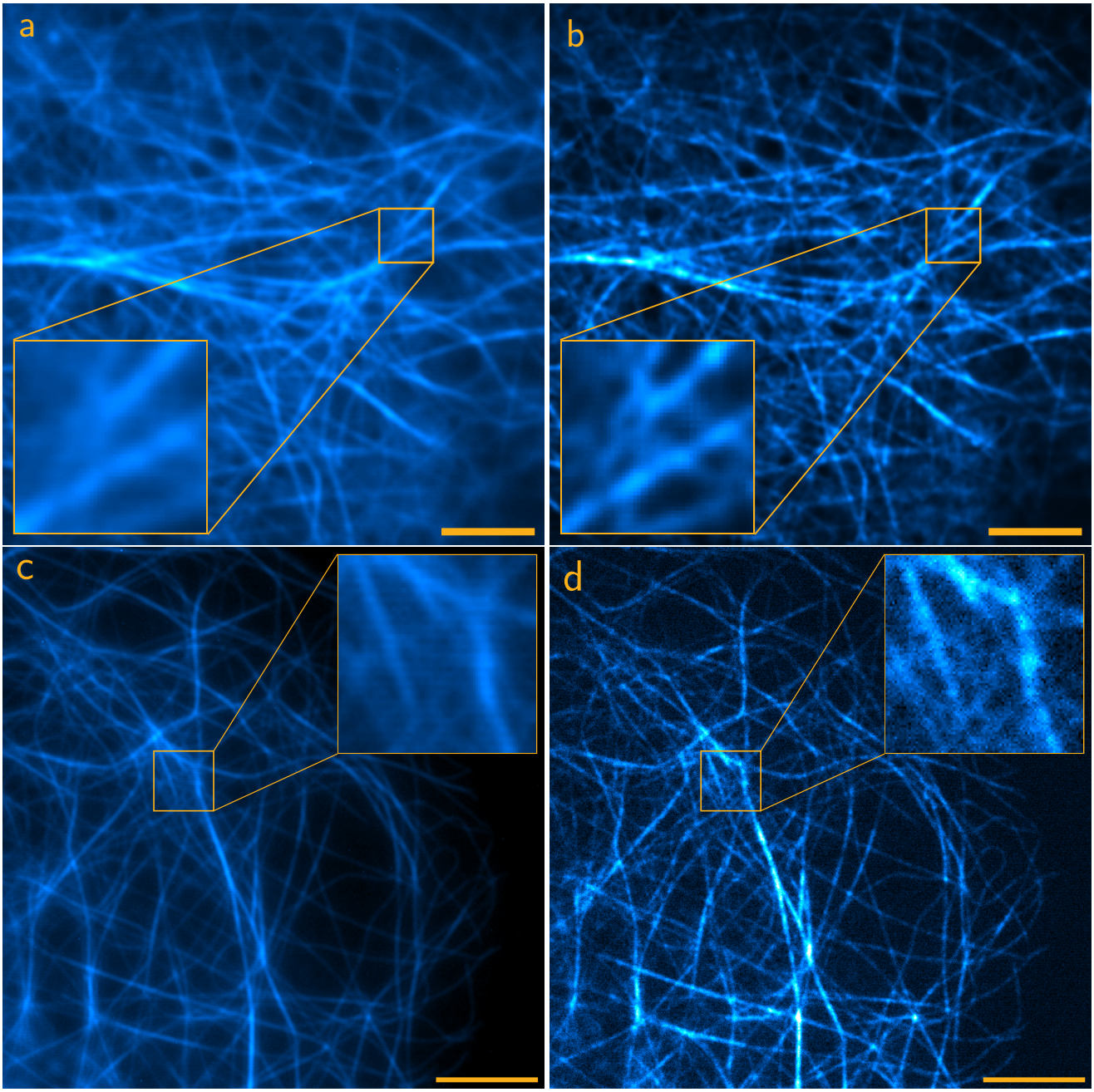

Figure 1. Comparison of 2nd order SOFI images of microtubules in cos-7 cells labelled with ffDronpa, acquired with an CMOS camera on both a commercial microscope system (a,b) and a home-build system employing free-standing optics (c,d). Averages image (a,c) and SOFI images (b,d) were acquired with 30 ms (a,b) and 10 ms (c,d) of exposure time. Both SOFI images were reconstructed using 350 consecutive frames. To maximise the SOFI signal, lag 1 and lag 2 computations have been performed for the datasets. Scale bar 5 μm, inset 3 μm.

