Fast, background-free, 3D super-resolution optical fluctuation imaging (SOFI)

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Super-resolution optical microscopy is a rapidly evolving area of fluorescence microscopy with a tremendous potential for impacting many fields of science. Several super-resolution methods have been developed over the last decade, all capable of overcoming the fundamental diffraction limit of light. We present here an approach for obtaining subdiffraction limit optical resolution in all three dimensions. This method relies on higher-order statistical analysis of temporal fluctuations (caused by fluorescence blinking/intermittency) recorded in a sequence of images (movie). We demonstrate a 5-fold improvement in spatial resolution by using a conventional wide-field microscope. This resolution enhancement is achieved in iterative discrete steps, which in turn allows the evaluation of images at different resolution levels. Even at the lowest level of resolution enhancement, our method features significant background reduction and thus contrast enhancement and is demonstrated on quantum dot-labeled microtubules of fibroblast cells.

1. The fluorescent label has to exhibit at least two different emission states. For example, these states can be a fluorescent and a nonfluorescent one, but in principle any two or more states that are optically distinguishable will do.
2. Different emitters have to switch between states repeatedly and independently from each other in a stochastic way.
3. For this approach, the image should be acquired with pixels smaller than the diffraction limit. Resolution less than the pixel size will be the topic of a future publication.

These conditions being met, the pixel value of a SOFI image (of the order $n$) is obtained from the $n$th-order cumulant of the original pixel time series. The signal in a pixel using conventional imaging applications is given by the superposition of the fluorescence originating from different, nearby emitters. The $n$th-order cumulant (a quantity related to the $n$th-order correlation function) filters this signal based on its fluctuations in such a way that only highly correlated fluctuations are left over. In practice the remitting signal is limited to emitters within the pixel. The fluorescence signal contribution of these emitters to neighboring pixels will non-linearly yield lower correlation values, leading to an increased resolution. The following section describes the underlying theory.

Theory

Given a sample composed of $N$ single, independently fluctuating emitters, located at position $r_k$ (Fig. 1A) and having a time-dependent molecular brightness $n_k(t)$, the resulting fluorescence source distribution is given by:

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The fluctuations are recorded in a movie. (C) Each pixel contains a time trace, which is composed of the sum of individual emitter signals, whose PSFs are reaching into the pixel. (D) The second-order correlation function is calculated from the fluctuations for each pixel. (E) The SOFI intensity value assigned for each pixel is given by the integral over the second-order correlation function. The second-order correlation function is proportional to the squared PSF, thus increasing the resolution of the imaging system by a factor of $\sqrt{2}$.

\begin{equation}
\sum_{k=1}^{N} \delta(r - r_k) \psi_k \sigma_k(t),
\end{equation}

where $\psi_k$ is the constant molecular brightness and $\sigma_k(t)$ is a time-dependent fluctuation.

We assume that the positions of emitters do not change during the image acquisition; temporal changes are caused only by changes in the fluorescent states of individual emitters (e.g., blinking). For reason of simplicity we assume further that the point spread function (PSF) does not vary locally because of e.g., aberrations or polarization effects. However, such effects can be taken into account.

The fluorescence signal $F(r, t)$ at position $r$ and time $t$ is given by the convolution of the system’s PSF $U(r)$ and the fluorescence source distribution (Eq. 1 and Fig. 1 B and C):

\begin{equation}
F(r, t) = \sum_{k=1}^{N} U(r - r_k) \psi_k \sigma_k(t).
\end{equation}

Assuming that the sample is in stationary equilibrium during acquisition, the fluctuations can be expressed as zero-mean fluctuations:

\begin{align*}
\delta F(r, t) &= F(r, t) - \langle F(r, t) \rangle,
\end{align*}

\begin{align*}
&= \sum_k U(r - r_k) \psi_k [s_k(t) - \langle s_k(t) \rangle],
\end{align*}

\begin{align*}
&= \sum_k U(r - r_k) \psi_k \delta s_k(t),
\end{align*}

where $\langle \cdots \rangle$ denotes time averaging. The second-order autocorrelation function $G_2(r, \tau)$ is then given by (see Fig. 1D):

\begin{align*}
G_2(r, \tau) &= \langle \delta F(r, t + \tau) \delta F(r, t) \rangle,
\end{align*}

\begin{align*}
&= \sum_{j,k} U(r - r_j)U(r - r_k)\psi_j \psi_k \langle \delta s_j(t + \tau) \delta s_k(t) \rangle,
\end{align*}

\begin{align*}
&= \sum_k U^2(r - r_k)\psi_k^2 \langle \delta s_k(t + \tau) \delta s_k(t) \rangle. \quad [4]
\end{align*}

In Eq. 4 we assumed that the emission of different emitters is not correlated in time so that all cross-correlation terms $\langle \delta s_j(t + \tau) \delta s_k(t) \rangle$ with $j \neq k$ vanish. The second-order autocorrelation function thus appears as a simple sum of the squared PSF, weighted by each emitter’s squared brightness and molecular correlation function $\langle \delta s_k(t + \tau) \delta s_k(t) \rangle$.

