
One-step nanoscale expansion microscopy reveals individual protein shapes

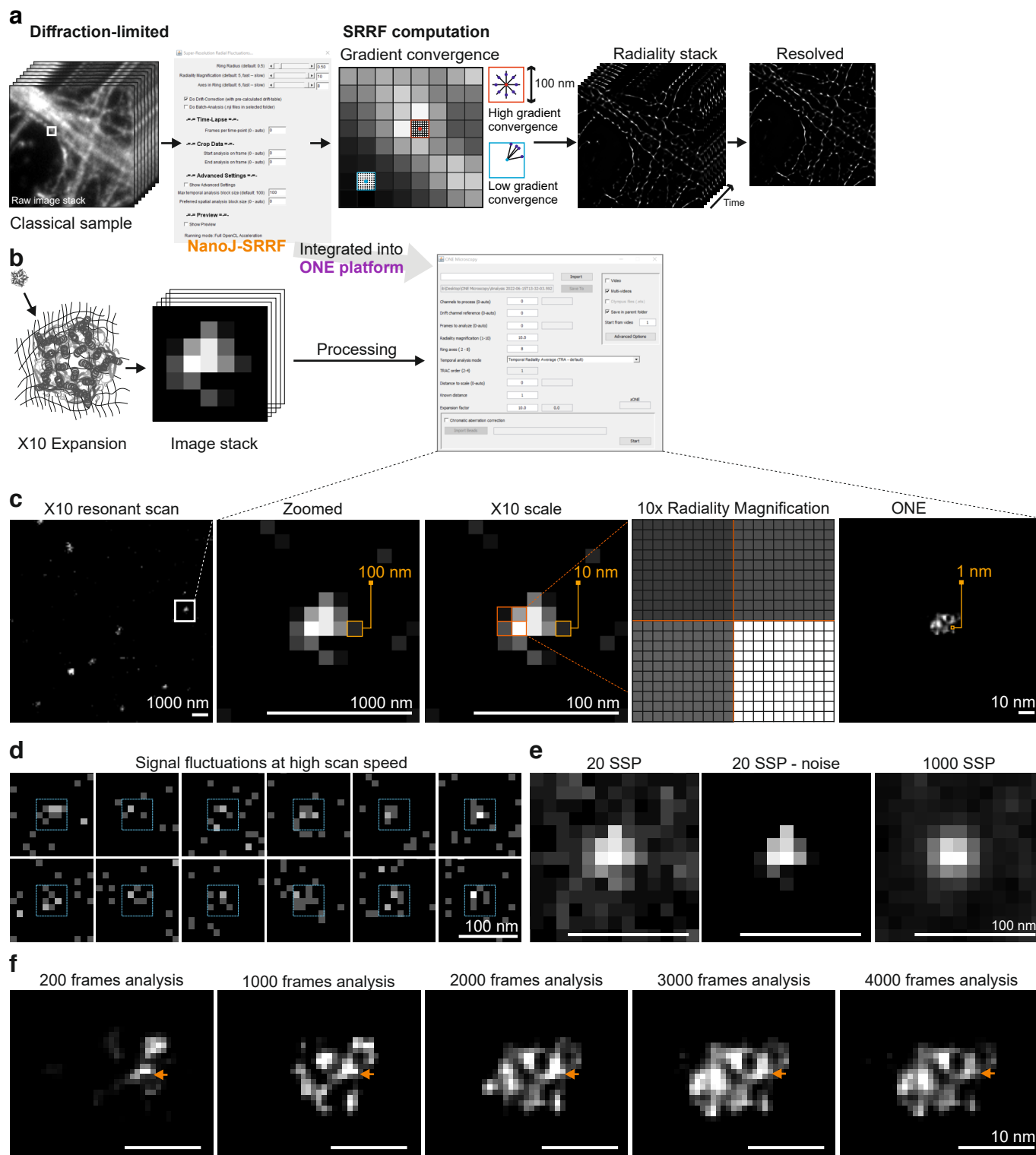
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Supplementary Information

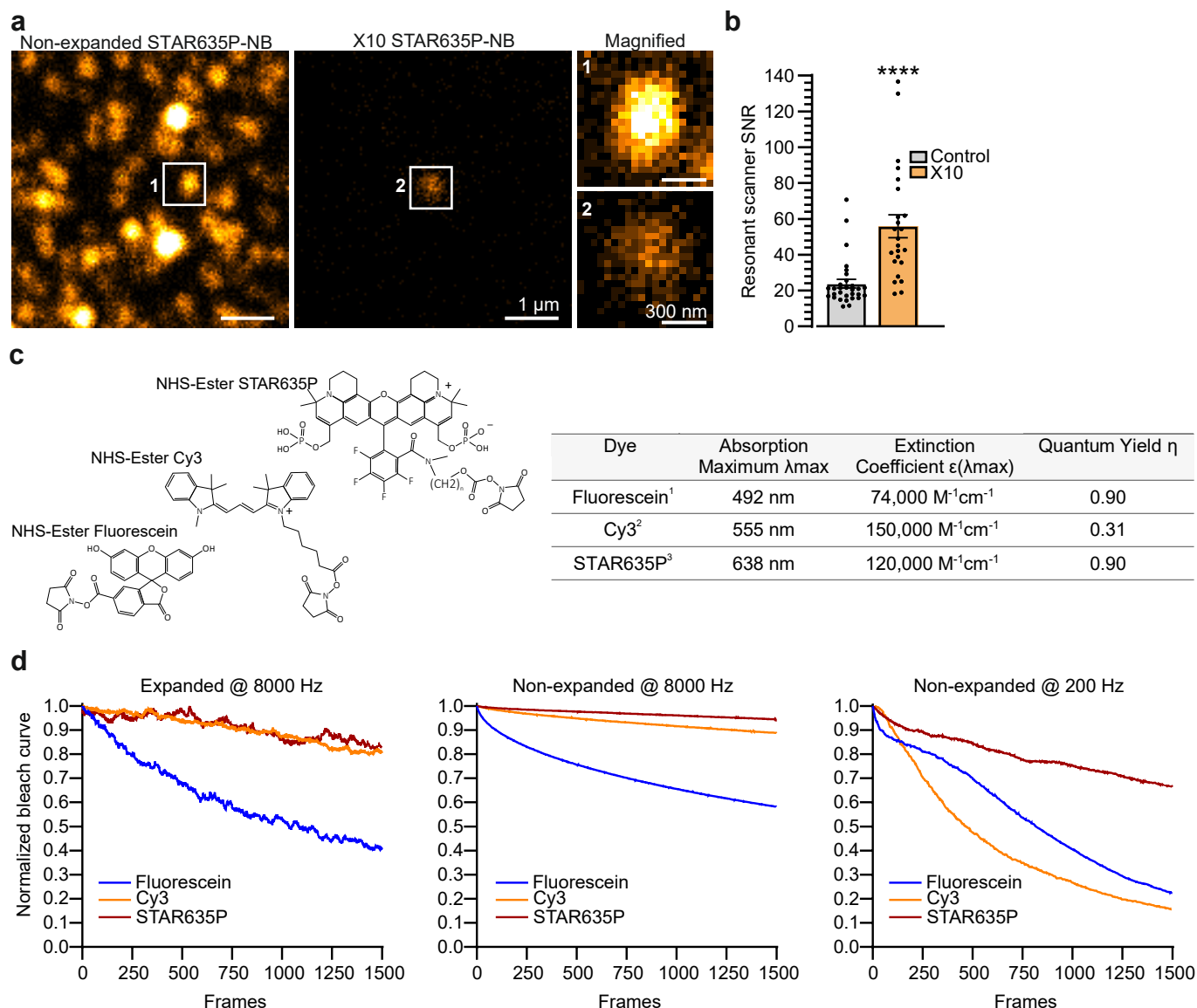
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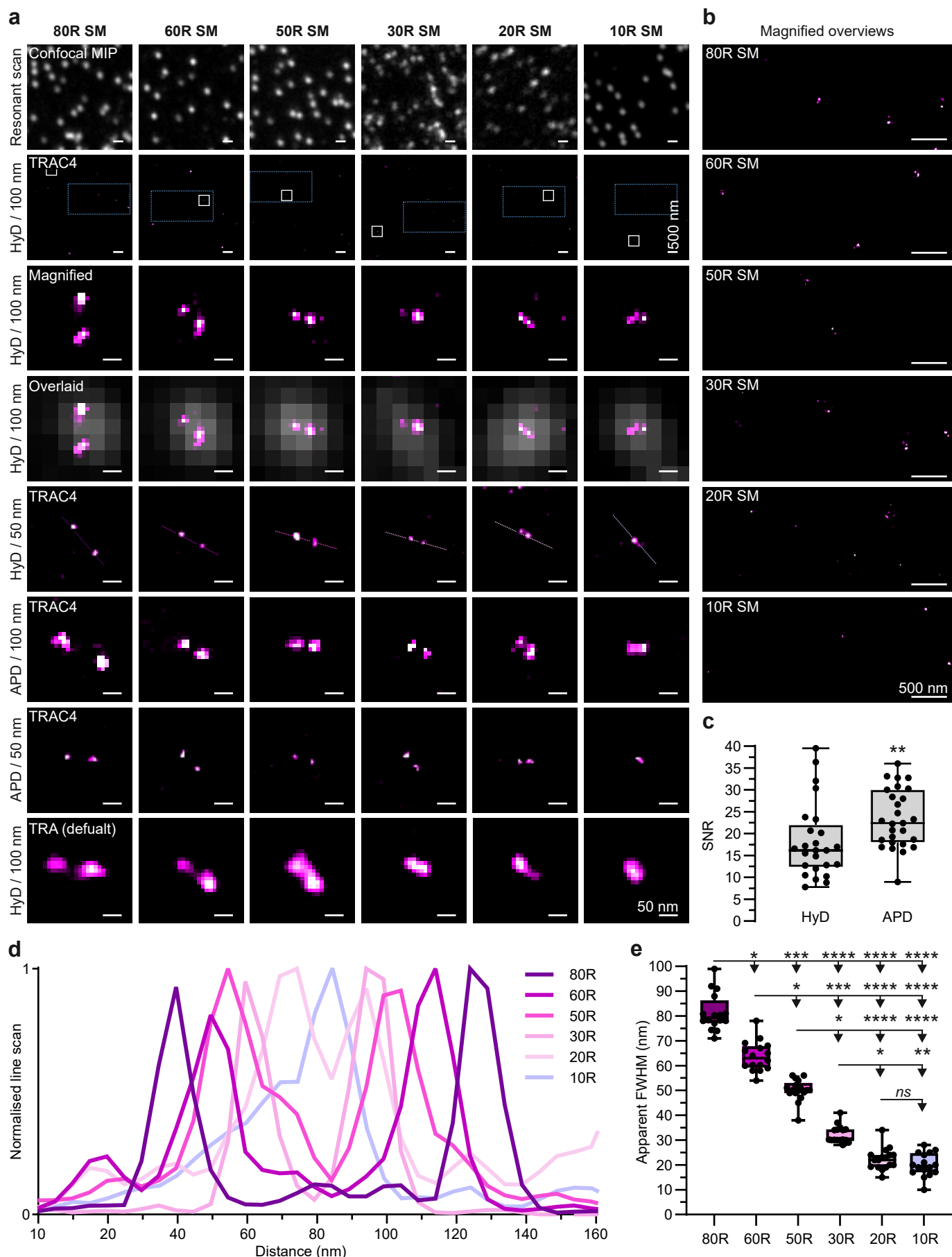
Supplementary Figures



