# Photometry unlocks 3D information from 2D localization microscopy data

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**Abstract**

We propose a straightforward photometric method called TRABI that allows users to extract 3D information from existing 2D localization microscopy data. TRABI uses the accurate determination of photon numbers within different regions of the emission pattern of single emitters to generate a *z*-dependent photometric parameter. The method can determine fluorophore positions up to 600 nm from the focal plane and can be combined with biplane detection to further improve axial localization.

Single-molecule localization microscopy (SMLM) methods such as photoactivated localization microscopy (PALM)[1] and *direct* stochastic optical reconstruction microscopy (*d*STORM)[2] have become widely used imaging tools. To facilitate 3D cellular imaging with enhanced isotropic resolution, different methods for 3D imaging have been successfully introduced such as astigmatism[3] or multiplane imaging[4,5], although at the cost of increased technical complexity[6-10].

Here, we present a simple 3D SMLM method based on photometric analysis of the fluorescence emission pattern of single emitters that uses an algorithm termed temporal, radial-aperture based intensity-estimation (TRABI). In combination with state-of-the-art localization software packages, it allows the accurate determination of single-molecule intensities.

We reasoned that the photon distribution within a diffraction-limited image (spot) of a single fluorophore, recorded on standard SMLM setups, normalized to the total detected light could be utilized to extract its axial position.. Using two circular apertures around the center of a spot with radii $r_1$ and $r_2$, while $r_1 > r_2$, the intensities within these areas and the photometric ratio $P = I_2 I_1^{-1}$ can be determined. According to a redistribution of photons by defocusing, $P$ then becomes a function of the axial position of the fluorophore (**Fig. 1a**).

Therefore, we first developed a method to accurately determine total photon numbers (TRABI), mainly inspired by classical aperture photometry (AP) (**Supplementary Note 1**)[11,12]. AP is based on subtracting the background from the total intensity signal to determine the fluorophore brightness. The background is estimated by analyzing the local area next to the emitter (**Supplementary Fig. 1**). TRABI advantageously uses fluorescent on- and non-fluorescent off-states of photoswitchable or photoactivatable fluorophores (**Supplementary Fig. 2-4**). In TRABI, a circular aperture with radius $r_1$ and area $A_1$ is encircling the center of the spot (**Fig. 1b**). The integrated signal within this aperture gives the raw intensity $I_{raw,1}$. In contrast to AP, the same aperture is then used to determine the background intensity $I_{BG}$ in a subsequent frame, when the fluorophore resides in its off-state (or bleached state). This way, the local background can be accurately determined to calculate the corrected intensity $I_1 = I_{raw,1} - I_{BG}$, without the need to expand the aperture as used in classical AP (**Supplementary Fig. 4**).

TRABI was implemented in ImageJ for analyzing SMLM image stacks (**Supplementary Software**, **Supplementary Notes 1** and **2**). It reads the localization file of well-established localization software packages such as rapidSTORM[13] and outputs a new text file, which is extended by $I_1$ of the spot as well as background information. In order to avoid contributions from adjacent fluorophores to the background signal, a circular exclusion zone around the spot with radius $2r_1$ is defined and the presence of other single-molecule coordinates checked (**Fig. 1b** and **Supplementary Fig. 2**). In the case of interference, the current frame is skipped and the next frame is analyzed in the same way for determining $I_{BG}$. TRABI also allows averaging over multiple apertures in a number of successive frames $n_{BG}$ to improve statistics for background estimation (**Supplementary Fig 3**). The analysis is complemented by a second circular aperture covering only the central part of the emission pattern ($I_2$) (**Fig. 1a**). For each single-molecule spot TRABI generates the photometric ratio $P = I_2 I_1^{-1} \times 100$ in %.

