Superresolution Imaging

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Superresolution Optical Fluctuation Imaging with Organic Dyes**

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Superresolution far-field microscopy has experienced a tremendous growth since the introduction of the first concept in 1994[1], broadening the scope and applications of fluorescence microscopy beyond the diffraction limit. Various powerful and excellent methods, such as stimulated emission depletion,[2] saturated-structured-illumination microscopy,[3] and stochastic single-molecule switching methods, such as STORM (STORM = stochastic optical reconstruction microscopy),[4] (f)PALM (PALM = photoactivated localization microscopy),[5,6] directSTORM (*d*STORM),[7,8] and GSDIM (GSDIM = ground-state depletion imaging)[9] have since been developed. Notably, the key to all superresolution methods lies in the exploitation of a two-state transition (e.g. a fluorescent 'on' and a nonfluorescent 'off' state) of the molecules. It is necessary to precisely control both the time a fluorophore spends in the fluorescent as well as in the dark state, especially for the stochastic single-molecule-switching methods (PALM, STORM etc.). In the *d*STORM concept, this control is achieved by irradiation of the sample with one or two wavelengths in the presence of reducing agents. In parallel, the invention of a reducing and oxidizing buffer system (ROXS) made the fluorescence intermittency rates of most organic dyes tunable to almost any degree.[10,11] The immediate consequences are increased photo-stability and 'nonblinking' fluorescent dyes, which have recently been exploited in increasing the performance of STED (STED = stimulated emission depletion).[12] Herein, we demonstrate that the reversible photoswitching of organic dyes, using similar conditions as in *d*STORM, can be utilized for another superresolution technique, namely superresolution optical fluctuation imaging (SOFI).[13]

SOFI makes use of random temporal signal fluctuations of single emitters and uses them to achieve fast, background-free, three-dimensional superresolution microscopy by means of higher order statistics (HOS). In contrast to single-molecule switching methods, many emitters can be 'on' at the same time in a diffraction-limited volume and still contribute to enhanced resolution. The SOFI procedure consists of recording a movie of the fluctuating signal on any kind of imaging platform. This movie is then processed by a software-based HOS analysis (that could also be implemented in hardware). So far, SOFI has been demonstrated to work with blinking quantum dots (QDs) and a resolution enhancement of a factor of two has been achieved in generic imaging applications.[14]

As almost all organic dyes exhibit fluorescence intermittency, owing to, for example, intersystem crossing to the dark triplet state, which gives rise to a fluctuating fluorescence signal, they could potentially be used for SOFI as well. The use of much-smaller and targetable organic dyes (as compared to QDs) could expand the scope of SOFI to a large array of live and fixed-cell imaging applications. On the other hand, using dyes for SOFI introduces many challenges: 1) the immanent photobleaching of dyes upon illumination limits the acquisition time and potentially compromises the SOFI algorithm (as it requires a temporally constant mean signal); 2) a lower signal-to-noise ratio (as compared to QDs) degrades the algorithm's performance; 3) typical intersystem crossing rates of conventional organic dyes result in microseconds 'on/off' blinking, whereas most movie acquisitions for SOFI are limited to millisecond timescales.

Herein, we apply experimental conditions similar to the ones used in the *d*STORM concept to adjust the reversible photoswitching of organic dyes to a suitable level for SOFI, using an EMCCD-camera acquisition. We chose the dye Alexa647 (which exhibits light-driven microsecond fluorescence intermittency) for these experiments (see the Experimental Section).[7] Note that similar experimental conditions were shown to control the blinking of a large selection of organic dyes,[8] and therefore our choice of a cyanine dye was almost arbitrary. The experiment was carried out on fixed COS-7 cells, whose β-tubulin network was immuno-labeled with Alexa647-conjugated antibodies. We recorded a movie of 1000 frames at a frame rate of 20 Hz (see the Supporting Information), and subsequently analyzed it with an extended SOFI algorithm.[14] This analysis enabled us to produce SOFI images that have four-times-more pixels than the original acquired image.

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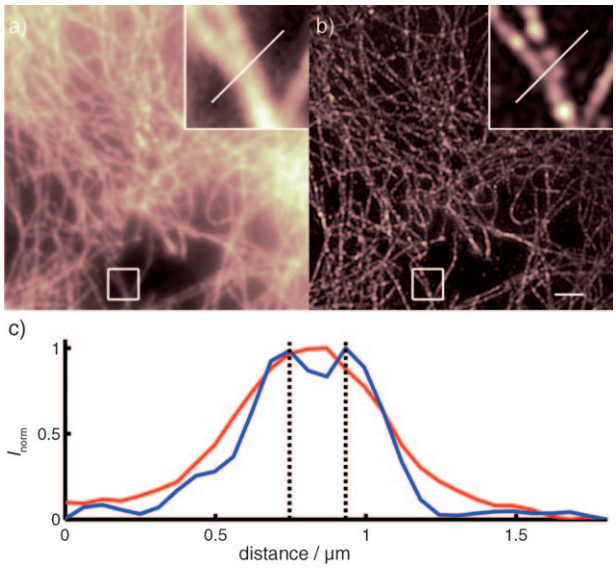

Figure 1. Fluorescence and corresponding SOFI images of ß-tubulin network of COS-7 cells. White boxes are magnified regions shown in the upper-right corner. An intensity cross-section (white line) was used to evaluate the resolution enhancement. a) Original fluorescence image as obtained by averaging over 1000 frames. b) SOFI image as obtained by the analysis of the same 1000 frames. Scale bar is 2 µm. c) Normalized intensity profile of the cross-sections taken in (a) and (b). Red: original image (a), blue: SOFI image (b). The resolved feature corresponds to 169 nm, which indicates the resolving power of SOFI in this image. The vertical lines indicate the centers of the peaks. The measured value is very close to the predicted Rayleigh limit of 145 nm (for an x2 higher resolution). Deviations from the theoretical Rayleigh limit might be accounted for by a slightly larger experimental point spread function (PSF) than the theoretically predicted one, which translates into a larger Rayleigh limit.

