# SUPPORTING INFORMATION

# Confocal Fluorescence-Lifetime Single-Molecule Localisation Microscopy

*Jan Christoph Thiele,[†] Dominic A. Helmerich,[‡] Nazar Oleksiievets,[†] Roman Tsukanov[†]*

*Eugenia Butkevich,[†] Markus Sauer,[‡] Oleksii Nevskyi[†,*] and Jörg Enderlein[†,¶,*]*

[†] III. Institute of Physics – Biophysics, Georg August University, Göttingen, Germany.

[‡] Department of Biotechnology and Biophysics, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany.

[¶] Cluster of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells" (MBExC), Georg August University, Göttingen, Germany.

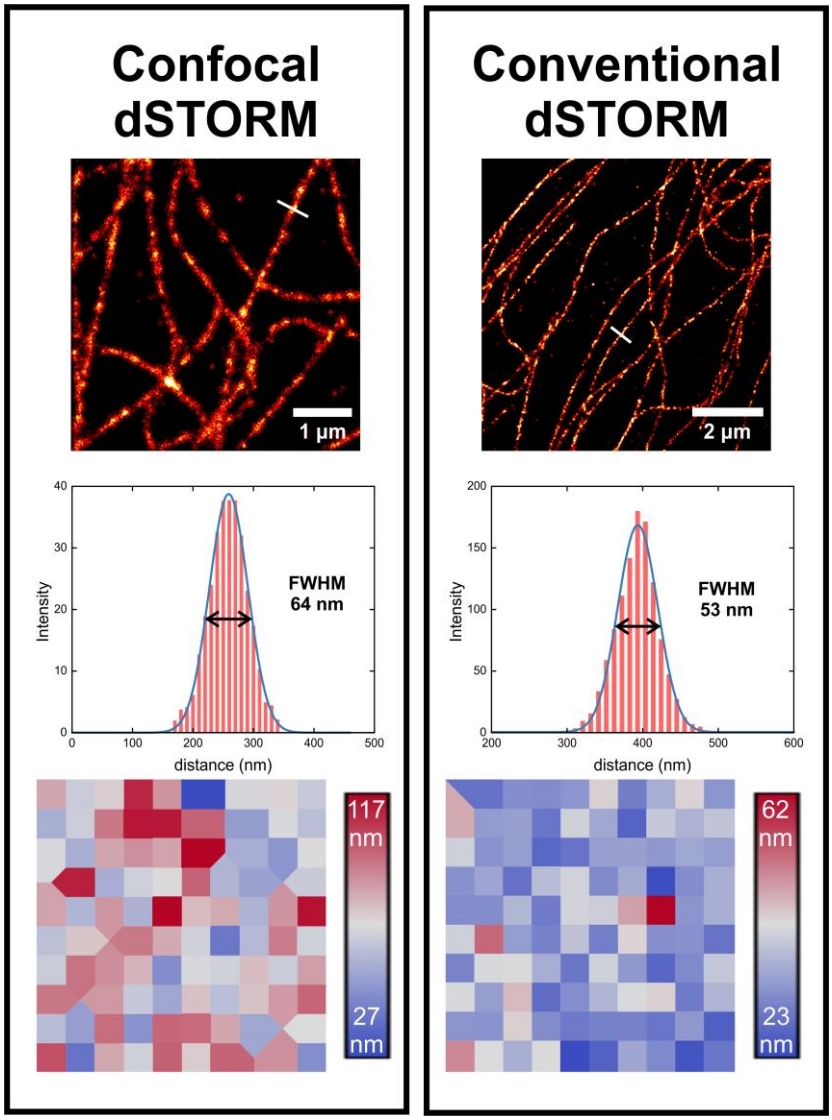


**Figure S1. dSTORM imaging of Alexa 647 labelled tubulin in hMS cells.** (**left**) Confocal dSTORM super-resolved image, intensity profile across the individual microtubule, FRC map of the corresponding region of interest. (**right**) Conventional dSTORM super-resolved image, intensity profile across the individual microtubule, FRC map of the corresponding region of interest.