Using simulations, optimal parameters were determined to reliably measure total spot intensities at high precision, i.e., $r_1 = 1.86 \times$FHWM and $n_{BG} = 7$ (**Supplementary Fig. 3**). Along a range of 500 – 35,000 simulated

photons per spot ($I_{True}$) and 1 – 50 noise photons (s.d.), TRABI provided accurate single-molecule intensities ($I_1 \approx I_{True}$) (**Supplementary Fig. 4**).

TRABI was then tested on experimental data originating from surface-immobilized Cy5 labeled DNA molecules. The total photon numbers ($I_1$) of the experimentally recorded five spots exemplarily shown in **Fig. 1c** were determined to 2,736 – 5,366, thus exceeding alternative intensity estimations by AP and Gaussian fitting. Single-molecule intensities obtained by Gaussian fitting ($I_{Fit}$) were calculated by the localization software, i.e., through determining the areal integral of the 2D Gaussian function that was fitted to the emission profile of the spots, and were significantly underestimated.

Next, we varied the distance of the objective from the single-molecule surface by a piezo scanner (**Fig. 1d**) and recorded every 100 nm 1,000 frames under *d*STORM conditions. For every spot the photometric ratio *P* was determined with $r_1$ = 1.86×FWHM and $r_2 = 5/13 \times r_1$. We generated histograms of *P* for every data set and found a strong z-dependence. Sharply in focus, the median of the distribution was 71.4%, placing the objective 300 nm and 600 nm further apart gave 51.6 and 31.4% (median), respectively (**Fig. 1d**). Because most experimental *P* distributions were not normally distributed (**Supplementary Figs. 5** and **6**), we stated the median as more robust estimator and determined the median absolute deviation (MAD) as error. Despite the symmetry around the focus, the total working range either above or below the focal plane could be determined to at least 600 nm (**Fig. 1e**). To test our approach, we inclined the single-molecule surface by 0.56 ± 0.02° thus creating an axial difference of 668 ± 29 nm within the field of view (i.e., 68.1 µm). As shown in **Fig. 1f** single-molecule events were robustly identified according to their *P* value. Experimentally, we could reproduce the angle of the tilt to 0.52 ± 0.10° (**Supplementary Fig. 7**).

By using the intensity determined by Gaussian fitting ($I_2 = I_{Fit}$) we found a similar z-dependence for *P*, albeit with reduced working range and enlarged relative error (MAD/Median) (**Fig. 1e**, **Supplementary Fig. 8**). Here, Gaussian fitting was performed with the PSF width as free fit parameter, which is a standard setting in most software packages where the 2D Gaussian is adapted to optimally fit the emission pattern. In other words, the size of the central aperture is erratic. Alternatively, we tested a stiff Gaussian fit with fixed width in rapidSTORM to determine $I_2$ (magenta curve in **Fig. 1e**), resulting in small relative errors of *P* and an extended working range of 750 nm (**Supplementary Figs. 8** and **9**). We reason that any geometric aperture can be used to determine $I_2$, including 2D fit functions such as Gaussians, which are less prone to pixelation artifacts. The effective working range is in principle limited by the analysis method, the position of the focal plane, and the emitter brightness (**Supplementary Note 2**).

In addition, we would like to note that albeit Gaussian fitting of well-established SMLM software underestimated single-molecule intensities in experimental data, it performed perfectly well on simulated data (**Supplementary Fig. 10**). The experimental deviation remained when imaging on microscope setups with different objectives and detector types (**Supplementary Fig. 11**). By averaging the single-molecule data into a single PSF, the deviation of intensity estimation was significantly reduced with $P > 96\%$ (**Supplementary Fig. 12**). Researchers should therefore bear in mind that standard simulations as well as more accurate models such as Gibson-Lanni[14] (**Supplementary Fig. 13**) might differ from experimentally recorded fluorescence spots due to optical aberrations that become noticeable by photometric analysis (see discussion in **Supplementary Note 1**).