The value of $G_2(r, \tau)$ for a time lag $\tau$ defines a SOFI image, the only difference between each image being the weighting of the squared PSF with the molecular correlation function. Note that the intensities of a SOFI image do not report directly on the fluorescence signal, but rather its brightness and its degree of correlation. Additionally, the PSF is replaced by a distribution that is the square of the original PSF.

If the original PSF of the optical system can be approximated by a 3D Gaussian distribution, it follows from Eq. 4 that the width of the “new” PSF is reduced by a factor of $\sqrt{2}$ along all three dimensions, thus increasing the optical resolution of the second-order SOFI image:

\begin{align*}
U(r) &= \exp \left(-\frac{x^2 + y^2}{2\omega^2_0} - \frac{z^2}{2\omega^2_z} \right),
\end{align*}

\begin{align*}
\Rightarrow \tilde{U}(r) &= \exp \left(-\frac{x^2 + y^2}{2\tilde{\omega}^2_0} - \frac{z^2}{2\tilde{\omega}^2_z} \right),
\end{align*}

with $\tilde{\omega}_z = \omega_0 / \sqrt{2}$ and $\tilde{\omega}_0 = \omega_0 / \sqrt{2}$.

Because the second-order correlation function involves the square of the PSF it is natural to look into higher-order correlation functions, generate higher powers of the PSF, and therefore further increase the resolution. The $n$th-order correlation function is given by:

\begin{align*}
G_n(r, \tau_1, \ldots, \tau_{n-1}) &= \langle \delta F(r, t) \delta F(r, t + \tau_1) \cdots \delta F(r, t + \tau_{n-1}) \rangle, \quad [6]
\end{align*}

$G_n$ can be easily computed in a straightforward manner, because the above formula states that the acquired signal fluctuations have to be multiplied for $n - 1$ time lags to obtain $G_n$. To generate SOFI images of higher orders, it is, however, necessary to transform the $n$th-order correlation functions into $n$th-order cumulant functions $C_n(r, \tau_1, \ldots, \tau_{n-1})$. The reason is that all cross-terms caused by lower-order correlation contributions are eliminated in cumulants, so that the $n$th-order cumulant consists only of terms containing the $n$th power of the PSF. This can be understood by considering the following example. To compute the fourth-order correlation function, one ultimately needs to correlate four photons in one pixel. These photons could originate from different emitters or from the same emitter. The latter case would straightforwardly yield super resolution. However, it is also possible that two of the four photons are coming from one emitter and two are from a different emitter. These pairs, too, would contribute to the fourth-order correlation but only with a squared PSF for each emitter (cross-terms of lower orders) concealing the fourth-power PSF contribution, which arises from fluctuations from only a single emitter. Cumulants do not contain these cross-terms. Thus, only the use of cumulants...
will ensure super resolution. This is expressed through the following equation:

$$C_n(r, \tau_1, \ldots, \tau_{n-1}) = \sum_\kappa U^n(r - r_\kappa) e^{\kappa w_\kappa(\tau_1, \ldots, \tau_{n-1})},$$

where $w_\kappa(\tau_1, \ldots, \tau_{n-1})$ is a correlation-based weighting function, depending on the specific fluctuation properties of each emitter. Note that the exact expression for $w_\kappa(\tau_1, \ldots, \tau_{n-1})$ depends on the order of the cumulants. Because the $n$th-order cumulant generates an image with an effective PSF that is the $n$th power of the original PSF, the resolution is enhanced by a factor $\sqrt{n}$ for a Gaussian PSF. For instance, the fourth-order cumulant results in a SOFI image that has a 2-fold increased resolution, whereas the 16th-order cumulant will result in a 4-fold resolution enhancement. Although there is no fundamental limit for resolution enhancement, there are practical ones. Because the PSF is raised to the $n$th power, so is the molecular brightness $e$ from each emitter. Thus, an emitter that has a 2-fold larger molecular brightness will appear $2^n$ times brighter in the $n$th-order SOFI image. Additionally, the weighting function $w_\kappa(\tau_1, \ldots, \tau_{n-1})$ can alter the apparent intensity in the SOFI image. An emitter that does not fluctuate over time will not yield any correlation [i.e., the weighting function $w_\kappa(\tau_1, \ldots, \tau_{n-1}) = 0$ and thus the emitter will not appear in the SOFI image], whereas an emitter that blinks yields a nonzero value for the weighting function and will show up in the SOFI image. The exact value for the weighting function is determined by the specific blinking behavior of the emitters. The product of the $n$th power of the molecular brightness and the weighting function of an individual emitter therefore determines its contribution to the SOFI image, which can lead to the masking of dim emitters that are in close proximity to bright emitters (see SI Text and Figs. S1 and S2). In other words, the resulting higher-order SOFI images have a very large dynamic intensity range. This effect reduces the apparent information content of the final SOFI image and is pronounced for orders $\geq 2$.