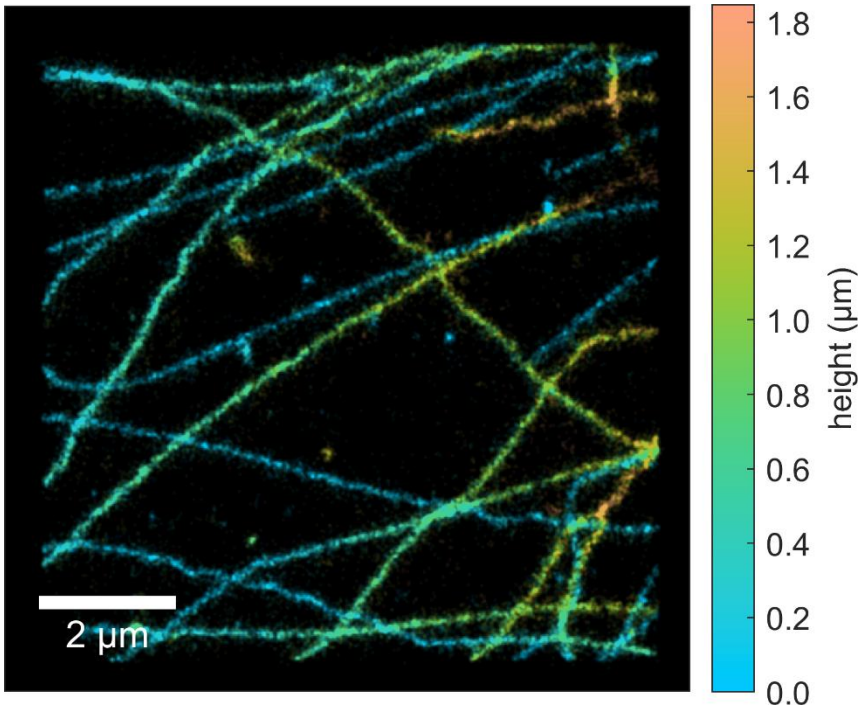


**Figure S2. Confocal sectioning.** 3D dSTORM image of Alexa647 labelled microtubules in fixed COS-7 cells. The localisations are colour-coded according to their *z* position. The image was generated from the corresponding 2.1 µm *z*-stack with the step of 300 nm.

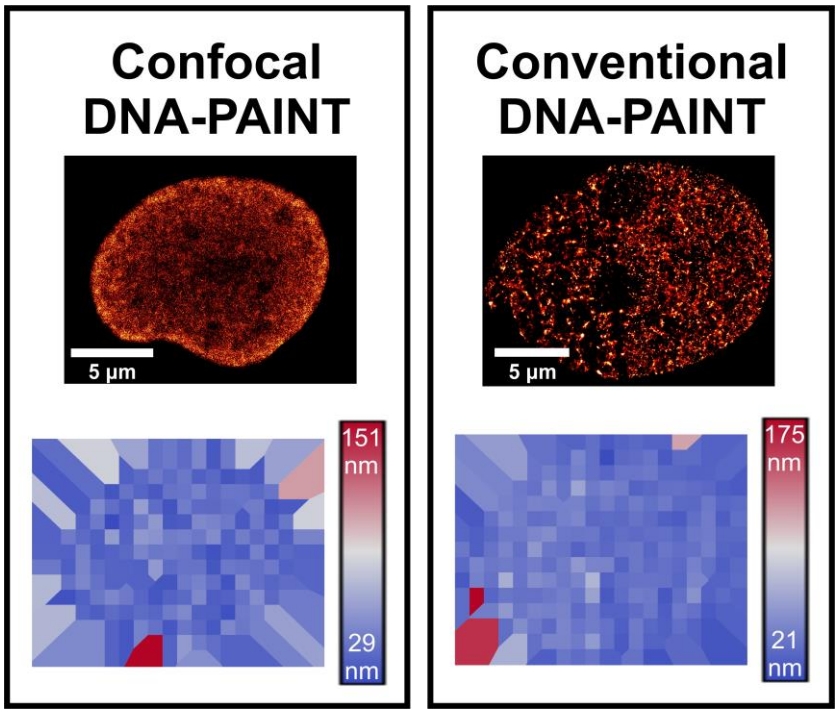


**Figure S3. DNA-PAINT imaging of cellular chromatin in COS-7 cells utilising DNA-labelled ATTO 655.** (**left**) Confocal DNA-PAINT super-resolved image and FRC map of the corresponding region of interest. (**right**) Conventional DNA-PAINT super-resolved image and FRC map of the corresponding region of interest.