Figure 1 clearly shows resolution enhancement, and, in addition, a striking reduction in background fluorescence was observed in the SOFI image. This reduction is due to the fact that the SOFI algorithm inherently eliminates the non-fluctuating background signal. Also, the inherent optical sectioning attribute of SOFI contributes to a 'cleaner' image, as out-of-focus light is suppressed even though imaging was done with a configuration close to total internal reflection (TIR). The resolution enhancement translated into a smaller Rayleigh limit of 169 nm (as compared to 290 nm for the conventional imaging), which was assessed by comparing the same line profile in Figure 1 a and 1 b (Figure 1 c).

To circumvent imaging artefacts that are due to dye photobleaching during acquisition (which would manifest as nonresolved features in the SOFI image), the acquired movie was analyzed piece-wise in blocks of frames. Within each block, the change of the mean signal because of photobleaching was negligible (see Experimental Section) and therefore the requirement for successful SOFI imaging was given for each movie block. SOFI images for all movie blocks were summed together before any further analysis.

In conclusion, we have demonstrated a convenient superresolution imaging method using conventional organic dyes with an unmodified TIRF-microscope. Compared to single-molecule superresolution methods based on switching, SOFI reached superresolution performance in comparable acquisition times (50 s for the data presented here). However, SOFI has the potential to run at even faster speeds (topic of current research). Note that even though we applied photoswitching conditions in order to tune the on/off times, the only SOFI prerequisite was to be able to monitor dye fluctuations. Therefore, it is likely that SOFI could be implemented in the future with faster-blinking dyes as faster cameras come online.[15,16] In contrast to PALM and STORM, SOFI does not require long off times or has to maintain a certain on/off time ratio. This advantage obviously points to future experiments, which will be performed in live cells, where tuning capabilities and measurement times are limited and fast acquisition is crucial. As the signal-to-noise ratio of dyes is considerably lower than for QDs, SOFI imaging is more demanding; that is, for QDs an arbitrary frame rate of the camera can be used (leading to an improved signal), whereas for dyes a fast frame rate is desirable as photobleaching limits the acquisition time. Photobleaching is also the limiting factor for applying higher-order correlations in order to improve resolution to a larger extent than demonstrated here.

Experimental Section

Cell preparation: African green monkey kidney COS-7 cells were plated in LabTek 8-well chambered coverglass (Nunc, Germany). After 12–24 h, the cells were fixed using 3.7 % paraformaldehyde in PBS for 10 min. The fixed cells were washed with PBS and permeabilized in PBS 0.5 % v/v Triton X-100 (Sigma–Aldrich, Germany) for 10 min. The microtubule network was stained by indirect immunochemistry using mouse monoclonal anti-ß-tubulin antibodies (Sigma–Aldrich, Germany) and Alexa Fluor 647 labeled secondary antibodies (Invitrogen Inc. USA), respectively, for 60 min. After each staining step, three washing steps were performed using PBS containing 0.1 % v/v Tween 20 (Sigma–Aldrich, Germany).

Imaging: SOFI imaging was performed on an inverted microscope (Olympus IX-71; Olympus, Japan) equipped with an oil-immersion objective (60x, NA 1.45, Olympus) and operated in total internal reflection (TIR) mode. The 647 nm and 514 nm laser lines from an argon–krypton laser (Coherent, Santa Clara, CA, USA) were selected by an acousto-optic-tunable filter (AAOptics, Paris, France), passed a dichroic beamsplitter (FF560/659; Semrock, Rochester, NY, USA) and focused onto the back focal plane of the microscope lens. The fluorescence light in the detection path was filtered using a bandpass (700DF75; AHF Analysentechnik) and a long pass (HQ665 LP, AHF Analysentechnik) filter, before being projected on an electron-multiplying CCD camera (Andor Ixon DV897; Andor, Belfast, Northern Ireland). Additional lenses were used to achieve a final imaging pixel size of 85 nm. Typical observation parameters were 1000 frames recorded with 20 Hz using irradiation intensities of 2 to 10 $kW\,cm^{-2}$ (647 nm) and 0.5 to 5 $kW\,cm^{-2}$ (514 nm) SOFI imaging was performed in PBS buffer solution (pH 7.4) with an oxygen scavenger and with 100 mM mercaptoethylamine (as described in Ref. [8]).

SOFI: The SOFI algorithm was performed according to previous reports,[13,14] making use of cross-cumulants and extracting the point spread function (PSF) for subsequent Fourier reweighting. The movie (1000 frames) was split into smaller blocks of 60 frames each, which were analyzed separately. All block-wise SOFI images were then summed together for the final SOFI image, shown in Figure 1. This modification to the previously published algorithm was done to compensate for photobleaching during acquisition. The length of the blocks was chosen so that the relative change of the temporal mean signal within each block (owing to bleaching) was no larger than

4.7%. The Fourier reweighting was done using a damping constant of 0.025.[14] The SOFI algorithm was performed using a self-written Matlab code (Mathworks Inc, USA).

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