Although there is no fundamental difficulty in computing higher-order cumulants, it is worth noticing, that the $n$th-order cumulant is an $(n-1)$-dimensional function of the $\tau_i$. Therefore, the computation time and memory requirements are growing as $n^3$, which may be rapidly become a limiting factor for the generation of higher-order SOFI images. In practice, it is most efficient to compute SOFI images by setting all time lags to zero:

$$C_n(r, 0, \ldots, 0) = C_n(r, \tau_1 = 0, \ldots, \tau_{n-1} = 0) = \sum_\kappa U^n(r - r_\kappa) e^{\kappa w_\kappa(0)}.$$  \[8\]

In this case the cumulant formulas can be expressed in a simplified form (e.g., the second-order cumulant becomes simply the variance of the signal), which is algorithmically easy to realize:

$$C_2(r, 0) = \langle F^2(r, 0) \rangle - \langle F(r, 0) \rangle.$$  \[9\]

This simplification, of course, does not eliminate shot noise of the signal and is most likely not applicable, when the signal-to-noise ratio is low, as, for example, when measuring with organic dyes. In this case cumulants should be calculated in a shot-noise free manner as discussed in SI Text and Figs. S1 and S3.
Results

We first demonstrate the concept of SOFI by using quantum dots (QDs) deposited on a coverslip. Because the QDs fluorescence on/off distributions obey a power law (18), they blink at all time scales, which allows the use of arbitrary camera frame rates. Data were recorded in series of 2,000 frames (100 ms per frame).

Because resolution is defined by the ability to differentiate between two close-by point sources, we first demonstrate resolution enhancement with a pair of QDs that are separated by a distance shorter than the diffraction limit. Fig. 2 shows the 2nd-, 4th-, 9th-, 16th-, and 25th-order cumulant SOFI images of two close-by QDs. It is clear from Fig. 2 that as the cumulant order is increased the PSF shrinks and the two QDs are better resolved. The dotted line in Fig. 2 denotes the cross-section through the PSF of a single QD. This intensity cross-section (Fig. 2, dotted line) was fitted with a 1D Gaussian model for all calculated cumulants orders. Fig. 3 shows the profiles (dots) and the fits (lines). Note that the PSF in the original (intensity) image comprises an offset caused by constant, uncorrelated background signal, which disappears in the SOFI images. Comparing the signal-to-background (S/B) ratios of the original image with the second-order SOFI image, we observe a 130-fold improvement (S/Boriginal = 1.9; S/Bsecond-order = 250). Fig. 3 shows the FWHM values of the Gaussian fits plotted versus the cumulant order. This curve was fitted with a power law: FWHM(n) = \( \omega_{50} \cdot e^{-\frac{n}{2}} \), \( \omega_{50} \) being the cumulants order and \( \omega_{50} \) being the FWHM value of the raw image. The fit yielded \( \omega_{50} = 289 \pm 2 \) nm and \( \mu = 0.51 \pm 0.01 \), in agreement with the expected square-root scaling of the resolution enhancement with the cumulant order. The 25th-order yielded an image with a FWHM = 55 ± 3 nm, corresponding to a 5-fold resolution enhancement in 2D. It is evident from Eq. 4 that the resolution enhancement takes place along all three dimensions.

Discussion

We have introduced a super-resolution technique and demonstrated that by using higher-order statistics the optical resolution is increased the PSF shrinks and the two QDs are better resolved. The isosurfaces for \( I_z \) axis (300-nm steps). Surfaces were smoothed with a Gaussian smoothing algorithm. The isosurfaces for \( I_{max} \cdot e^{-\frac{n}{2}} \) are shown. Starting from the outer-most isosurface, the original PSF is shown followed by orders 2nd, 3rd, 4th, and 16th. PSF aberrations are noticeable at the top of the original PSF image.