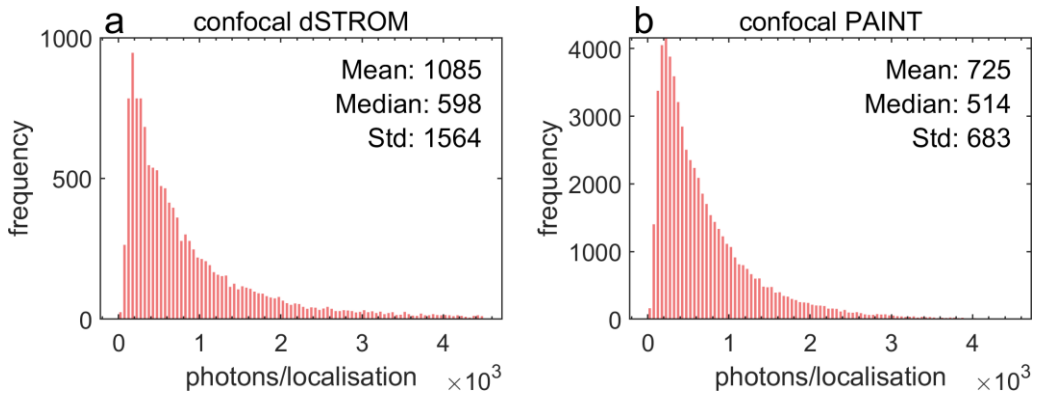


**Figure S4. Histograms of detected photons for cell measurements shown in Fig. 2. (a)** Confocal dSTORM utilizing Alexa 647 labelled secondary antibodies. **(b)** Confocal DNA-PAINT utilising DNA-labelled ATTO 655. The given number of photons is based on the amplitude and width of the fitted Gaussian above the background.

| Name | Sequence 5′→3′ | 5′ Modification | 3′ Modification |
|---|---|---|---|
| Strand 1 for Beads (SB1) | GCAGCCACAACGT TATCATCGATT | - | Alexa 647 / ATTO 655 |
| Strand 2 for Beads (SB2, complementary to SB1) | AATCGATGATAGACGTTGTGGCTGC | biotin | - |
| PAINT Strand 1 (PS1) | GTAATGAAGA | - | ATTO 655 |
| Docking Strand 2 (PS2, complementary to PS1) | TCTTCATTAC | Nanobody | - |