Due to increased precision and working range, we used fixed PSF fitting (300 nm as fixed FWHM; **Supplementary Fig. 14**) to calculate *P* with TRABI. We applied TRABI on conventionally recorded two-dimensional SMLM data and generated virtual 3D super-resolution images, color-coded in *P*(z) (**Fig. 2**, **Supplementary Figs. 15** and **16**). Synaptonemal complexes (SCs), which are meiosis-specific multiprotein complexes that are essential for synapsis, recombination, and segregation of homologous chromosomes, were recently studied using 2D *d*STORM[15]. The SC protein component SYCP3 consists of two lateral elements, which are arranged as two cables separated by ~220 nm[15]. Using TRABI, the twist of the cables, in 2D seen as superposition (**Fig. 2a**), could axially be separated by using *P* (**Fig. 2b**, **Supplementary Video 1**). Further, the bending of one cable surrounding the other became visible in the *P*(*z*)-*y* projection.

In addition, we reanalyzed 2D *d*STORM data on the presynaptic protein Bruchpilot in *Drosophila* larva, which is a major functional component of the active zone cytomatrix[16]. The virtual 3D TRABI image clearly visualized the three-dimensional organization of active zones in different layers, emphasizing the inclined axial arrangement of active zones that are not seen *en face* in 2D (**Fig. 2c**, **Supplementary Video 2**). Finally, we used TRABI on filamentous Actin, which was imaged in a PALM like imaging modality with caged near-infrared Si-rhodamine dyes[17]. The conventional 2D super-resolution image was successfully transformed into a virtual 3D image with additional axial information on the structural organization of F-Actin (**Fig. 2d**, **Supplementary Fig. 15** and **Supplementary Video 3**). We propose that any 2D SMLM data where the focal plane is predominantly set below or above the structure of interest can be reanalyzed with TRABI to create virtual 3D images (**Supplementary Note 2**). The FWHM information of spots, which is extractable by free fitting, could be used in a similar manner but with substantial restriction in sensitivity (**Supplementary Figs. 8e** and **14e**).

To further increase the axial precision and scope of TRABI based 3D imaging, we applied a biplane scheme (BP)[4,5] allowing to distinguish spots appearing above and below the focal plane, i.e., $z = 0$ nm. Therefore, we used an image splitter and separated the focal planes of the two channels by ~300 nm and measured a calibration curve with *P* as a function of *z* for each channel (**Supplementary Fig. 8**). Thus, we were able to retrieve absolute axial coordinates over a range of ~800 nm. We then imaged AF647 labeled microtubules by *d*STORM and analyzed both image stacks with rapidSTORM and TRABI. We sorted the localizations that appeared on both channels, derived the axial position of each molecule using the calibration curve, corrected the coordinates for the refractive index mismatch[3,18] and reconstructed a 3D *d*STORM image (**Fig. 3a**). Enlarged regions and axial cross section profiles of adjacent microtubule filaments clearly demonstrate improved resolution (**Fig. 3b-e, Supplementary Videos 4** and **5**). In this experiment, we determined the axial precision of BP-TRABI to 16.6 nm (s.d.). This precision can be further increased by applying higher photon thresholds to 12.6 nm, which is over 1.4 fold higher than classical biplane imaging using the FWHM information of the spots (BP-FWHM) (**Supplementary Fig. 17**). In contrast to BP-FWHM, with BP-TRABI we can fully separate adjacent filaments in axial direction at a distance of 94 nm (**Fig. 3e**). Depending on the sample, the overall increase in precision of BP-TRABI over BP-FWHM can be even higher (**Supplementary Figs. 18** and **19**).

3D SMLM is a powerful research tool, but often requires complex calibration procedures. TRABI can be directly applied on any 2D SMLM data acquired by e.g. *d*STORM, PALM or PAINT to unlock previously unused 3D information without the need for additional optical instrumentation. This might already offer new biological interpretations of many conventionally recorded data sets. TRABI can be used platform

independently in ImageJ, but we expect that this algorithm can be also implemented easily in existing localization software packages to provide corrected spot intensities along with additional axial information. Besides 3D imaging, TRABI allows researchers to determine exact total photon numbers independent of the axial position, making it a versatile tool also for the characterization of novel dyes and switching buffers.