Supplementary Fig. 1. A detailed view of the ONE procedure. **a**, Processing a stack of diffraction-limited images with SRRF, based on the analysis of a gradient of convergence of sub-pixels over a radiality stack, results in super-resolved images with resolutions varying between 50-70 nm. **b**, The ONE procedure adapts the SRRF algorithm to expanded gels. **c-f**, A detailed explanation of the analysis procedure. **c**, A sample was fixed and expanded using a 10-fold expansion protocol (X10). The sample was then imaged using a resonant scanner on a confocal microscope. The zoomed-in view indicates one bright spot, whose size in real space is limited by diffraction to ~200-300 nm, but represents a 10-fold smaller size in the pre-expansion space (see scale bars in the middle panels). Every pixel is then subjected to a 10-fold radiality magnification and is then subjected to the procedure explained in panels d-f, which provides the final, high-resolution image (right-most panel). **d**, Signal fluctuations are measured by imaging the sample repeatedly, using the resonant scanner (here at 8 kHz). **e**, A view of the overall signals, obtained by summing 20 of the fluctuating images (raw in the left-most panel, background-subtracted in the middle panel), or by summing 1000 images. **f**, Each image from series obtained as in panel b is subjected to a temporal analysis of fluctuating fluorophores, based on radiality magnification, thereby providing a super-resolved image whose level of detail becomes optimal after ~1500 frames. These results are typical of 753 images acquired from 5 independent experiments.

