Additional file 1: Theory, algorithm, protocols and estimation accuracies

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Theory

The intensity distribution measured over time on a detector pixel $\vec{r}$ of a sample composed of M independently fluctuating emitters can be written as

$$I(\vec{r},t) = \sum_{k=1}^{M} \epsilon_k U\left(\vec{r}-\vec{r}_k\right) s_k(t) + b\left(\vec{r}\right), \tag{1}$$

where ϵ_k, $\vec{r}_k$ and s_k denote the emitter's brightness, its position and its normalized temporal fluctuation signal. $U(\vec{r})$ is the system's point-spread function (PSF) and b represents a temporally constant background term. Taking the n-th order temporal cumulant without time lags leads to

$$\kappa_n(\vec{r}) = \sum_{k=1}^{M} \kappa_n\left\{\epsilon_k U\left(\vec{r}-\vec{r}_k\right) s_k(t)\right\}_t + \kappa_n\left\{b\left(\vec{r}\right)\right\}_t = \sum_{k=1}^{M} \epsilon_k^n U^n(\vec{r}-\vec{r}_k)\omega_{n,k} \tag{2}$$

with $\omega_{n,k} = \kappa_n\left\{s_k(t)\right\}_t$ a weighting factor depending on the blinking properties of the emitter k. If we approximate $U(\vec{r})$ by a Gaussian, $U^n(\vec{r})$ yields a Gaussian PSF with a $\sqrt{n}$-times reduced size in all dimensions leading to a resolution improved by a factor $\sqrt{n}$. Furthermore, applying a simple reweighting scheme in the Fourier domain of the cumulant enables a final resolution improvement that scales linearly with the cumulant order [1].

In addition to structure, higher-order cumulants contain information on the photo-physics of individual emitters. This information is contained in the weighting factors $\omega_{n,k} = \kappa_n\left\{s_k(t)\right\}_t$ and can be extracted by combining several cumulant orders.

In the simplest case, the emitter fluctuates between two states only, i.e. a bright S_{on} and a dark state S_{off}. The relative durations of S_{on} and S_{off}, can be defined as

$$\rho_{\text{on}} = \frac{\tau_{\text{on}}}{\tau_{\text{on}} + \tau_{\text{off}}} \tag{3}$$

$$\rho_{\text{off}} = \frac{\tau_{\text{off}}}{\tau_{\text{on}} + \tau_{\text{off}}} = 1 - \rho_{\text{on}}, \tag{4}$$

where τ_{on} and τ_{off} are the characteristic lifetimes of S_{on} and S_{off}. We may define the fluctuation signal to be 1 when the emitter is in state S_{on} and $\xi \in [0, 1[$ if it is in state S_{off}. The weighting factors $\omega_{n,k}$ then become

$$\begin{aligned}
\omega_{1,k} &= (1-\xi_k)\rho_{\text{on},k} \\
\omega_{2,k} &= (1-\xi_k)^2\rho_{\text{on},k}(1-\rho_{\text{on},k}) \\
\omega_{3,k} &= (1-\xi_k)^3\rho_{\text{on},k}(1-\rho_{\text{on},k})(1-2\rho_{\text{on},k}) \\
\omega_{4,k} &= (1-\xi_k)^4\rho_{\text{on},k}(1-\rho_{\text{on},k})(1-6\rho_{\text{on},k}+6\rho_{\text{on},k}^2) \\
&\vdots \\
\omega_{n,k} &= (1-\xi_k)^n f_n(\rho_{\text{on},k}),
\end{aligned} \tag{5}$$

with $f_n(\rho_{\text{on},k}) = \rho_{\text{on},k}(1-\rho_{\text{on},k})\frac{\partial f_{n-1}}{\partial \rho_{\text{on},k}}$ the n-th order cumulant of a Bernoulli distribution with probability $\rho_{\text{on},k}$.

