Precisely and accurately localizing single emitters in fluorescence microscopy

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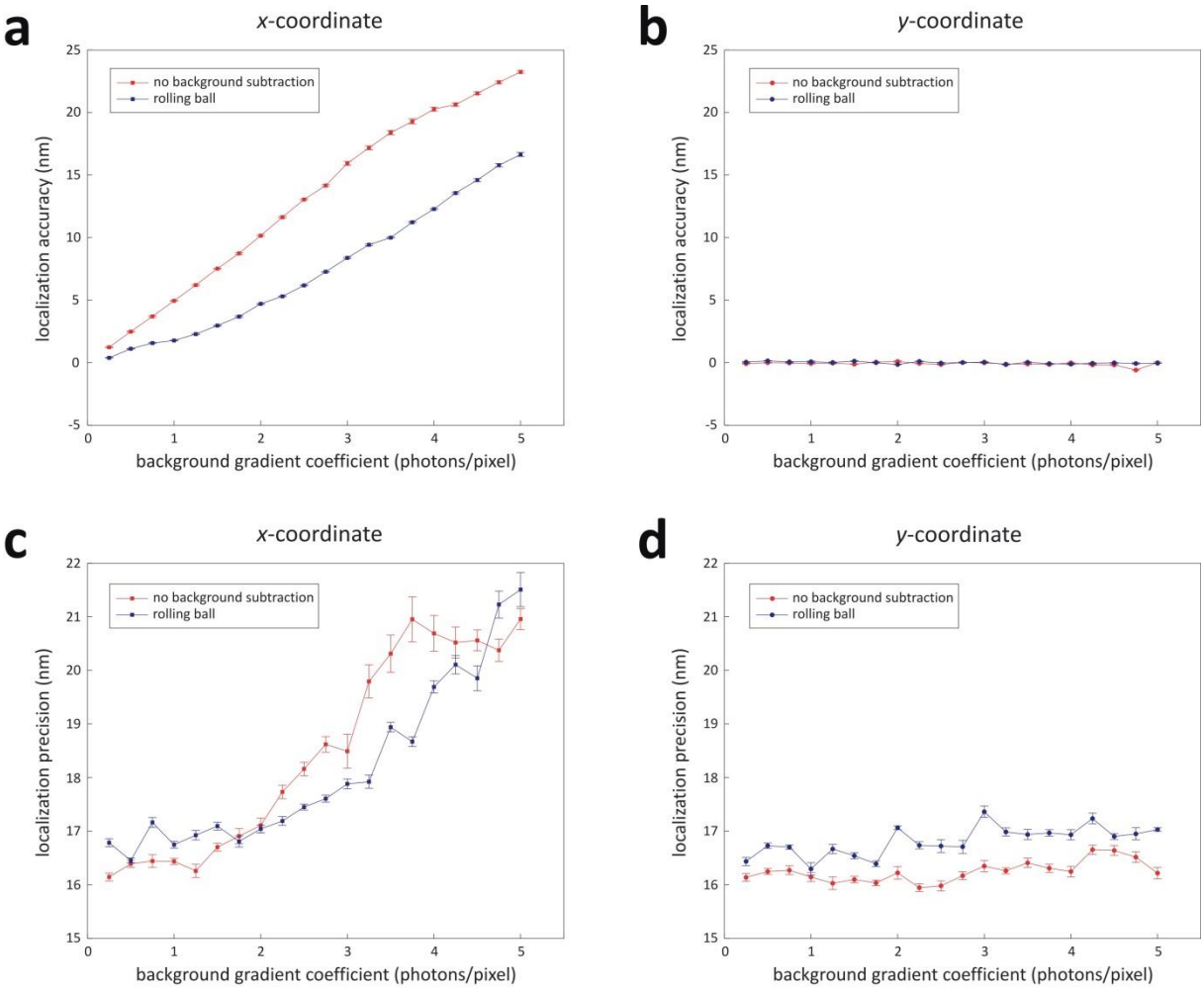
Nature Methods: doi:10.1038/nmeth.2843

Supplementary Figure 1



Supplementary Figure 1 | The influence of a heterogeneous photon background on the localization accuracy and precision. (**a**-**d**) Images of single isotropic emitters at $x = 0$ with a photon background $B = 20 + \beta x$ were simulated for different values of the background gradient coefficient β. The emitter positions were obtained by fitting of a 2-D Gaussian function, which allowed to determine the localization accuracy in the *x*-direction (**a**), the localization accuracy in the *y*-direction (**b**), the localization precision in the *x*-direction (**c**), and the localization precision in the *y*-direction (**d**). In **a**-**d**, the localization precision and accuracy are calculated in case of either background subtraction using the rolling ball algorithm (blue line) or no background subtraction (red line). The error bars show the 95% confidence intervals.