**Table S1. DNA sequences with modifications**

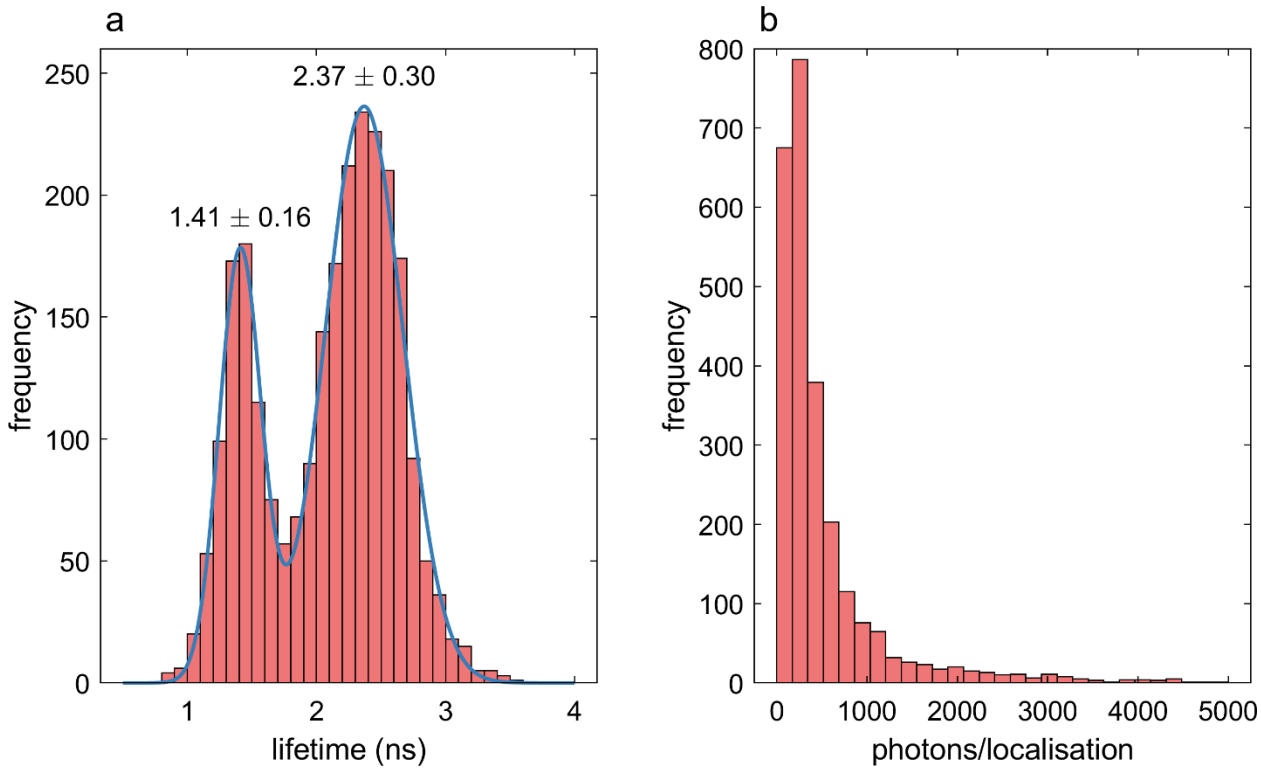


**Figure S5. Histograms corresponding to the localisations in the bead measurement shown in Fig. 3. (a)** Histogram of the fluorescence lifetimes. **(b)** Histogram of the number of photons.

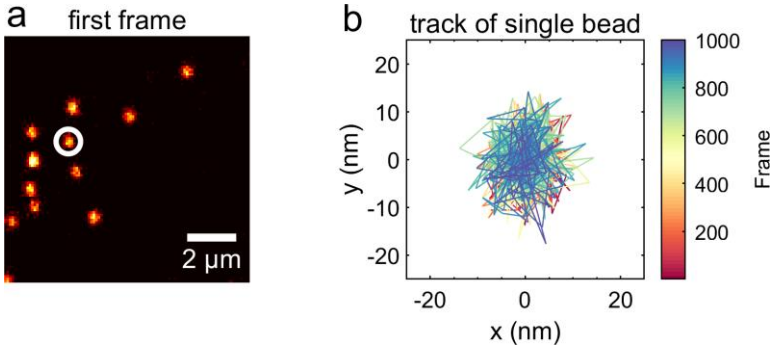


**Figure S6. Scanning confocal measurement of TetraSpeck microspheres. (a)** First frame of the stack. **(b)** Drift corrected position of the highlighted bead.

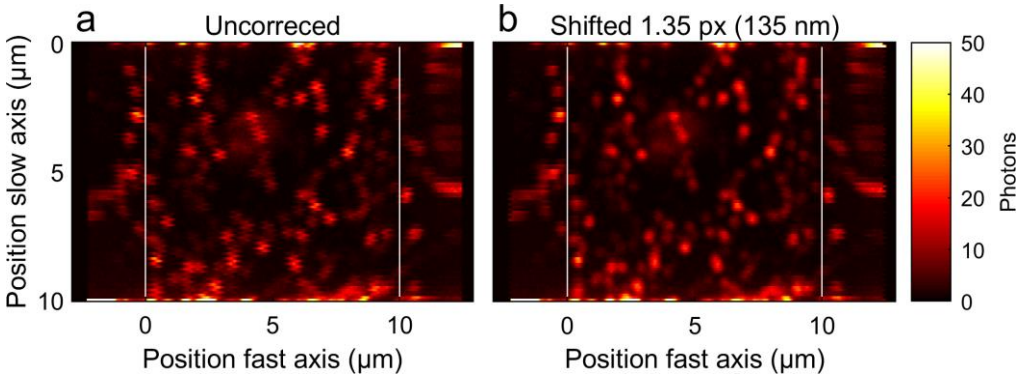


**Figure S7. Correction of line mismatch between forward and reverse scan.**

**(a)** Uncorrected image. **(b)** Corrected image. For the localisation, the central part between the white lines is used and the image is generated with quadratic pixels.

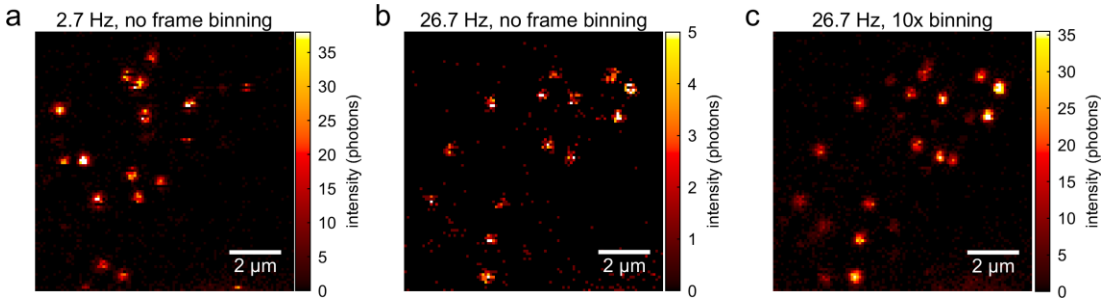


**Figure S8. Influence of the scan speed and frame binning.**

Single frame from a measurement of the same ROI at a frame rate of **(a)** 2.7Hz and **(b, c)** 27 Hz. In **(c)**, 10 subsequent scans were summed (binned) to create one frame. The intensity scale in each panel was adjusted to cover 0 to the 0.999 quantile. Sample: Alexa 647 labelled β-tubulin (COS7 cell) in $D_2O$ with 20 mM MEA.