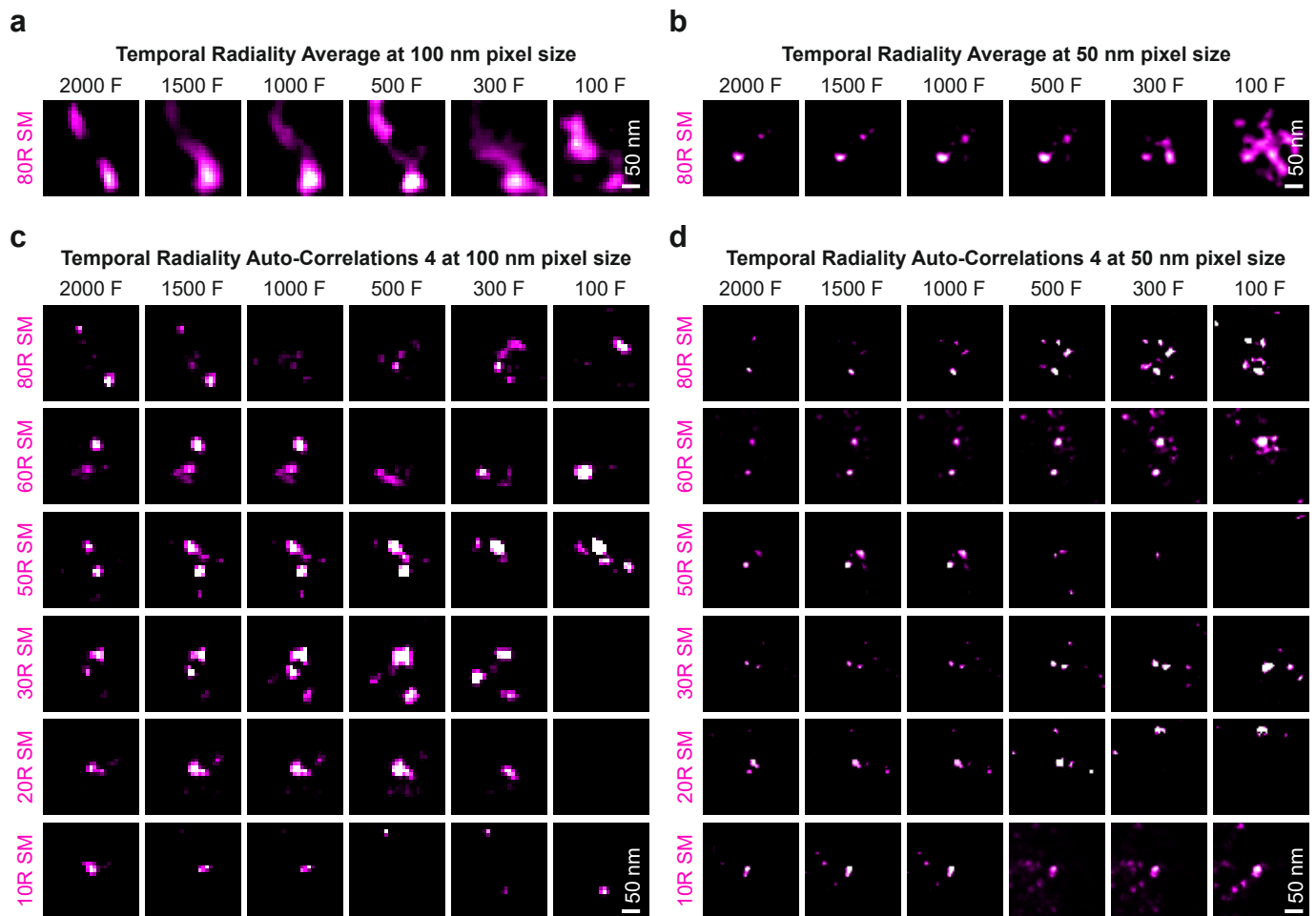


Supplementary Fig. 3. Expansion microscopy results in a higher signal-to-noise ratio. Expansion microscopy, which separates proteins of interest and removes much of the other cellular components (e.g. lipids, metabolites) should result in a higher signal-to-noise ratio (SNR). **a**, To test this, we analyzed here the simplest possible sample, consisting of Star635P-conjugated nanobodies on glass coverslips, or in expanded gels, using confocal microscopy, relying on analysis using a resonant scanner. **b**, The SNR of these samples displayed as an average bar graph with standard error of mean, increases by 2-fold, on average, after expansion. $N = 30-24$, from 3 independent measurements. $P = 0.000001$, Two-tailed Mann-Whitney Ranksum test. **c**, Bleaching properties of fluorescein, Cy3, and STAR635P. A representation of the structures of each of the used dyes, followed by a table of their properties. The molecule structures and properties were reproduced from measurements of commercial providers: ¹<https://broadpharm.com/product/bp-23900>, ²<https://broadpharm.com/product/bp-22535>, and ³<https://abberior.shop/abberior-STAR-635P>. **d**, Normalized bleach curves from expanded specimens at 8000 Hz, and non-expanded specimens at 8000 Hz and 200 Hz.

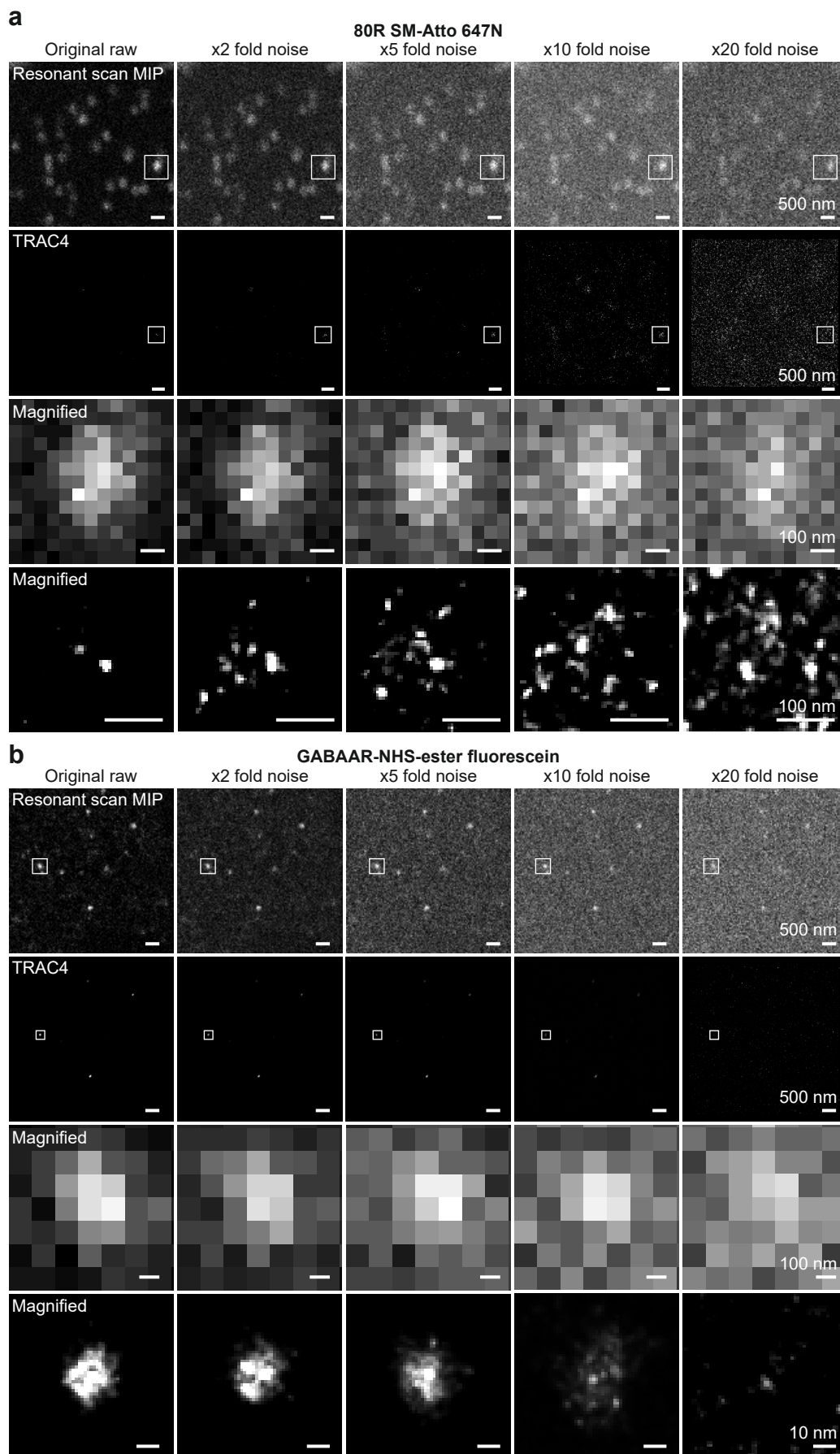


Supplementary Fig. 4. Evaluating SRRF analysis performance using DNA origami nanorulers, in non-expanded samples. **a**, Nanorulers with single Atto647N molecules (R SM) were generated by GATTAquant carrying fluorophores on each end of DNA structures of 80, 60, 50, 30, 20 and 10 nm in length. ...

... They were then imaged using a confocal resonant scanner, without expansion procedures. The first panel shows confocal maximal intensity projections (MIPs) for each of the rulers. The second panel shows temporal radiality averaging (TRA) analysis overviews. White boxes indicate the magnified regions displayed in the third panel. The fourth panel shows a temporal radiality auto-correlations of fourth order (TRAC4) analysis, overlaid with the respective confocal MIPs. The remaining panels show different ruler examples, acquired at different starting pixel sizes, using either a hybrid detector (HyD) or an avalanche photodiode detector (APD), and analyzed in different SRRF modalities. This analysis is shown in the fifth panel for 50 nm pixel size, using a HyD and analyzed using TRAC4. The sixth and seventh panels show rulers acquired at 100 and 50 nm pixel sizes, using an APD and analyzed using TRAC4. The eighth panel shows rulers that were acquired at 100 nm pixel size and were analyzed using default SRRF settings (TRA). **b**, Magnified overviews of selected regions (indicated by blue rectangles) from each of the ruler exemplary images to the left. **c**, Signal-to-noise ratio (SNR) box plot analysis showing mean, minimum and maximum whiskers of HyD and APD detectors, $N_{\text{nanorulers}} = 25$ and 30 independent measurement for HyD and APD, respectively. Two-tailed Mann Whitney test, $p = 0.004$. **d**, Normalized line scans across the different ruler images, as indicated in the respective panels in **(a)**. **e**, Apparent FWHM of the different rulers. $N = 17, 17, 18, 17, 18$ and 17 for 80, 60, 50, 30, 20, and 10 R SM DNA origami nanoruler, respectively. Three independent measurements were carried out. A Kruskal-Wallis test was applied, followed by Dunn's *post hoc* test; p values for 80R vs. 60R, 50R, 30R, 20R and 10R = 0.0367, 0.0006, <0.0001, <0.0001, and <0.0001, respectively. p values for 60R vs. 50R, 30R, 20R and 10R = 0.031, 0.0004, <0.0001 and <0.0001, respectively. p values for 50R vs. 30R, 20R and 10R = 0.0286, <0.0001 and <0.0001, respectively. p values for 30R vs. 20R and 10R = 0.0121 and 0.004, respectively. p value of R20 vs. R10 = 0.1757. p values > 0.05 not significant (*ns*), $p \leq 0.05$: *, $p \leq 0.01$ **, $p \leq 0.001$ ***, $p \leq 0.0001$ ****.