## METHODS

Methods and any associated references are available in the online version of the paper.

## DATA AVAILABILITY

Data are available upon request.


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## AUTHOR CONTRIBUTIONS

C.F. and S.v.d.L. designed the TRABI algorithm, developed software, performed experiments and evaluated the data; C.F., M.S. and S.v.d.L. discussed results and commented on the manuscript; S.v.d.L. conceived the project and wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests due to a provisional patent application.

## Editor's Summary

3D localization microscopy can yield important biological insights. A photometric approach is described that allows users to gain 3D information from existing 2D images and to improve axial resolution obtained with existing biplane setups.

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## ONLINE METHODS

### Code availability

TRABI is provided as Supplementary Software. Updates will be available from http://bcp.phys.strath.ac.uk/photophysics/super-resolution/software/

### Sample preparation

Single-molecule surfaces were prepared on LabTek chambered coverslips (Nunc) that were previously cleaned with 2% Hellmanex (Sigma) for one hour by incubating each chamber with approximately 10 mg $mL^{-1}$ bovine serum albumin (BSA) and 0.2 mg $mL^{-1}$ biotinylated BSA (all from Sigma) overnight at 4°C. Afterwards, chambers were treated with 0.2 mg $mL^{-1}$ NeutrAvidin (Sigma) for 30 min. Finally, a DNA with the sequence 5'-ATC GTT ACC AAA GCA TCG TAA ATC GCA TAA TAG CAC GTT AAT TTA GCA CGG ACG ATC GCC-3', modified with Cy5 and biotin at the 5' and 3' end, respectively[19], was hybridized with an unmodified antisense strand (IBA lifesciences) and used at a concentration of $10^{-7}$ and $10^{-10}$ M for a 1-5 min incubation period. Between each step, the sample was gently washed three times with PBS (Sigma). All solutions were prepared in PBS.

Immunostainings of microtubules, actin, and the nuclear pore complex were performed as previously described[2,13,20,21]. Microtubules shown in **Fig. 3** were labeled with mouse anti β-tubulin antibodies (QL2122823, Sigma) and AF647 labeled goat anti-mouse $F(ab')_2$ fragments (1692984, Invitrogen). Single-molecule surfaces and microtubule structures shown in **Fig. 3** and **Supplementary Figs. 18** and **19** were imaged in 100 mM mercaptoethylamine (MEA) at pH 7.4 applying an enzymatic oxygen scavenger system, i.e., 5% (w/v) glucose, 10 U $ml^{-1}$ glucose oxidase, 200 U $ml^{-1}$ catalase[21]. Samples stained with Alexa Fluor 647 (AF647), AF488, AF532 and AF568 were embedded in PBS containing 100 mM MEA (Sigma) at pH 7.4–8.6 (**Supplementary Figs. 5** and **6**). COS-7 and U2OS were purchased from CLS Cell Lines Service GmbH (Germany).

### Imaging

Single-molecule and *d*STORM imaging was performed on a custom-built objective-type TIRF setup as described elsewhere[2,20,22], employing highly inclined illumination. We used an inverted microscope (IX71, Olympus) equipped with a nosepiece stage (IX2-NPS, Olympus) and a 60× oil objective (NA 1.45 PlanApo, Olympus). Biplane imaging was performed using a two channel image splitter with twofold magnification (TwinCam, Cairn Research) equipped with a 50/50 beamsplitter (Cairn Research) and two EMCCD cameras (Ixon 897 and Ixon Ultra 897, Andor). Cameras were synchronized by a pulse generator (DG535, Stanford Research Systems). 3D calibration experiments were performed by moving the objective with a piezo scanner (Pifoc, Physik Instrumente) driven with a LVPZT servo controller (E-662, Physik Instrumente). The inclined surface was created by using three high precision coverslips with 170 µm ± 5 µm thickness (Carl Roth).