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Supplementary Note 1

The effect of a heterogeneous photon background on the localization accuracy and precision

To investigate the effect of a heterogeneous photon background on the localization accuracy, 10^4 images of individual isotropic emitters were simulated, using Matlab (MathWorks, Natick, MA, USA). The image of each emitter was a 2-D Gaussian with a standard deviation determined by the diffraction-limited resolution for an objective lens with NA = 1.4 and a wavelength λ = 560 nm of the detected light. The amount of emitted photons was 400 in each image and the size of the pixels was 0.1 µm. The background B added to each image varied as a linear function of the x-position ($B = 20 + \beta x$) where the background gradient coefficient β was tested from 0 to 5 photons per pixel in the x-direction, and $x = 0$ was chosen as the center of the image, which itself was 9 × 9 pixels (*i.e.* 0.9 µm × 0.9 µm) in area. Poisson noise was added to the pixel values within the image as appropriate for the number of detected photons in each pixel. A 2-D Gaussian function was then fitted to these simulated images, including free parameters for amplitude, width, x- and y-coordinate of the center position, and a constant offset.

The mean of the fitted coordinates was subtracted from the actual coordinates to yield the localization accuracy, the results are shown in **Supplementary Fig. 1a,b**. The localization accuracy along the x-direction decreases with the background gradient coefficient β. The rolling-ball background subtraction technique with a ball radius of 6 pixels was employed to compensate for the background levels, this improved the localization accuracy for all values of β. The localization precision was calculated as the standard deviation of the distribution of the fitted coordinates, the results are shown in **Supplementary Fig. 1c,d**. The localization precision decreases as a function of the background gradient coefficient β. The localization precision along the x-direction is lower than for the y-direction from $\beta > 2$, for both background subtracted and no background subtraction. The results for $\beta = 0$ agree with the expected localization precision for the given conditions[1, 2].

References

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Supplementary Note 2

Experimental images illustrating the influence of localization precision and labeling density on the resolution in localization microscopy

HeLa Cells were grown under standard conditions in a humidified 5% CO_2 balanced-air atmosphere at 37°C. Cells were incubated for 30 min at room temperature in blocking buffer (phosphate buffered saline containing 3% normal bovine serum albumin and 0.1 % Triton-X-100) and then incubated for one hour in primary monoclonal mouse anti-α-tubulin antibody (Sigma Aldrich, St. Louis, MO, USA) that was diluted 1000× in the blocking buffer. Cells were rinsed three times before incubation with the secondary antibody Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes®, Eugene, OR, USA) that was diluted 500× in the blocking buffer. Finally, the cells were rinsed three times in phosphate buffered saline for 5 min and mounted in the 18 mm low profile Quick Change Chamber (Warner Instruments, Hamden, CT, USA) and embedded in a buffer solution (containing 0.1 M β-mercaptoethylamine).

STORM imaging is performed using a Nikon N-STORM microscope (Nikon, Tokyo, Japan) equipped with four laser lines: a Cube 405 nm (Coherent Inc., Santa Clara, CA, USA), a Sapphire 488 nm (Coherent Inc., Santa Clara, CA, USA), a Sapphire 561 nm (Coherent Inc., Santa Clara, CA, USA), and a 647 nm laser (MPB Communications Inc., Montreal, Quebec, Canada). An Ixon 897 BV EMCCD camera (Andor Technology, Belfast, UK) was used at a frame rate of 45 Hz to collect signal from single emitters. The position of each single emitter was determined after background subtraction through a Gaussian fitting procedure using the STORM macro in NIS Elements (Nikon, Tokyo, Japan). The image was reconstructed by plotting the emitter positions as Gaussian spots, where the standard deviation represents the localization precision.

In order to demonstrate the effect of a decreasing localization precision on image quality and resolution, images were created with an effective lateral localization precision:

$$\sigma_{xy} = \sqrt{\varepsilon_{xy}^2 + \delta^2}$$

Here, ε_{xy} is the localization precision of the original experimental image, and δ is the standard deviation of normally distributed displacements that were added at random to the experimental positions, using Matlab (MathWorks, Natick, MA, USA). The effect of this simulated decrease in localization precision is shown in **Fig. 6a**. The analysis can be stopped when a defined number of molecules have been localized, to show the influence of the label density, as shown in **Fig. 6b**.

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