Since parameters such as the molecular state lifetimes and the molecular brightness often depend on the local environment (for instance the concentration of reducing and oxidizing agents influences the dark-state lifetime of organic fluorophores), we assume region-dependent but locally constant on-time ratios and amplitudes. The overall cumulants can then be approximated by

$$\begin{aligned}
\kappa_1(\vec{r}) &= \widetilde{\epsilon}(\vec{r})\rho_{\text{on}}(\vec{r})\sum_{k=1}^{N} U(\vec{r}-\vec{r}_k) + b(\vec{r}) \approx N(\vec{r})\mathcal{E}_V\left\{U(\vec{x})\right\}\widetilde{\epsilon}(\vec{r})\rho_{\text{on}}(\vec{r}) + b(\vec{r}) \\
\kappa_2(\vec{r}) &\approx N(\vec{r})\mathcal{E}_V\left\{U^2(\vec{x})\right\}\widetilde{\epsilon}^2(\vec{r})\rho_{\text{on}}(\vec{r})\left(1-\rho_{\text{on}}(\vec{r})\right) \\
\kappa_3(\vec{r}) &\approx N(\vec{r})\mathcal{E}_V\left\{U^3(\vec{x})\right\}\widetilde{\epsilon}^3(\vec{r})\rho_{\text{on}}(\vec{r})\left(1-\rho_{\text{on}}(\vec{r})\right)\left(1-2\rho_{\text{on}}(\vec{r})\right) \\
\kappa_4(\vec{r}) &\approx N(\vec{r})\mathcal{E}_V\left\{U^4(\vec{x})\right\}\widetilde{\epsilon}^4(\vec{r})\rho_{\text{on}}(\vec{r})\left(1-\rho_{\text{on}}(\vec{r})\right)\left(1-6\rho_{\text{on}}(\vec{r})+6\rho_{\text{on}}^2(\vec{r})\right) \\
&\vdots \\
\kappa_n(\vec{r}) &\approx N(\vec{r})\mathcal{E}_V\left\{U^n(\vec{x})\right\}\widetilde{\epsilon}^n(\vec{r})f_n(\rho_{\text{on}};\vec{r}),
\end{aligned} \tag{6}$$

where $\mathcal{E}_V\{U^n(\vec{x})\} = 1/V \int_V U^n(\vec{x})d\vec{x}$ is the expectation value of $U^n(\vec{x})$ or the n-th moment of $U(\vec{x})$ (see [2] for some examples) and $N(\vec{r})$ is the number of molecules inside a detection volume V centered at $\vec{r}$. For a 3D Gaussian PSF, the n-th order moment can be written as

$$\mathcal{E}_V\{U^n_{\text{3D Gauss}}(\vec{x})\} = \frac{c(\sigma_{\text{x,y}}, \sigma_z)}{n^{3/2}}, \tag{7}$$

with $c(\sigma_{\text{x,y}}, \sigma_z)$ a constant depending on the spatial extent of the PSF. In our present case, with a total internal reflection (TIR) illumination and a Gaussian approximation of the detection PSF, we find

$$\mathcal{E}_V\{U^n_{\text{TIR Gauss}}(\vec{x})\} = \frac{c(\sigma_{\text{x,y}}, \sigma_z, d_z)}{n^2}, \tag{8}$$

with d_z the characteristic penetration depth of the TIR illumination. Finally we can write

$$\kappa_n(\vec{r}) \approx N(\vec{r})\frac{c(\sigma_{\text{x,y}}, \sigma_z, d_z)}{n^2}\widetilde{\epsilon}^n(\vec{r})f_n(\rho_{\text{on}}; \vec{r}), \tag{9}$$

which enables the pixel-wise estimation of N, $\widetilde{\epsilon}$ (related to the spatial distribution of the average molecular amplitude) and $\rho_{\text{on,off}}$ by considering three or more cumulant orders. To estimate the parameters of a two-state system, the cumulant orders two to four can be used to build the ratios

$$\begin{aligned} K_1(\vec{r}) &= \frac{3^2\kappa_3}{2^2\kappa_2}(\vec{r}) = \widetilde{\epsilon}(\vec{r})\left(1 - 2\rho_{\text{on}}(\vec{r})\right) \\ K_2(\vec{r}) &= \frac{4^2\kappa_4}{2^2\kappa_2}(\vec{r}) = \widetilde{\epsilon}^2(\vec{r})\left(1 - 6\rho_{\text{on}}(\vec{r}) + 6\rho^2_{\text{on}}(\vec{r})\right) \end{aligned} \tag{10}$$