Comparative measurements on single-molecule surface were also performed on a Nikon Eclipse Ti-E with 100x PlanApo λ NA 1.45 oil immersion objective equipped with a sCMOS camera (Zyla, Andor) and a Zeiss-Observer Z1 with 63x LD C-Apochromat NA 1.15 water immersion objective equipped with an EMCCD (iXon Ultra, Andor).

**Simulations**

Using a custom-written Python script, single-molecule spots were simulated by distributing photons randomly[23,24] on a pixel grid, according to a two dimensional probability distribution[25] according to the PSF of the microscope (Eq. 1);

$$\mathrm{PSF}(D) = \left(\frac{2J_1(D)}{D}\right)^2 \qquad (1)\,,$$

with $D(x, \lambda, n_1, n_2) = 2\pi n_1 x/(\lambda n_2)$, where $J_1$ is the Bessel function of first kind and first order, $x$ the radial coordinate, $n_1$ and $n_2$ are the refractive indices of immersion and sample medium, respectively, and $\lambda$ is the emission wavelength. For every frame, coordinates for single photons where drawn according to Eq. (1) with sub-nanometer precision until the target amplitude was reached. Then, the resulting emission pattern was subsequently binned into a 50×50 pixel-grid, where every pixel represented (100 nm)$^2$. Additional noise was added according to a Poisson distribution, where the target noise level was set as its mean value. To perform the TRABI analysis, a pseudo-realistic blinking behavior was achieved by inserting pure noise frames in between subsequent frames. Standard simulation parameters were $n_1$ = 1.45, $n_2$ = 1.33, and $\lambda$ = 670 nm. For every amplitude-noise set 2,000 frames were simulated, consisting of each 1,000 emission and pure noise frames.

Three-dimensional emission patterns were simulated according to the Gibson-Lanni model[14] using ImageJ[26]. Parameters were set to $n_1$ = 1.5, $n_2$ = 1.33, lateral FWHM = 340 nm, NA = 1.45. The $z$-dependent PSF was used as probability density matrix while photons and noise were distributed as described above.

**PSF averaging**

Image stacks were processed with custom-written software in Python. Single spots were identified according to the localization file in rapidSTORM, centered within a 23×23 pixel region of interest around the pixel containing the molecule's center, cropped, and binned into 1 nm sub-pixels. Each spot was then centered within the pixel-grid according to the rapidSTORM localization. Finally, all emitters were averaged into a single image, which was binned to 100 nm and 25 nm prior to photometric analysis.

**Aperture Photometry**

AP was written and implemented in ImageJ and is described in **Supplementary Note 1.**

**TRABI**

Temporal, Radial-Aperture Based Intensity-estimation (TRABI) was written and developed in ImageJ. For a detailed description see **Supplementary Notes 1** and **2** and **Supplementary Fig. 2**.

**Localization software**

We used well-established single-molecule localization software packages[27], i.e., rapidSTORM 3.3[13] as stand-alone software as well as ThunderSTORM[28] and PeakFit[29] as up-to-date ImageJ plugins using standard settings. The PSF width was a free fit parameter in ThunderSTORM and PeakFit with fit window radii of 5.0 and 4.5 camera pixels, respectively. Fitting in rapidSTORM was performed with the PSF width (FWHM) either as free (free fit) or fixed parameter (fixed fit). If not otherwise stated a PSF FWHM of 300 nm was used for fixed fitting in rapidSTORM (**Figs. 1e**, **2**, **3, Supplementary Figs. 7-9, 11, 13, 15-19**).

**Calibrated 3D imaging with BP-TRABI and BP-FWHM**

3D calibration curves were generated by imaging a single-molecule surface under *d*STORM conditions with a biplane detection scheme. Using a piezo scanner, we varied the relative axial position from -2 µm to +2 µm with a frequency of 0.00125 Hz at duty cycle of 0.999. Both cameras were triggered with a frequency of 20 Hz, thus generating a *z*-step width of 0.25 nm per camera frame.