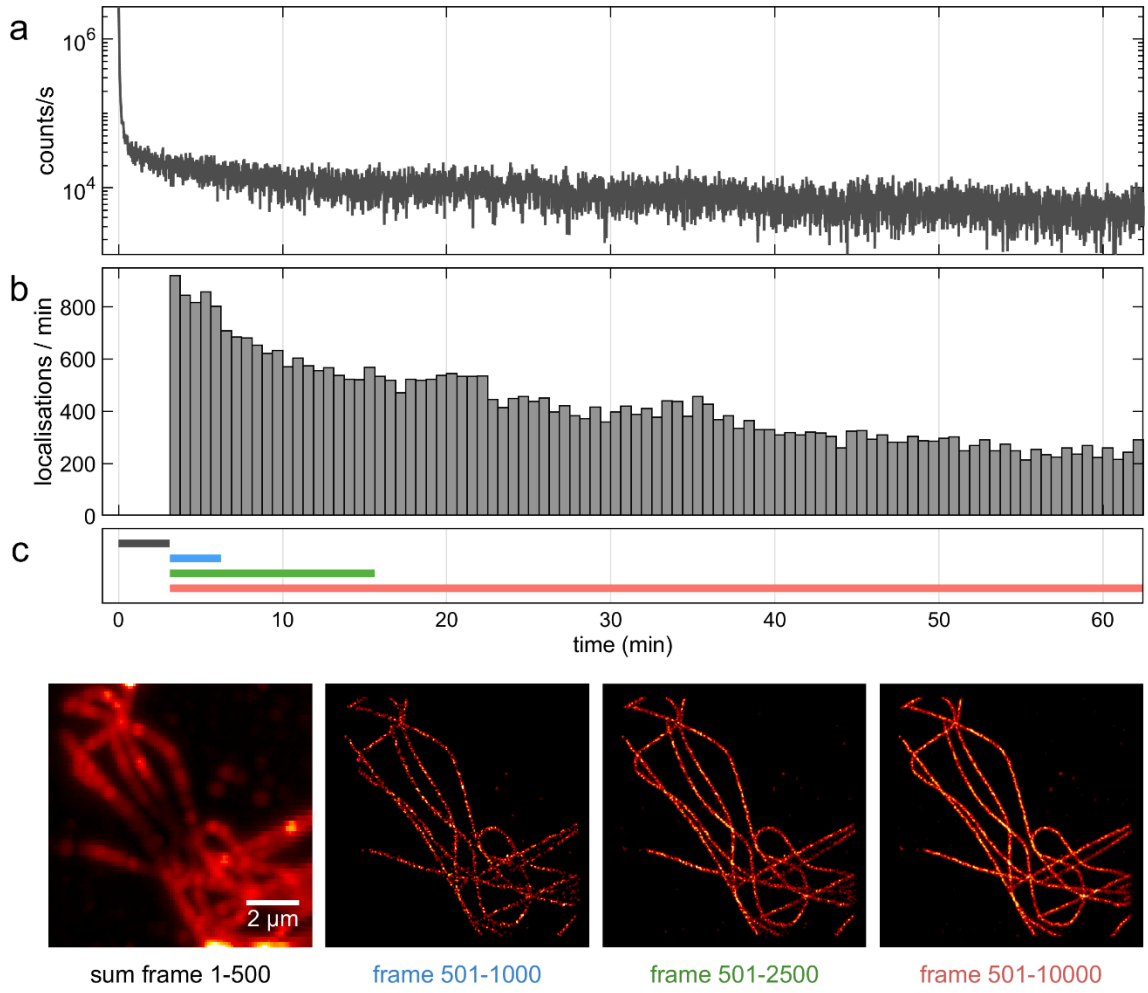


**Figure S9. Reconstructions for different measurement durations, development of count rate and number of localisations over time.**

**(a)** Photon count rate over the course of the measurement. **(b)** Number of localisations over time. During the first several minutes, the fluorophores are switched off. Therefore, the first 500 frames are excluded from this analysis. **(c)** Sum of the first 500 frames and reconstructions including localisation from an increasing number of frames. The intensity scale in each panel was adjusted to cover 0 to the 0.999 quantile. Sample: Alexa 647 labelled β-tubulin (COS7 cell) in $D_2O$ with 20 mM MEA, analysed with 10x frame binning.