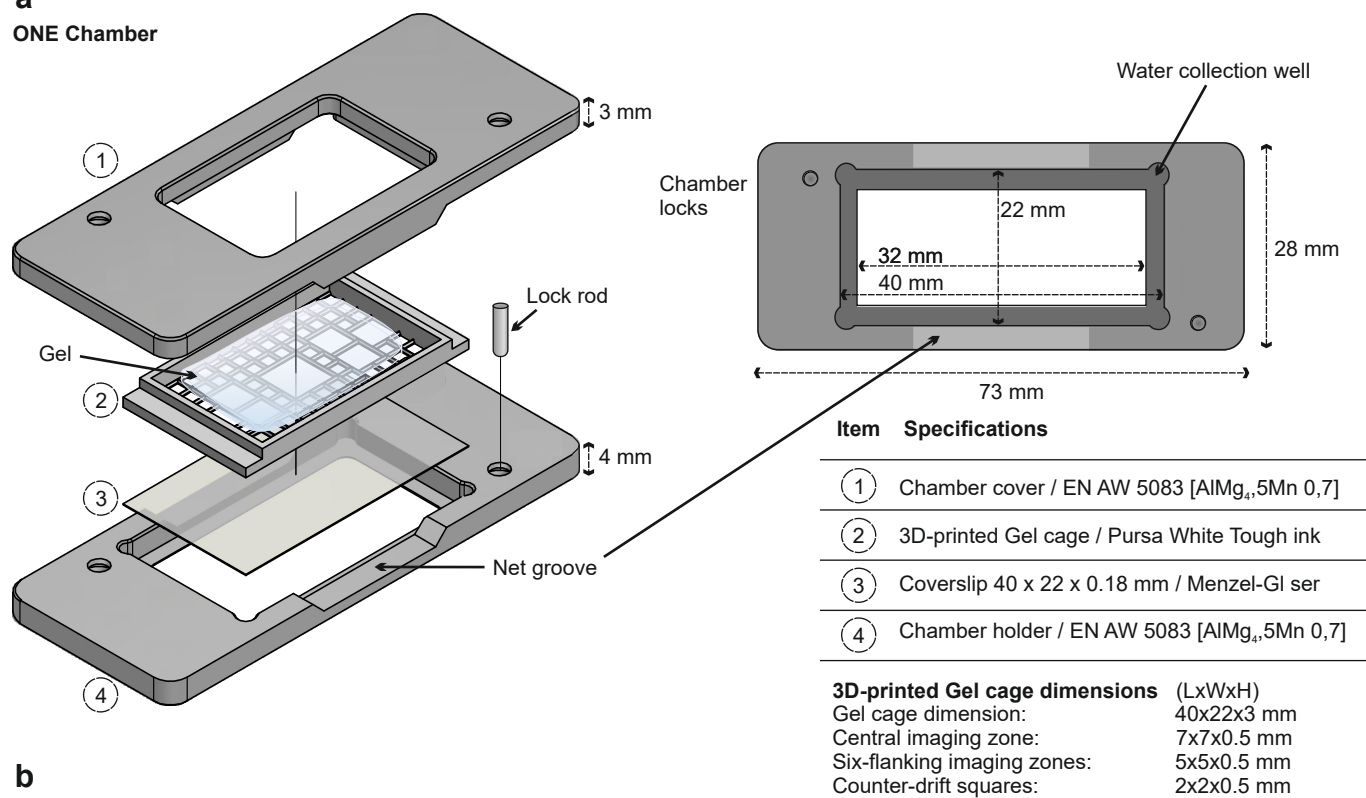
Supplementary Fig. 5. The effect of frame number on SRRF analysis. **a & b**, 80 nm rulers were imaged at 100 and 50 nm pixel sizes, and were then analyzed with the default SRRF parameter (temporal radiality average, TRA), using varying frame counts (termed F in the figure), from 100 to 2000. **c & d**, The same procedure was repeated using temporal radiality auto-correlations (TRAC4) for rulers of 10 to 80 nm. The frame count does not affect the TRA analysis as much as it affects TRAC4. The TRA performance, which is the parameter reported in most publications, is far poorer than that of TRAC4, when sufficient frames are analyzed. These experiments were repeated at least three times each.



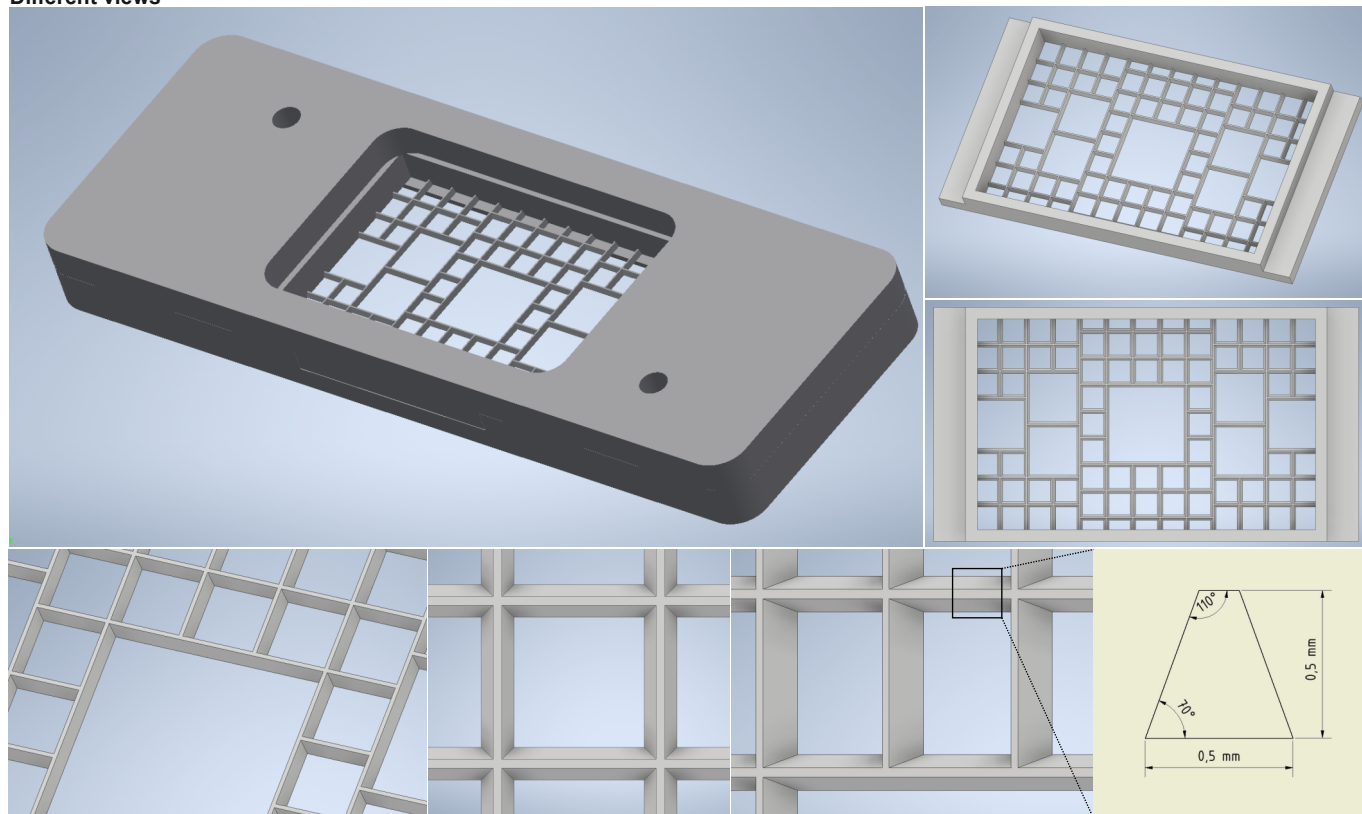
Supplementary Fig. 6. SNR effect on SRRF performance. **a**, The top panel shows an overview of 30 frame-MIPs of an 80 nm ruler, followed by MIPs of the same ruler that were subjected to 2-fold, 5-fold, 10-fold and 20-fold increase in noise. Noise was added artificially, using a Matlab routine. The initial SNR was 27.84...

