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Optical fluctuation microscopy based on calculating local entropy values

Idir Yahiatène [a,b], Simon Hennig [b], Thomas Huser [a,b,*]

[a] Center for Biophotonics Science and Technology, University of California, Davis, Sacramento, 2700 Stockton Blvd, 95817 CA, USA
[b] Biomolecular Photonics, Department of Physics, University of Bielefeld, 33615 Bielefeld, Germany

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ABSTRACT

We demonstrate a novel and easy-to-use method to dramatically reduce noise and background contributions in advanced fluorescence microscopy experiments. The underlying idea is that the entropy value increases for systems with a large number of accessible energy states. Intensity fluctuations originating from photophysical or photochemical effects lead to an increased information content. Calculating the pixel-wise entropy value results in an enhancement of the signal-to-noise ratio by a factor of 90–100. Comparing ECI (entropy-based contrast-enhanced imaging) to superresolution methods such as STORM and SOFI, we find that this technique also bears substantial potential for enhancing fluctuation-based superresolution microscopies.

1. Introduction

In fluorescence microscopy one of the most widely encountered problems is poor contrast or poor signal-to-noise ratios (SNR) where the SNR is defined as $\mathrm{SNR} = \frac{I_{\mathrm{signal}}}{I_{\mathrm{noise}}}$. Poor SNRs might prevent one from distinguishing fluorescence signals from autofluorescent background or inelastically scattered light. Here, we define 'noise' as any signal which does not originate from the sample, including electronic shot-noise and background fluorescence (such as out-of-focus contributions). In order to overcome poor SNR values, one typically has to find ways to remove or minimize noise from the images, which typically requires changes in experimental conditions by experimenting with different substrates, or choosing fluorophores with different spectral properties. Image processing steps, such as applying thresholding methods can also be utilized without losing relevant information. A common thresholding algorithm is based on multi – or bimodal histogram-formation of all pixels to obtain two separate mean values. One value corresponds to the average intensity of the noise, while the other value represents the mean signal intensity. Therefore, a clear separation between the two is possible by setting the threshold to a value above the noise level. Another straightforward method for noise reduction is a simple mean filter. This algorithm relies on averaging the values of two (or more) neighboring pixels to increase the overall SNR. This typically results in a blurred, but noise-reduced image. Based on this method, a GAUSSIAN blur algorithm can also be applied where the input image is convolved with a GAUSSIAN function leading to an overall smoothed image. A more recent algorithm for noise reduction in image processing was published by Ooi et al. [1], who describe the Toboggan method which is also based on GAUSSIAN filtering. In essence, this method receives the GAUSSIAN-smoothed images as input data and groups the pixels according to their local gradient magnitude. Pixels from the same group are then all assigned the same intensity value. Other, more advanced techniques make use of spatial averaging together with temporal information and utilize adaptive algorithms in order to retain the high spatial resolution [2-4]).

The more widespread, common noise-reduction techniques using averaging filters tend to be very simple and inefficient resulting in less than desirable outcomes, while the more sophisticated ones techniques tend to be computationally very expensive. Here, we present a method that makes use of the different statistical nature of signal and noise to remove noise from images and improve SNRs. Our method is based on the symmetry information contained in intensity distributions of time-dependent signals. The degree of intensity fluctuations is measured by evaluating the entropy information contained in the pixel time traces of frames acquired as part of many-frame fluorescence microscopy movie files to distinguish fluorescent molecules from noise.

2. Methods

To enhance the contrast in fluorescence microscopy images we developed a method based on thermodynamics and information theory considerations [5]. After acquiring a rapid sequence of images (i.e., a movie) of fluorophores exhibiting random, time-dependent fluorescence fluctuations (as used e.g. in super-resolution microscopies [6-9] as implemented in the STORM/PALM or SOFI methods

Abbreviations: SNR, signal-to-noise ratio; ECI, entropy-based contrast-enhanced imaging; QD, quantum dot; PSF, pointspread function.

* Corresponding author. Address: Biomolecular Photonics, University of Bielefeld, Universitätsstraße 25, 33615 Bielefeld, Germany.

E-mail addresses: iyahiate@physik.uni-bielefeld.de (I. Yahiatène), shennig@physik.uni-bielefeld.de (S. Hennig), thomas.huser@physik.uni-bielefeld.de (T. Huser).