Incoming fluorescence signal was split by a 50/50 beamsplitter, where the focal planes were separated by ~300 nm [5,30]. In the following, reflected and transmitted light are referred to as channel 1 and 2, respectively. Images of both channels were aligned by linear transformation.

*d*STORM image stacks were analyzed in rapidSTORM. We used four different settings for 3D imaging:

1) The determination of $I_1$ and $P$ with TRABI, where
    a. $I_2$ was determined with a second circular aperture in TRABI,
    b. $I_2$ was obtained through free PSF fitting in rapidSTORM,
    c. $I_2$ was obtained through fixed PSF fitting in rapidSTORM.
2) The determination of the spot FWHM with rapidSTORM by free PSF fitting (classical biplane).

For 1a) – c), we used the same aperture to calculate $I_1$. A fit window radius of 400 nm (3.0 px) was used for free fitting (1b), fixed fitting was performed with 300 nm FWHM and a fit window radius of 1,000 nm (1c), whereas for 3D biplane analysis a fit window radius of 1,500 nm (11.3 px) was applied (2) (**Supplementary Fig. 8**). For the data sets of 1b) and 2), the localizations were processed with an additional filter of 200 nm < FWHM < 1,200 nm in rapidSTORM. Only values of $P > 0\%$ and $P < 100\%$ were analyzed. This filtering was also applied to the data shown in **Figs. 2** and **3** and **Supplementary Figs. 15, 16, 18, 19**.

Because of its high precision and ease of operation we used TRABI on fixed PSF fitting to determine $I_2$ for the visualization of the data shown in **Figs. 2**, **3** and **Supplementary Figs. 15, 16, 18, 19**.

Calibration curves were calculated from localizations that were found in both channels. This was done by a nearest neighbor analysis, i.e., localizations further apart than 300 nm after channel transformation were discarded. For BP-TRABI, the percentage ratio $P_{1,2}$ from each localization pair was calculated as

$$P_{1,2} = \frac{P_1 - P_2}{P_1 + P_2} \ ,$$

where $P_1$ and $P_2$ stand for the photometric ratio determined with TRABI ($P$) for each spot in channel 1 and 2, respectively (**Supplementary Fig. 8**). Similarly, for BP-FWHM the ratio of spot widths ($w_{1,2}$) was calculated as

$$w_{1,2} = -\frac{w_1 - w_2}{w_1 + w_2} \ ,$$

where $w_1$ and $w_2$ are the FWHM of each spot in channel 1 and 2, respectively.

$P_{1,2}$ and $w_{1,2}$ were fitted with a fourth order polynomial to generate *z*-lookup tables with a *z*-resolution of 0.25 nm (**Supplementary Fig. 8d**).

Experimental data shown in **Fig. 3** were analyzed according to the following protocol:

1) rapidSTORM analysis (for BP-TRABI: 300 nm fixed PSF FWHM and 1,000 nm fit window radius; for BP-FWHM: PSF width as free fit parameter and 1,500 nm fit window radius);
2) TRABI evaluation of the data to determine *P*;
3) Nearest neighbor assignment of the two channels for both datasets, BP-TRABI and BP-FWHM, to calculate $P_{1,2}$ and $w_{1,2}$ of each localization, respectively;
4) Final *z*-coordinates were assigned from the lookup tables, corrected for the refractive index mismatch and visualized with rapidSTORM and ImageJ.

*z*-coordinates were corrected for the refractive index mismatch by a scaling factor of 0.71 (refractive index of buffer with 5% glucose $n_b$ = 1.34 and substrate (glass) $n_s$ = 1.52, numerical aperture of NA = 1.45, emission wavelength $\lambda_{em}$ = 680 nm, assuming isotropic emission)[31,32].