... The second panel shows TRAC4 analyses of the data. The third and fourth panels show a magnified region from the resonant scan MIPs, and their respective TRAC4 analysis results. **b**, The same analysis was performed on expanded GABAAR. Note that the receptor pore disappears at a 5-fold noise level in TRAC4-resolved images. The nanoruler image is corrupted far more strongly by a 2-fold increase in noise than that of the GABAAR, owing to the substantially higher original SNR of the receptor image (76.72). These results are typical of 753 images acquired from 5 independent experiments.

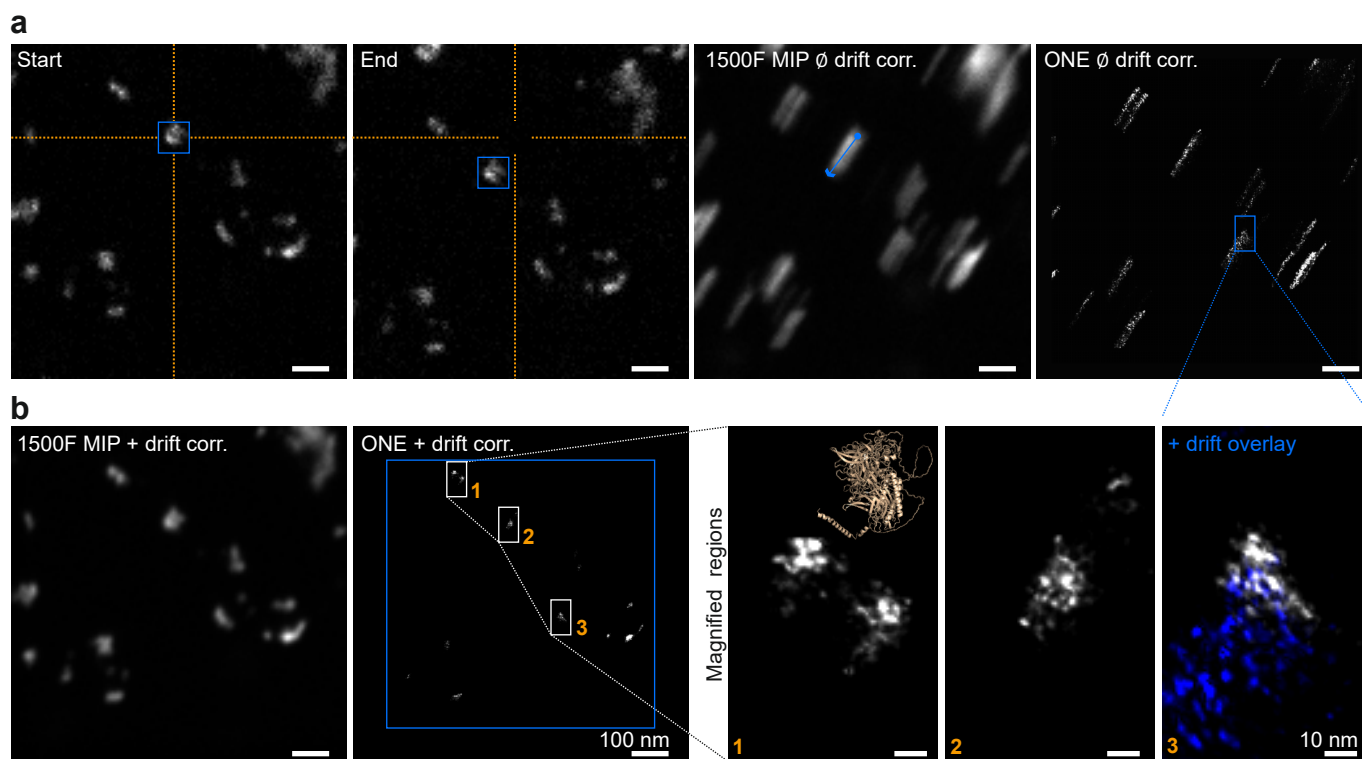
a
ONE Chamber



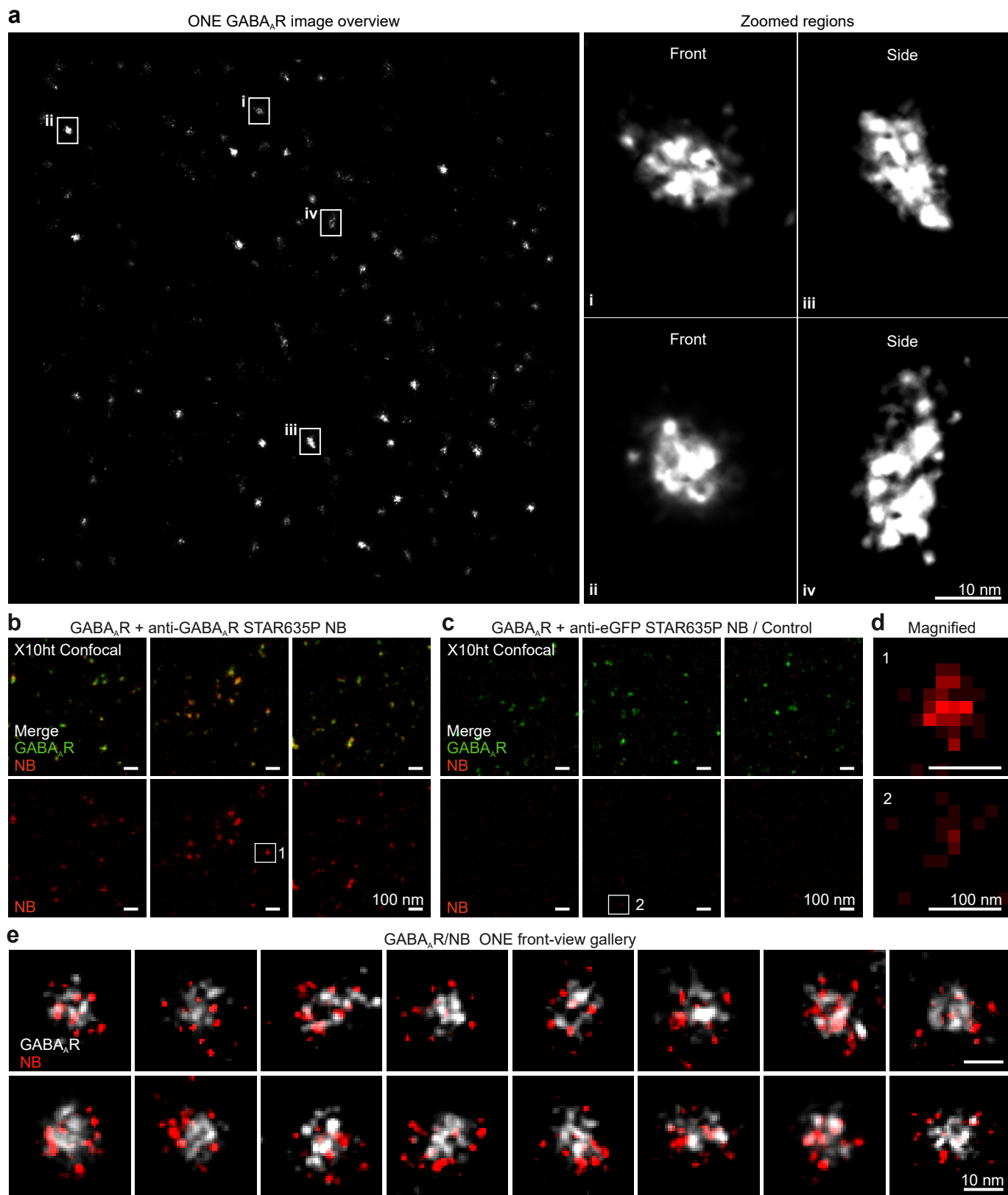
b
Different views



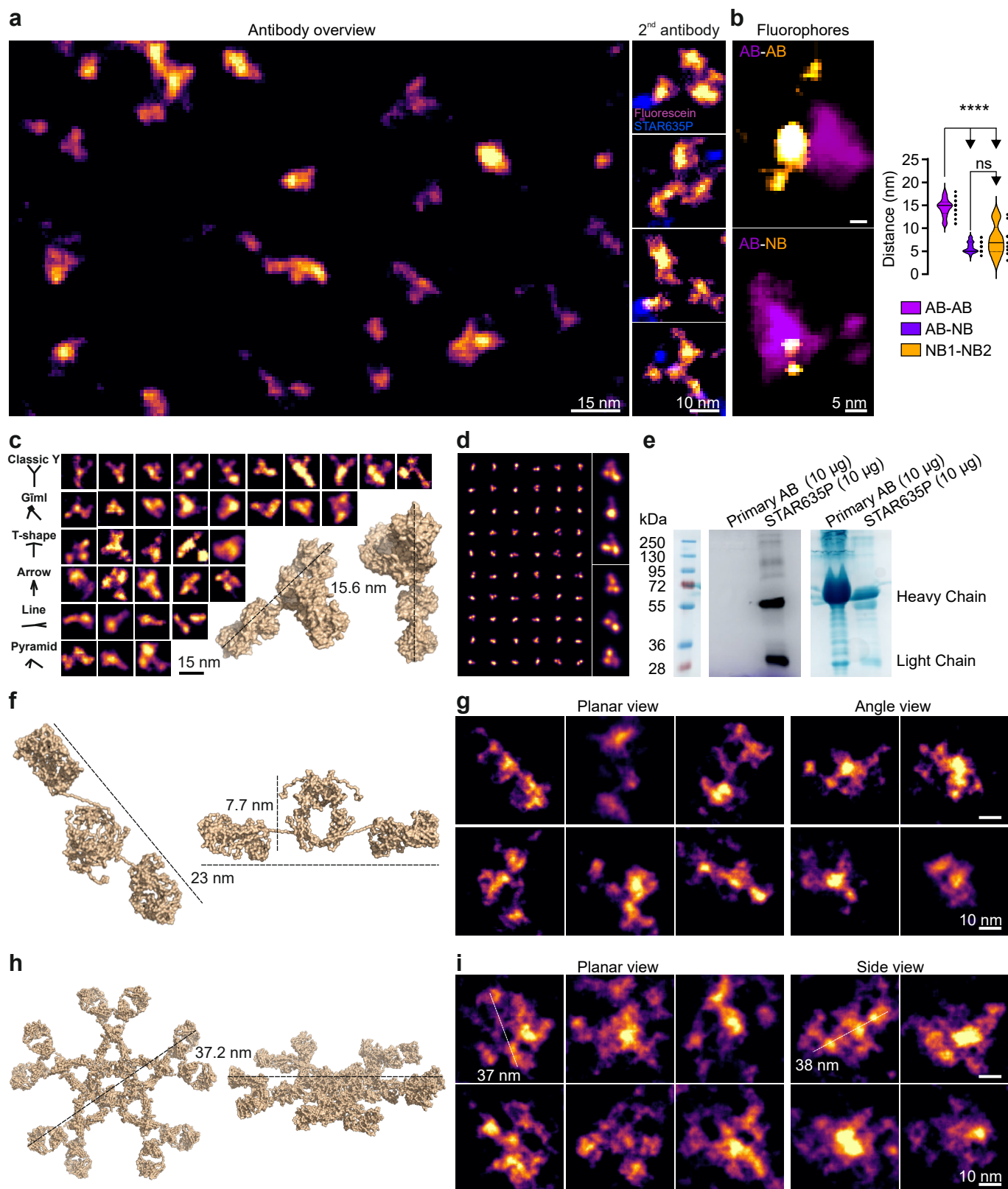
Supplementary Fig. 7. Technical scheme of the stabilization chamber used in this work. **a**, The exact measurements and materials for the stabilization chamber are included in the figure text. The 3D-printed gel cage patterning can be organized according to the user's preferred design. Only a suggested design is included here in **b**, many others work equally well. The design files can be obtained from the corresponding authors, to produce this chamber in any facility.



Supplementary Fig. 8. Drift compensation. **a**, A resonant confocal X10 image of otoferlin molecules at the start of a 1500-frame time series recording (first panel) and at its end (second panel). The third panel shows a maximum intensity projection of the resonant confocal scan. The blue arrow indicates the direction of drift. The fourth panel shows ONE processing without drift correction. A streak artefact is evident as a result. **b**, Applying drift correction, using the SRRF software, to the same acquisition and maximum intensity projection yields an image (first panel) similar to the first image in (**a**). The second panel shows the result of the ONE processing with drift correction application. The last set of panels show magnified regions of otoferlin molecules. An otoferlin AlphaFold cartoon is presented for comparison (not drawn to scale). In panel 3, the ONE image is overlaid with its counterpart from the same dataset, processed without drift correction (blue). This is a typical observation for all otoferlin and GABA_AR 945 images acquired from at least of 9 independent experiments.

