STOCHASTIC OPTICAL FLUCTUATION IMAGING

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Abstract—**The presentation gives an introduction and overview of our recently developed Stochastic Optical Fluctuation Imaging microscopy technique. The theoretical foundations are given and the method is exemplified on several model systems. Its advantages and limitations are discussed, and many applications of SOFI in cell microscopy are presented.**

Keywords-super-resolution imaging, fluorescence correlation

I. INTRODUCTION

Fluorescence microscopy has evolved into one of the most important research tools in the life sciences. Due to the high sensitivity of fluorescence detection (down to the detection of single molecules), and its spectral specificity, fluorescence microscopy allows for unsurpassed elucidation of structure and dynamics within living cells and tissue with molecular specificity. Moreover, the last two decades have seen a revolution in the emergence of new fluorescence microscopy techniques, in particular techniques concerned with the breaking of the Abbe's classical diffraction limit of resolution.

The first technology which could indeed overcome the classical resolution limit was STimulated Emission Depletion alias STED microscopy [1]. Although STED is by now even commercially available, it remains to be a technologically complex and personnel-intensive method, which requires also the usage of special fluorescence labels and, in general, high illumination intensities of the STED-beam which may lead to irreparable photo-damage of the studied sample. Nonetheless, STED is an enormously powerful microscopy method which, when applicable, delivers images with enormously enhanced spatial resolution at high image acquisition speed.

A second large group of new super-resolution microscopy techniques is based on the physical principle that the center position of the image of a single fluorescing molecule (or any other emitter) on a recording camera can be determined with much higher localization precision than the characteristic diameter of this image, which is determined by the optical resolution of the used imaging optics. In this second group of super-resolution techniques, one uses furthermore the property of special fluorescent markers which are able to switch between fluorescent and non-fluorescent states. This opens the possibility that, at any given time, only very few molecules with nicely separable locations are switched on into a fluorescing state, so that their center positions can be determined with nanometer precision. By sequentially recording many images of the same sample, but with different subsets of fluorescent molecules switched on, the center positions of all molecules can be determined with highest accuracy (typically 10-30 nm localization accuracy) and, in this way, one obtains a pointillistic image of the whole sample. Exactly this is the working principle of PhotoActivable Localization Microscopy (PALM) [2] and STochastic Optical Reconstruction Microscopy (STORM) [3], whereby the sole difference between both methods is the kind of used fluorescence labels and the number of on/off switching events. The enormous advantage of PALM and STORM are their principal simplicity and the moderate light intensities required for illumination. Their disadvantage is their sequential character which limits the overall image acquisition speed.

Recently, we have developed a third approach towards super-resolution microscopy, which was termed Stochastic Optical Fluctuation Imaging or SOFI microscopy [4]. SOFI employs the temporal fluctuations in the emission intensity of emitters for improving the spatial resolution of imaging. In the next section, we give a brief description of the working principle of SOFI.

II. PRINCIPLE OF SOFI

The physical foundation of SOFI is the fact that the temporal fluctuations of different fluorescing emitters are statistically independent. Examples are the fluctuations caused by triplet/single-state transitions in dyes, or the so-called blinking of fluorescent quantum dots. Thus, if one denotes the time-dependent fluorescence signal generated by the j^{th} emitter in a sample by $s_j(t)$, one finds that the n^{th}-order cumulant $C[s_{j1}(t_1), s_{j1}(t_2), ..., s_{j1}(t_n)]$ as defined by the time average

$$C[s_{j1}(t_1), s_{j2}(t_2), ..., s_{jn}(t_n)] = \langle [s_{j1}(t+t_1) - \langle s_{j1}(t)\rangle] \cdot [s_{j2}(t+t_2) - \langle s_{j2}(t)\rangle] \cdots [s_{jn}(t+t_n) - \langle s_{jn}(t)\rangle]\rangle$$

is only non-zero if all input signals come from one and the same emitter, i.e. if $j1 = j2 = ... = jn$. In the last equation, the angular brackets denote the time-average over time t. Thus, calculating a cumulant cancels any cross-terms involving signals from different sources. When recalling that a microscopy image $F(\mathbf{r}, t)$, at any given time t and as a function of position $\mathbf{r}$, is formed by the convolution of the point spread function (PSF) $U(\mathbf{r})$ with the sample's fluorescence emission distribution, i.e. by