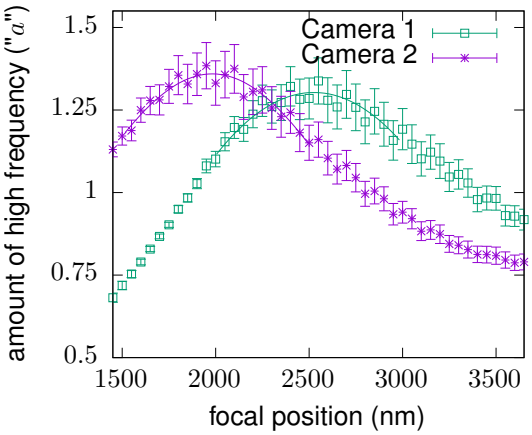

Figure 2. Focus alignment for the bi-plane detection system. To obtain this alignment, a layer of fluorescent beads was prepared (see methods section) and imaged. The objective lens was moved along the z-axis in steps of 50 nm via a piezo-electric mount. At each focal position, an image was acquired with each cameras. An exponential function $b \cdot \exp(-k_x \cdot a) + c$ was fitted to the power spectrum of each of the images to estimate their frequency distribution. The approach here is than when the beads come in focus, more high frequency signal is picked up by the microscope and thus a increases towards best focus. Empirically, this approach seems to work well and surpasses observing single beads in terms of noise and stability. By plotting a against the objective lens position for both cameras, and finding the maximum of both curves, the focus position is found for both cameras, with $f_{\text{cam0}} = 1982 \pm 8\text{nm}$ and $f_{\text{cam1}} = 2525 \pm 13\text{nm}$, which yields a focal plane distance of $543 \pm 15\text{nm}$ between the cameras. We find this value consistently for different test datasets.

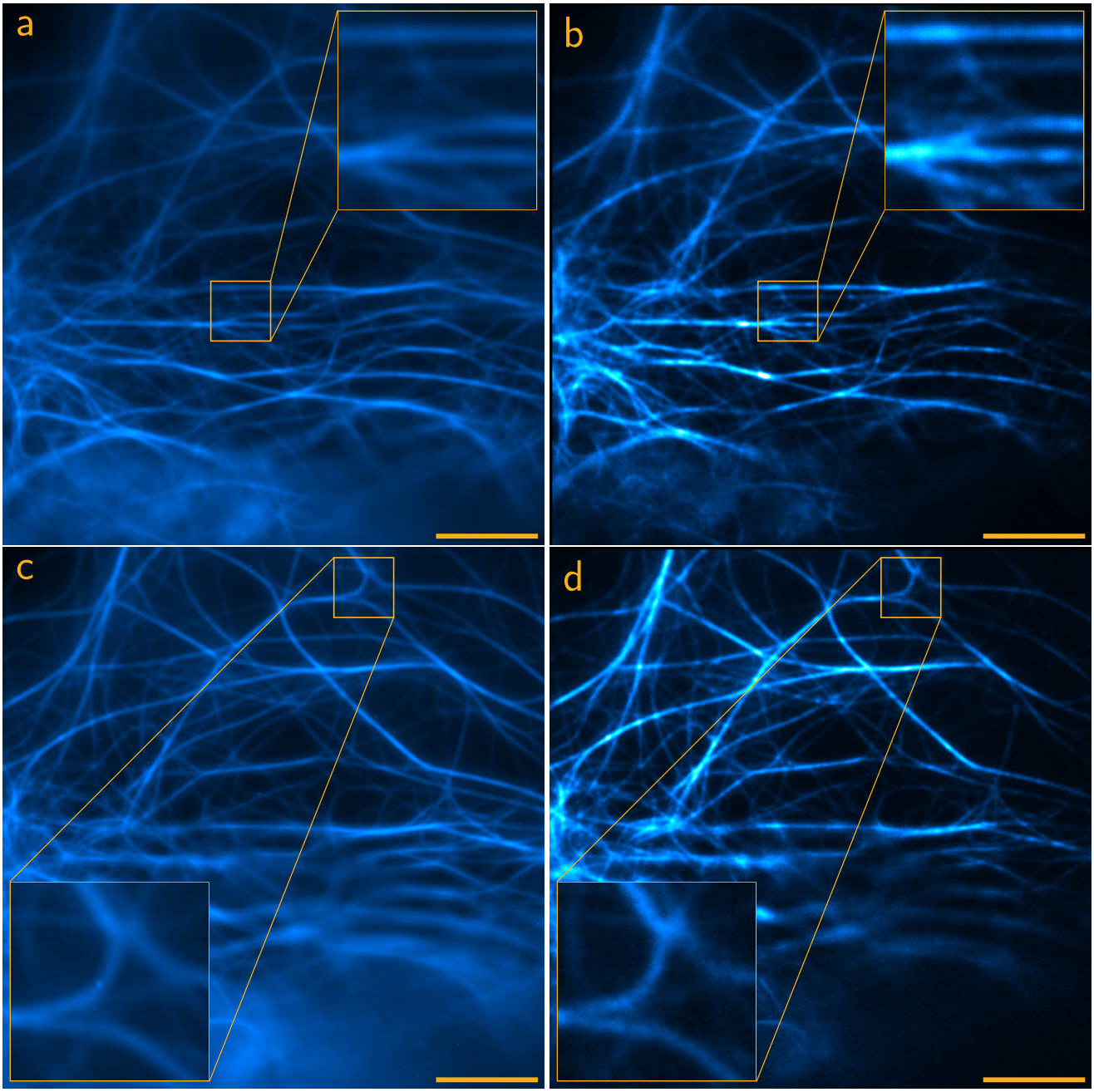

Figure 3. 2nd order SOFI images of microtubules in cos-7 cells labelled with ffDronpa in two different planes, with plane 1 shown in (a,b) and plane 2 in (c,d), spaced approx. 541 nm apart (see fig. 2). Data was acquired on a free-standing, home-build biplane setup (see suppl. fig. 1), using an CMOS detector (exposure time: 20 ms). For the SOFI analysis 350 frames were used. (a) and (c) show average images and (b) and (d) the SOFI images. To maximise the SOFI signal, lag 1 and lag 2 computations have been performed for this data set. Scale bar 5 μm, inset 3 μm.