and solving for the amplitude

$$\widetilde{\epsilon}(\vec{r}) = \sqrt{3K_1^2(\vec{r}) - 2K_2(\vec{r})}, \tag{11}$$

the on-time ratio

$$\rho_{\text{on}}(\vec{r}) = \frac{1}{2} - \frac{K_1(\vec{r})}{2\widetilde{\epsilon}(\vec{r})}, \tag{12}$$

and the number of particles

$$N(\vec{r}) = \frac{\kappa_2(\vec{r})}{\widetilde{\epsilon}^2(\vec{r})\rho_{\text{on}}(\vec{r})\left(1 - \rho_{\text{on}}(\vec{r})\right)}. \tag{13}$$

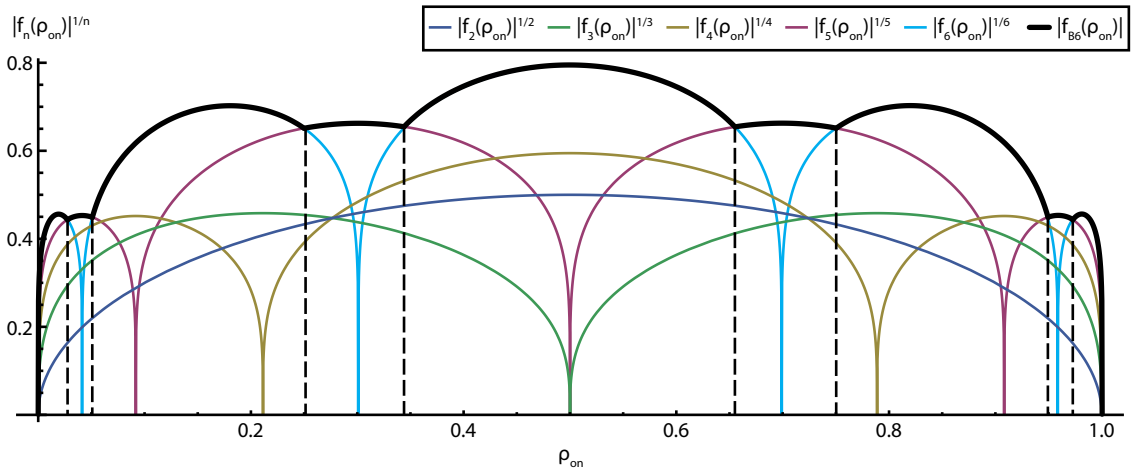

Figure 1: Determination of on-ratio thresholds using $|f_n(\rho_{\text{on}})|^{1/n}$ for combining different cumulant orders (exemplified with the 6th order balanced cumulant in black).

Software

We used a custom MATLAB (The MathWorks, Inc.) code for computing higher-order cross-cumulants according to an algorithm presented previously [3]. For the Lucy-Richardson deconvolution of the resulting cumulants we used the MATLAB implementation `deconvlucy`. The cumulant balancing after deconvolution and brightness linearization involves the combination of cumulant orders n and $n-1$ and first requires an interpolation step to obtain the same number of pixels for both orders. We then used the accordingly interpolated on-ratio map for identifying the image regions exhibiting an $f_n(\vec{r})$ close to zero. The thresholds have been chosen according to Figure 1, where it is shown for the 6th order balanced cumulant.

Estimation accuracy

In order to get an idea on the accuracies of estimating molecular parameters with the proposed method and for a two-state system under different conditions, we implemented a simulation of randomly blinking fluorophores with a certain background offset and Poisson noise evaluated on a single point.

On-ratio estimation

Among all parameters that can be extracted from the data, the estimation of the on-ratio is the most accurate. Figure 2 illustrates the accuracy of the on-ratio estimation for different single molecule peak signal-to-noise ratios (pSNR). Down to a pSNR of only 10dB, the estimation shows reliable results for all $\rho_{\text{on}} \in (0,1)$. At a very low pSNR of 0dB the estimation still works in the range of $0.1 < \rho_{\text{on}} < 0.9$.