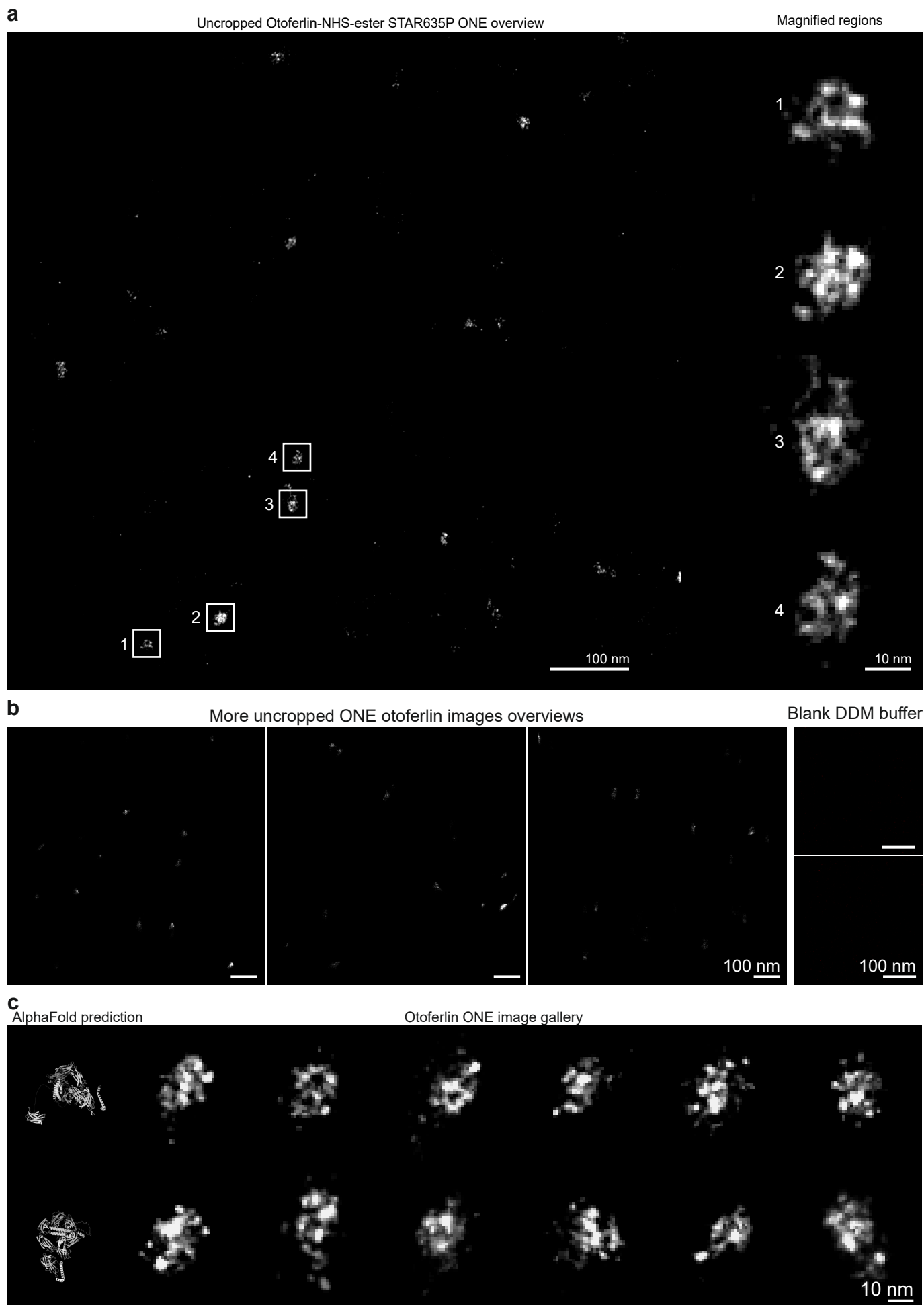


Supplementary Fig. 9. GABAAR ONE overview and nanobody labeling. **a**, Uncropped exemplary ONE GABA_AR image overview followed by four magnified regions. This is a typical overview from 105 image acquisitions from 3 independent experiments. **b**, Confocal images of expanded GABAAR labelled with anti-GABAAR nanobodies (NBs) conjugated to STAR635P. **c**, Confocal images of expanded GABAAR mixed with anti-eGFP nanobodies, which only induce little non-specific background. **d**, Magnified regions of single receptor either labelled with anti- GABAAR or anti-eGFP NBs. **e**, A gallery of ONE images showing GABAAR in white and anti-GABAAR NBs in red. A 648 GABA_AR+NBs images were acquired from 3 independent experiments.



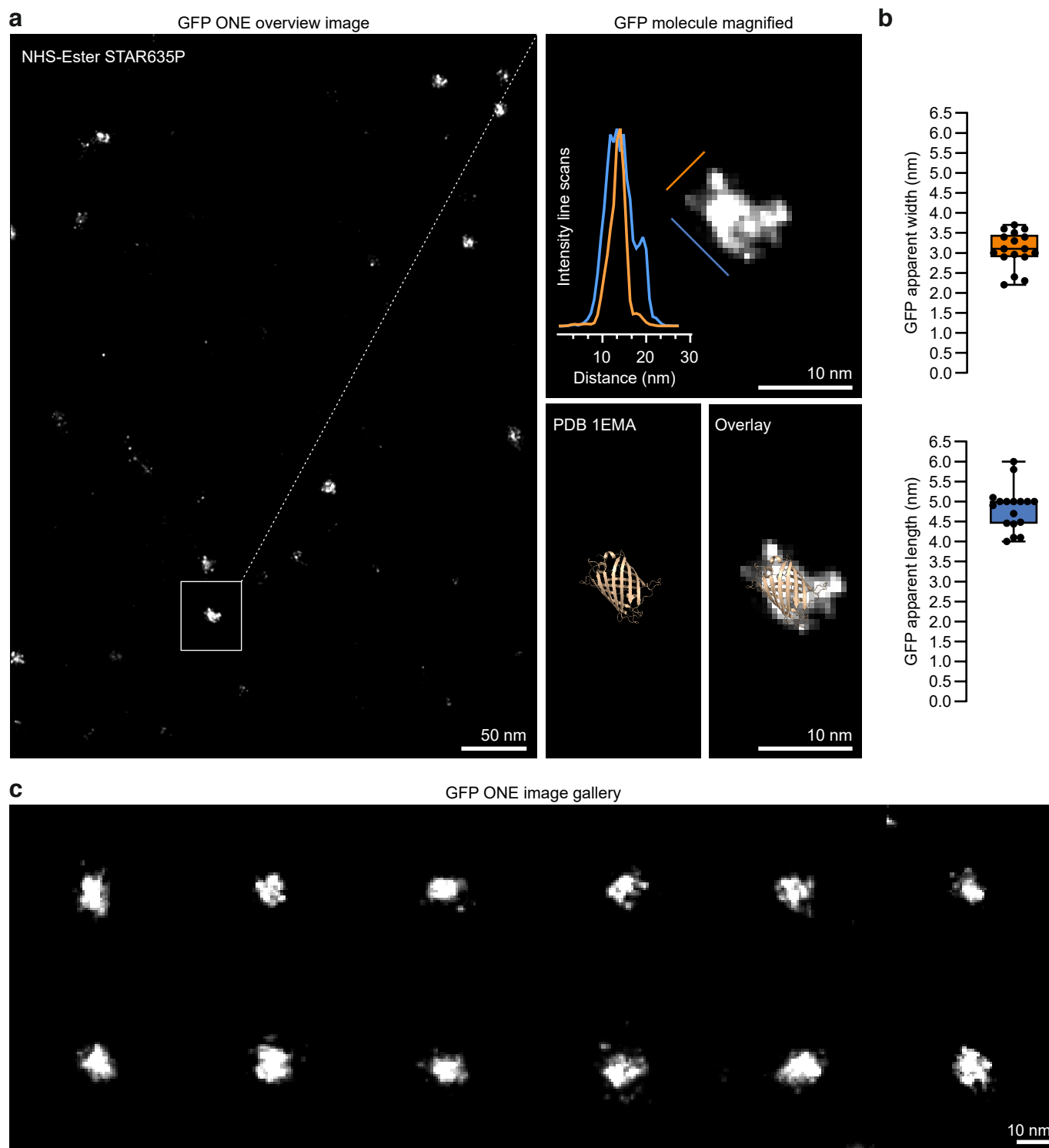
Supplementary Fig. 10. Further ONE examples of immunoglobulin imaging. **a**, An overview of a field showing IgG antibodies labelled using NHS-fluorescein (left), along with a few zoom-in images of fluorescently-conjugated secondary IgG antibodies (right; Abberior Star635P conjugation shown in blue). **b**, Distances between fluorescently-conjugated IgGs and fluorescently-conjugated secondary antibodies (top) or secondary nanobodies (bottom), $N = 20$ AB-AB and AB-NB signals from 3 independent gels for AB:AB/AB:NB molecules, two-tailed Mann-Whitney test, $p = 0.0001$. **c**, Several examples of IgG antibodies imaged in different positions and perspectives. **d**, A gallery of the expected antibody shapes, obtained by convoluting a PDB IgG structure with a ONE point-spread-function, after revolving the IgG molecules in 3D space randomly. A few enlarged views are shown, along with a multitude of small-sized views, to explain how IgG molecules should appear when they are visualized in fluorescence in random orientations. The typical IgG views are similar to the modeled ones. **e**, Fluorescence (Abberior Star635P) and Coomassie SDS-PAGE gels indicating...

... the size distribution of antibody fragments. A mouse monoclonal primary antibody was run on the gels, along the secondary antibody imaged in panel a. The gel was first imaged under a fluorescence (Cy5 channel) and then total proteins were revealed with Coomassie brilliant blue staining. The results from this one gel suggest that numerous small fragments are expected for both primary and secondary antibodies in the ONE images, not only full antibodies, due to impurities being present in the commercial antibody samples. **f & g**, An overview of IgA molecules. **h & i**, A similar overview of IgM molecules. The antibody structures are shown using Pymol representations from PDB structures 1HZH, 1IGA, and 2RCJ.