$$F(\mathbf{r}, t) = \Sigma_j U(\mathbf{r} - \mathbf{r}_j)\, s_j(t),$$

where $\mathbf{r}_j$ denotes the position of the j^{th} emitter, then it is obvious that due to the above mentioned property of cumulants the n^{th}-order temporal cumulant of the image $F(\mathbf{r}, t)$ factorizes into a sum over contributions of the separate emitters, namely

$$C[F(\mathbf{r}, t_1), F(\mathbf{r}, t_2), ..., F(\mathbf{r}, t_n)] = \Sigma_j U^n(\mathbf{r} - \mathbf{r}_j)\, c_j(t_1, t_2, ..., t_n)$$

where $c_j(t_1, t_2, ..., t_n)$ is the n^{th}-order temporal cumulant of the signal from the j^{th} emitter. The important point is that in the above equation, the effective PSF for the n^{th}-order cumulant image is given by the n^{th} power of the original PSF $U(\mathbf{r})$! If one assumes, for simplicity, that the original PSF can be approximated by a Gaussian with width σ, then the resulting n^{th}

order cumulant image is generated by convoluting the sample's emitter distribution with an effective PSF that is a Gaussian with a width of $\sigma/\sqrt{n}$.

As will be explained during the presentation, the actual improvement in spatial resolution is even better, not only following a square-root as just described but a linear dependence on the order n. This linear resolution enhancement is achieved by reweighting the Fourier mode amplitudes of the raw cumulant image [4]. Moreover, by employing more sophisticated cross-correlation algorithms that correlate the recorded signals not only in time but also between pixels on a CCD, the final resolution of a cumulant or SOFI image can even be better than the hard-ware resolution of a CCD as defined by the pixel size of the CCD-chip [5].

III. Applicability of SOFI

One of the significant advantages of SOFI is its technical simplicity and wide versatility, combined with robustness against aberrations and other optical imperfections. The two pre-requisites for SOFI are

(i) The fluorescent labels have to exhibit at least two different emission states. For example, these states can be a fluorescent and a non-fluorescent one, but in principle any two or more states which are optically distinguishable will do.

(ii) Different emitters have to switch between states repeatedly and independently from each other in a stochastic way.

From a hardware point-of-view, SOFI requires a standard wide-field fluorescence microscope with sufficiently sensitive detection. In the past, we could show that it successfully works with fluorescent quantum dots [4], organic dyes [6], fluorescent proteins, and even with scattering rotating nanoparticles.

Besides improved spatial resolution, SOFI offers two additional important advantages:

(i) SOFI lives on the intensity fluctuations in a sample, so that any signal which is non-fluctuating will be filtered out in the final SOFI image. This helps to dramatically increase image contrast in samples with large auto-fluorescent background or scattering.

(ii) Because the effective PSF of an n^{th}-order SOFI image corresponds to the n^{th} power of the PSF of a widefield microscope, SOFI has intrinsic 3D-sectioning capability. For example, second-order SOFI has the same 3D-sectioning capability as a conventional confocal laser scanning microscope, although the setup employed for taking the raw images is a wide-field microscope.

The mathematical algorithms used in SOFI are standard correlation routines which do not necessarily need *a priori* knowledge about the used detection optics, nor do they involve complicated fitting routines as typically required in PALM or STORM. This makes SOFI fast and reliable. More importantly, any spatial overlap between emitters in a sample does not affect SOFI performance, in contrast to PALM/STORM, so that it can work at a 10 to 100 times higher efficient label density (density of simultaneously emitting labels) than PALM/STORM [7]. This makes SOFI an interesting alternative for fast imaging in live cells.

IV. Conclusion

SOFI is a new super-resolution imaging modality which is easy to implement, giving two to four-fold increased spatial resolution, applicable to any fluctuating emitter labeling, and easy to implement for multi-color and live-cell imaging.

Acknowledgment

We thank Thomas Dertinger (Berlin), Theo Lasser (EPFL Lausanne), Stephan Jacobs (University Medicine Göttingen), Alexander Egner (Laser Lab Göttingen), Omar Ramirez, Felipe Santibanez, Steffen Härtel (University Santiago de Chile), and Peter Dedecker (KU Leuven) for many fruitful discussions and contributions. Financial support by the Center for the Molecular Physiology of the Brain (Göttingen) and the Bernstein Center for Computational Neurobiology (Göttingen) is gratefully acknowledged.

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