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**Figures legends**

**Fig. 1** Photometry based 3D super-resolution imaging. **a**) The *z*-dependent photometric parameter *P* is created by determining the intensities within two apertures of the molecule's emission pattern according to $P = I_2\ I_1^{-1} \cdot 100$. **b**) Principle of TRABI, which uses a circular aperture with radius $r_1$ (solid circle) and an exclusion zone defined by $2r_1$ (dashed circle); $I_1$ is determined by subtracting background ($I_{BG}$) from raw intensity ($I_{raw,1}$); $I_{raw,1}$ and $I_{BG}$ are not estimated in frames where localizations from neighboring spots interfere. **c**) *left*: *d*STORM imaging of surface-immobilized Cy5 labeled DNA; *right:* Number of photons obtained by fitting using rapidSTORM (free fit; black), classical aperture photometry (AP; $r_1$ = 865 nm, $r_2$ = 1,131 nm; grey), and TRABI ($r_1$ = 865 nm, $n_{BG}$=7; magenta). **d**) Histograms of *P* for different axial positions of the single-molecule surface adjusted with a piezo-scanner. Dashed lines indicate the median of the distribution. **e**) *P* as a function of the axial position. Dots represent the median of the distribution, solid lines are used to guide the eye; $I_1$ was determined by TRABI ($r_1$ = 865 nm), $I_2$ was either determined with TRABI ($r_2$ = 333 nm, black curve), or through free (green) or fixed fitting (magenta) in rapidSTORM. **f**) Reconstructed *d*STORM image of a single-molecule surface tilted by an angle of 0.56°, color-coded by *P*. Arrowheads indicate *x*-positions of magnified single-molecule localization patterns (insets). Scale bars, 1 µm (**c**), 5 µm (**f**, top), 100 nm (**f**, insets).

**Fig. 2** Virtual 3D imaging obtained by TRABI from 2D *d*STORM data on different structures. **a**) Conventional 2D *d*STORM image of the synaptonemal complex (SC) by staining SYCP3 with AF647 labeled antibodies[15] (left panel); virtual 3D image, i.e., *x-y-P(z)*, obtained by TRABI by analyzing the conventional 2D data (right panel). **b**) Magnified views of insets (*i*, *ii*) shown in **a**; arrowheads indicate twists of the lateral element of the SC (*i*). Magnified views of *ii* are shown as *x-y* and *P*(*z*)*-y* projections (**Supplementary Video 1**); **c**) 3D analysis of active zones in the neuromuscular junction of *Drosophila* larva, stained with Cy5 labeled antibodies, showing the presynaptic protein Bruchpilot in rab3 mutants[16] (**Supplementary Video 2**); the inset shows the *P(z)* projection of one active zone; upper left corner shows the conventional *d*STORM image. **d**) 3D analysis of conventional 2D data on F-actin in COS-7 cells, stained with Si-rhodamine labeled phalloidin[17] (**Supplementary Video 3**); upper left corner shows the conventional *d*STORM image; Scale bars, 5 µm (**a**), 1 µm (**b**), 200 nm (**b**, bottom panel), 500 nm (**c**), 2 µm (**d**).

**Fig. 3** 3D super-resolution imaging of microtubules in U2OS cells, stained with AF647 labeled antibodies. **a**) 3D *d*STORM image using BP-TRABI, color-coded in *z*. **b**) Axial localization precision (s.d.) of BP-TRABI and BP-FWHM were determined to 17 nm and 23 nm, respectively. **c, d, e**) Magnified views of insets shown in **a**. **c**) *x-z* projection of the entire *x-y* view. **d**) *x-z* projection of the boxed region of the *x-y* view. **e**) *z-y* projection of the boxed region in *x-y*; cross-section profile of adjacent filaments as seen with BP-TRABI and BP-FWHM; solid lines indicate double Gaussian fits. Using BP-TRABI, indicated filaments were resolved with highest resolution and axially separated by 94 nm (averaged in *y* as indicated by dashed boxes). Regions shown in **c** and **d** are available as **Supplementary Videos 4** and **5**. Scale bars 5 µm (**a**), 200 nm (**c**, **d**) and 100 nm (**e**).