![Fig. 4. 3D SOFI. Rendered 3D PSF composed of 2D sections taken along the z axis (300-nm steps). Surfaces were smoothed with a Gaussian smoothing algorithm. The isosurfaces for \( I_{max} \cdot e^{-\frac{n}{2}} \) are shown. Starting from the outer-most isosurface, the original PSF is shown followed by orders 2nd, 3rd, 4th, and 16th. PSF aberrations are noticeable at the top of the original PSF image.](image-url)

![Fig. 5. SOFI images of cells. Wide-field image of QD625 labeled 3T3 cells. (A) Original image generated by time averaging all frames of the acquired movie (3,000 frames, 100 ms per frame). (B) The image in A deconvolved. (C) Second-order SOFI image. (D) The image in C deconvolved. (E–H) Magnified views of the boxed regions in A–D. (Scale bars: A–D, 2 μm; E–H, 500 nm.)](image-url)
of a conventional microscope can be increased far beyond the diffraction limit (\( \sqrt{\pi} \) times the FWHM of the PSF, where \( \pi \) is the statistical order) while simultaneously enhancing the image contrast. We argue that no other super-resolution microscopy technique can compete with the simplicity of the SOFI approach and its undemanding requirements with regard to fluorescent labels, optics, and other hardware. The experimental procedure essentially amounts to taking a movie of a fluctuating signal. Other correlation-based imaging methods such as image correlation spectroscopy (ICS) and related methods such as Raster ICS and spatiotemporal (STICS) (19, 20, 23), diffusion imaging (21), OLID, or fluorescence cumulant analysis (22) do not exploit the super-resolution imaging potential of higher-order statistics. Comparing SOFI specifically with STICS reveals a striking analogy in formulas, because both methods use image series and temporal correlations. However, in STICS spatial correlation images are calculated from each image (or parts thereof) combined with a temporal correlation on these spatial correlation images. The behavior of this spatiotemporal correlation image series allows the quantification and separation of molecular transport properties, although no super-resolution imaging. Lidke et al. (24) have developed a super-resolution imaging method that is based on independent component analysis and blinking statistics of QDs. They demonstrated that this method is capable to resolve QDs that are closely spaced below the diffraction limit. As pointed out by Lidke et al., underestimating or overestimating the number of QDs can affect the accuracy in determination of the loci of the (incorrect number of) emitters. In SOFI no such a priori knowledge is necessary.

Also dynamic speckle illumination microscopy (DSI) (25–27) is based on the evaluation of fluctuations in the observed signal, but ones that are induced by the excitation and subsequently evaluated in an analogous way to SOFI. Even though this approach yields sectioning along the optical axis, the fact that the fluctuations are not stemming from independent microscopic (i.e., subdiffraction sized) emitters but from diffraction limited speckles imposes a fundamental limit on DSI resolution, because it is diffraction limited. The similarities between SOFI and the above-mentioned methods have their origin solely in the common mathematical concept of correlation functions. However, a detailed analysis of these approaches reveals dramatic differences in capabilities and resulting effects.

We verified that the SOFI concept leads to an increase in optical resolution along all three directions. Furthermore we demonstrated that SOFI is suitable for biological imaging applications as shown on fluorescently labeled, fixed 3T3 fibroblasts, achieving background-free, contrast-enhanced, super-resolution images. An additional benefit of the SOFI images is that deconvolution algorithms tend to perform better on these background-free images.

The main limitation of SOFI is the brightness scaling of the images; the higher the order, the larger initial differences in brightness will grow. This results in very large dynamic range images and can lead to masking effects of dim emitters in proximity to bright emitters. SOFI can be regarded as a single-molecule technique, because it relies on the detection of fluctuations from single emitters. Even though SOFI theoretically eliminates any kind of noise (noise is per definition not correlated over time), it is sensitive to the signal-to-noise ratio because acquisition times are limited (e.g., caused by photobleaching). Limited measurement times lead to a “correlation” noise in SOFI, which is not any different from other correlation-based methods such as fluorescence correlation spectroscopy (FCS). There are several publications characterizing noise in FCS measurements (as, for example, refs. 28 and 29). To reduce noise in a SOFI image, measurement times have to be extended or the signal-to-noise ratio has to be improved until the desired image quality is achieved.

SOFI has the potential to perform super-resolution imaging, at high frame rates. The acquisition speed in this work was limited mainly by the blinking behavior of QDs. Because of their power-law fluorescence on/off distribution (18, 30–32), some QDs exhibit strong blinking, whereas others might remain in the on state for a few seconds of movie acquisition, leading to very different brightness values in the SOFI images. By choosing probes displaying a uniform blinking rate, one should be able to drastically shorten the acquisition times. Also, a more uniform blinking could help to resolve the brightness scaling problem of SOFI (because SOFI brightness depends on the molecular brightness and the molecular blinking behavior). In terms of flexibility, SOFI is not limited to work with high numerical aperture microscope objectives and thus could be used in imaging applications, where its main advantage could be background reduction. Last, SOFI is not limited to blinking between fluorescent on and off states. Any (even nonfluorescent) fluctuating objects, such as rotating dipoles, or blinking of celestial objects, such as binary stars, could be imaged and superresolved by SOFI.

**Methods**

All experiments were carried out using QD625 secondary antibodies (Invitrogen) QD. 3T3 cells were fixed and labeled by using a standard immunostaining protocol (for details refer to SI Text).
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