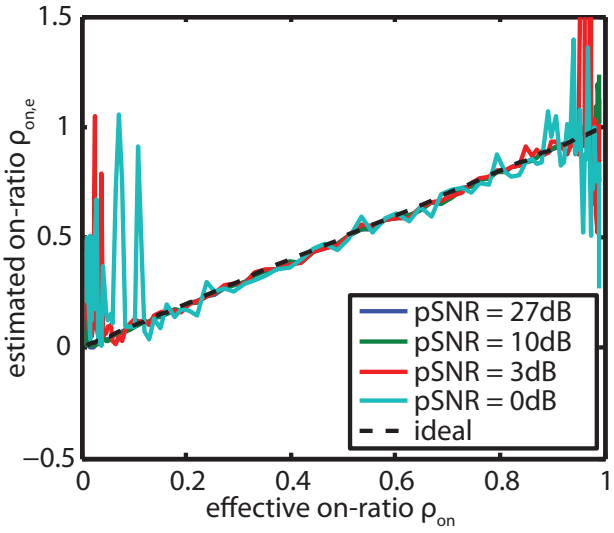

Figure 2: Estimated on-ratio $\rho_{\mathrm{on,e}}$ of a single molecule as a function of the simulated on-ratio ρ_{on} for several pSNRs. Acquisition length: 5000 blinking periods with a temporal double oversampling.

In Figure 3(a) N molecules of equal intensity are hypothetically superposed on the same point. The accuracy of the estimation decreases for a higher number of superposed molecules and improves for low on-ratios. Here, the on-ratio is estimated assuming intentionally and wrongly a 3D molecular distribution and a 3D Gaussian approximation of the PSF (according to equation 8). As the molecules are all located on a single point, this would correspond to a zero-dimensional distribution with $\mathcal{E}\left\{U^n(\vec{r})\right\} = 1$. As a result, there is a small bias barely visible in Figure 3(a) for on-ratios $\rho_{\mathrm{on}} < 0.1$ and $\rho_{\mathrm{on}} > 0.9$.

In Figure 3(b), the additional molecules are uniformly distributed in three dimensions. For more than 10 molecules, the vanishing bias confirmed the correcting moments (equation 8).

Estimation of molecular brightness and density

Figure 4(a) shows the estimation of the brightness in the case of two superposed molecules of different intensities. The simulation demonstrates that the estimated intensity tends towards the value of the brightest molecule. Consequently, the estimation of the absolute number of molecules works well only when the intensities of the molecules are similar at the point of interest (Figure 4(b)). The molecules are counted as long as they are within the detection range with respect to their pSNR. In general, the estimation of the molecular brightness and density is less accurate than the on-ratio estimation and may be used primarily as a qualitative measurement.