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[10-18], the final contrast-enhanced image will be reconstructed using the entropy information as new pixel values. Every new image pixel value is calculated using the Shannon-definition of entropy

$$H_0 = \sum_{i=1}^{n} p_i \cdot I(p_i) = -\sum_{i=1}^{n} p_i \cdot \log_{10}(p_i) \quad (1)$$

which is the expectation value of the information content of a time-dependent series defined as

$$I(p) = -\log_{10}(p), \quad (2)$$

where p is the probability (or the relative number) of a specific intensity value to occur in this time-dependent signal. Practically speaking, all intensity values are binned and their relative frequency is calculated, leading to a histogram of the probability for any intensity value. A schematical representation of this procedure is depicted in Figure 1. Here we would like to point out that this entropy-based method does not necessarily require a time-dependent and continuous signal as input data to operate. However, all data points of the series represent independent snapshots of energetic states of a given thermodynamic system. Using the entropy values, in principle, any set of equations that deals with energy considerations can potentially be beneficial to improve our method further. Analyzing data with the algorithm described here is fairly straightforward. As a first step, data acquisition is achieved by imaging a fluorescent sample using a standard inverted widefield microscope and saving the data as an image sequence. Subsequently, the registered intensities will be binned using a constant bin width along the time axis and the relative number of the intensity values is calculated to estimate the probability of this value's occurrence. As a last step the entropy is calculated pixel wise according to Eq. (1) or if the degree of denoising needs to be set, Eq. (3) has to be applied. Since only one sum has to be computed the image can be reconstructed in linear time depending on the bit depth of the input image sequence and therefore the maximum intensity of the current pixel trace.

The basic idea of entropy-based contrast-enhanced imaging (ECI) is the fact that the number of thermodynamically accessible states of a given system increases with higher entropy values. In this interpretation the greater the number of different intensity values are which are registered in a pixel, the higher is its entropy. Every photophysical process that generates time-dependent intensity fluctuations and thus a broad distribution of intensity values results in high entropy values. As the number of sources leading to intensity fluctuations is much higher than the sources for noise (with constant mean), the resulting entropy values originating from a fluctuating emitter are high and can easily be discriminated from noise. In principle this method is not shot-noise limited. Image sequences containing only camera/detector noise are reconstructed as black ECI images.

Semiconductor nanocrystals or quantum dots as fluorescent probes exhibit blinking on all time scales which turns them into ideal fluorescent probes for ECI applications [19,20]. The large number of different intensity values for qdots leads to a broad distribution and thus to a large standard deviation of the underlying distribution. We can use higher order powers of the empirical standard deviation

$$s = \sqrt{\frac{1}{t-1}\sum_{i=1}^{t}(I_i - \bar{I})^2}$$

as an entropy weighting factor, where t is the number of frames acquired, I_i the current pixel intensity value and $\bar{I}$ the average pixel intensity of the particular pixel trace. One can then define the degree (or: order) of denoising and thus the SNR improvement by modifying expression (1) to

$$H(n) = H_0 \cdot s_{k,j}^{n} \quad (3)$$

where k and j denote the coordinates of the current pixel trace being processed.

The computational effort (Figure S1) to reconstruct an ECI image basically scales linearly with the bit depth of the original image stack and the bin width according to the histogramming step of the ECI algorithm. The number of iterations can be drastically decreased with an increased bin width due to a decreasing number of bins. For a constant bit depth (and a constant number of bins) the time to reconstruct one pixel in the ECI image is almost independent of the ECI order and thus constant.

3. Results and discussion

To characterize the capabilities of ECI we first performed ECI analyses of simulations of images containing intensity-fluctuating molecules. The point spread function of the simulated molecules is represented by a 2D GAUSSIAN density function

$$I_{2D}(x,y) = I_0 \cdot \exp\left(-\left(\frac{(x-x_0)^2}{2s_x^2} + \frac{(y-y_0)^2}{2s_y^2}\right)\right) \quad (4)$$

where I_0 is the maximum intensity at position x_0, y_0 in the image. s_x and s_y denote the standard deviations (which are proportional to the FWHM) in the x and y direction. For all simulations we assumed that $s_x = s_y$. The simulated data was generated and analyzed using custom-written code (C++, Code::Blocks IDE Ver. 10.05) including the freely available image library CImg for image data processing. For all simulations we additionally added Poissonian noise

$$P_L(k) = \frac{\lambda^L}{k!} e^{-L}$$

according to the statistical nature of light, where k is an integer and L denotes the expectation value and the variance of the distribution.

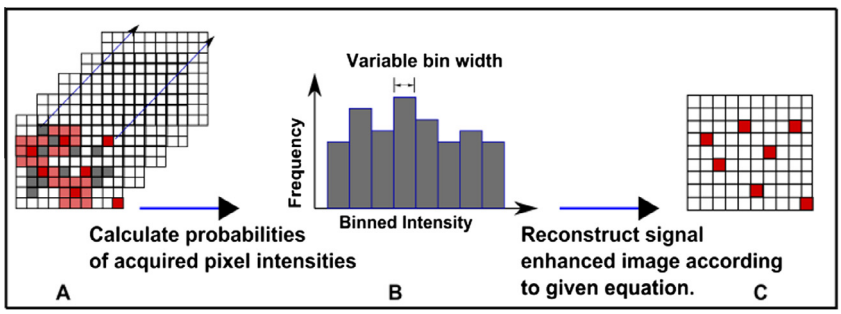

Figure 1. Demonstration of the ECI scheme. Calculation of the intensity probabilities by histogramming and subsequent pixel-wise computation of the Shannon entropy value to reconstruct the SNR-enhanced image.