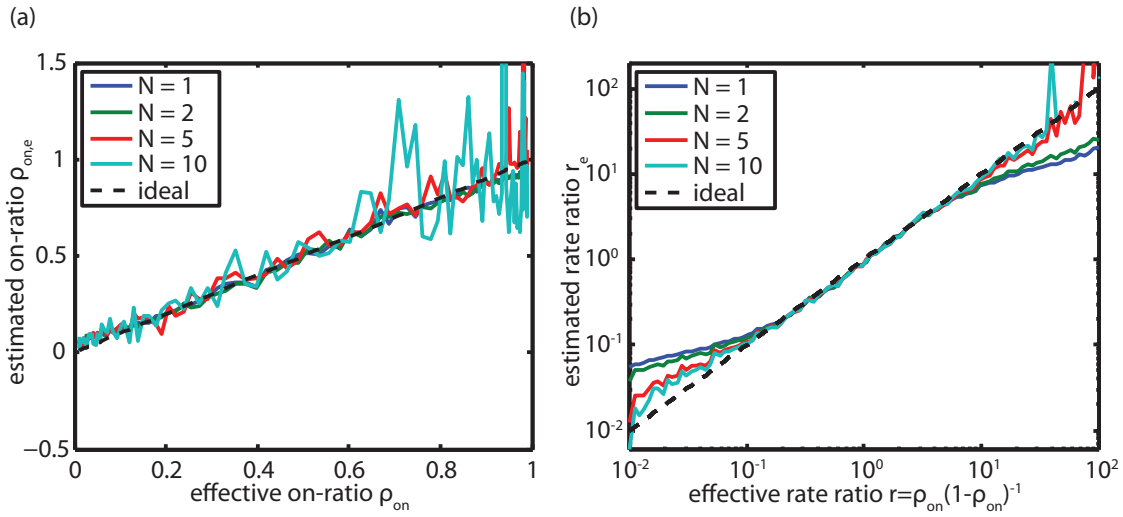

Figure 3: Influence of several superposed molecules on the estimation of the rate ratio **(a)** without and **(b)** with spatial distribution. The on-ratio is estimated using the correcting moments of a 3D PSF (equation 8). **(b)** In the case of a three dimensional molecular distribution, the bias vanishes for more than ≈ 10 molecules.

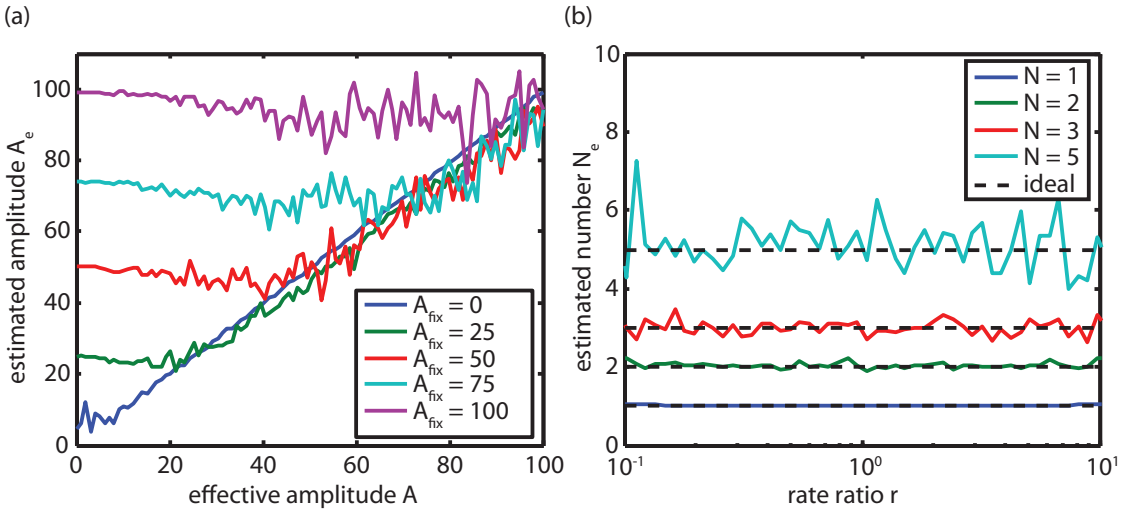

Figure 4: **(a)** Estimation of the molecular brightness A_e for two superposed molecules with the same rate ratio $r = 1$ and different intensities A and A_{fix}. The molecule with the higher intensity is predominant. **(b)** The estimation of the number of molecules N_e of N superposed molecules with equal brightness as a function of the rate ratio.

Protocols

Cell staining

HeLa cells were cultured on glass coverslips. The cells were washed once in PBS prior to fixation with paraformaldehyde (PFA, 4%), and then rinsed 3 times 5 minutes in PBS. The cells were incubated overnight at 4°C with a primary antibody for alpha-tubulin (DM1A, mouse monoclonal, Sigma Aldrich, 1:140) in TBST (Tris-buffered saline and 0.1% Tween20) containing 10% fetal calf serum (FCS).

Cells were washed 3 times 5 minutes in TBST prior to a 2 hours incubation at 4°C with a secondary antibody (donkey anti-mouse, Alexa Fluo 647, diluted 1:400) in TBST containing 10% FCS and washed 3 times 5 minutes in TBST.

The samples were then kept in PBS and stored in the fridge (4°C) for further use.

Imaging buffer

The samples were imaged using a combination of an oxygen scavenger and a thiol. The solutions were prepared using phosphate-buffered saline (PBS) (Sigma Aldrich), 0.5mg/ml glucose oxidase (Sigma Aldrich), 40μg/ml catalase (Sigma Aldrich), 6.6%w/v glucose and 50mM cysteamine (Sigma Aldrich).

References

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