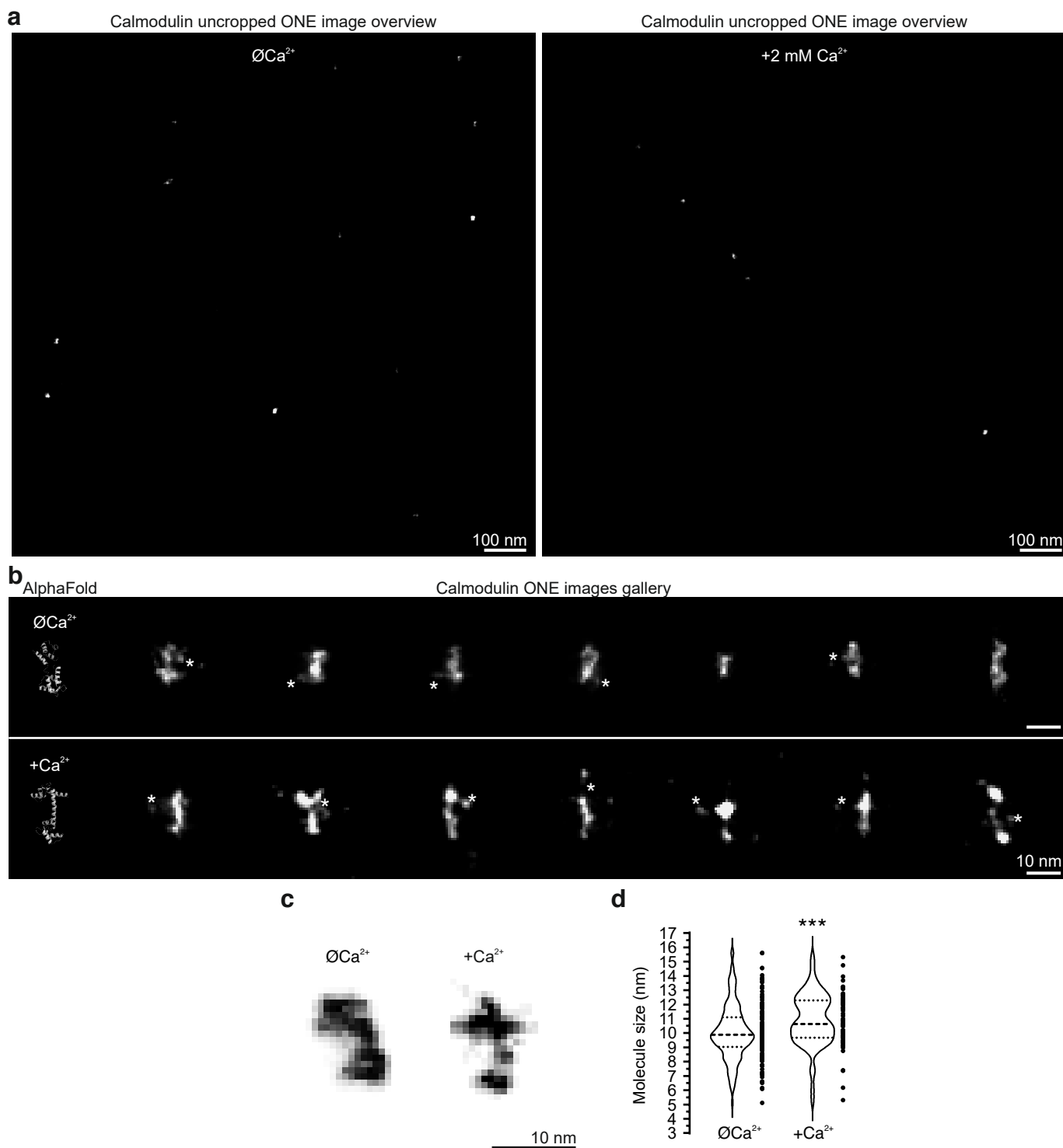


Supplementary Fig. 11. Otoferlin galleries. **a**, An overview of otoferlin followed by 4 magnified regions. **b**, More ONE...

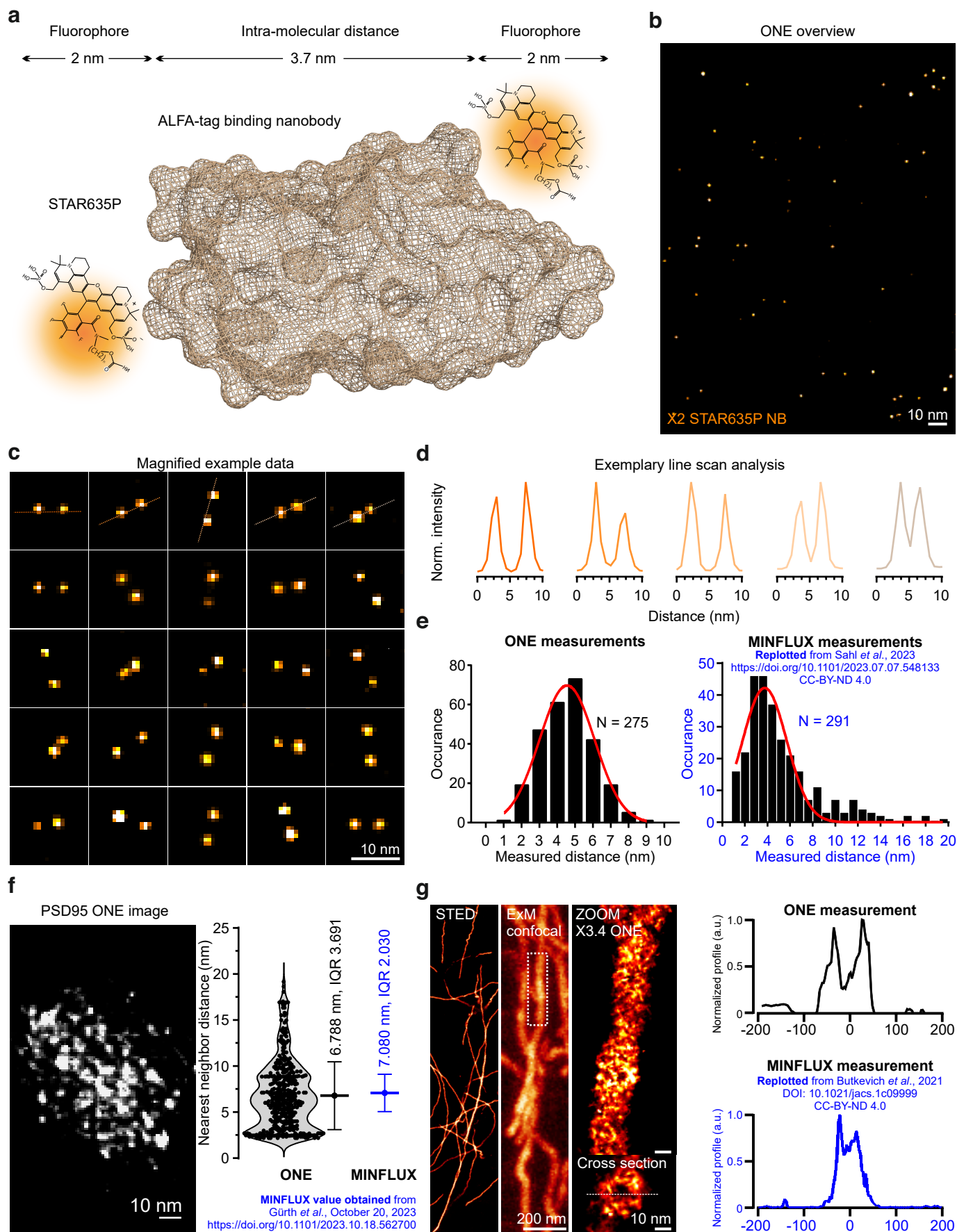
... images of uncropped otoferlin overviews and their respective blank control. **c**, A gallery of magnified otoferlin molecules. These images are typical of 150 otoferlin image acquisition from at least 3 independent experiments.



Supplementary Fig. 12. ONE imaging of purified eGFP molecules. **a**, The first panel shows a ONE overview of eGFP molecules labeled with NHS-Ester STAR635P. The second panel shows a magnified area. The third panel shows the eGFP 1EMA PDB structure. The fourth panel shows a PDB/fluorescence overlay. **b**, A box plot displaying the mean and the minimum and maximum whiskers for a measurement of the apparent width and length of the molecules, from line scans as the examples shown in panel a, in blue and orange. A total of 17 single molecules were measured from an n of 3 independent gels. **c**, A gallery of eGFP molecules.

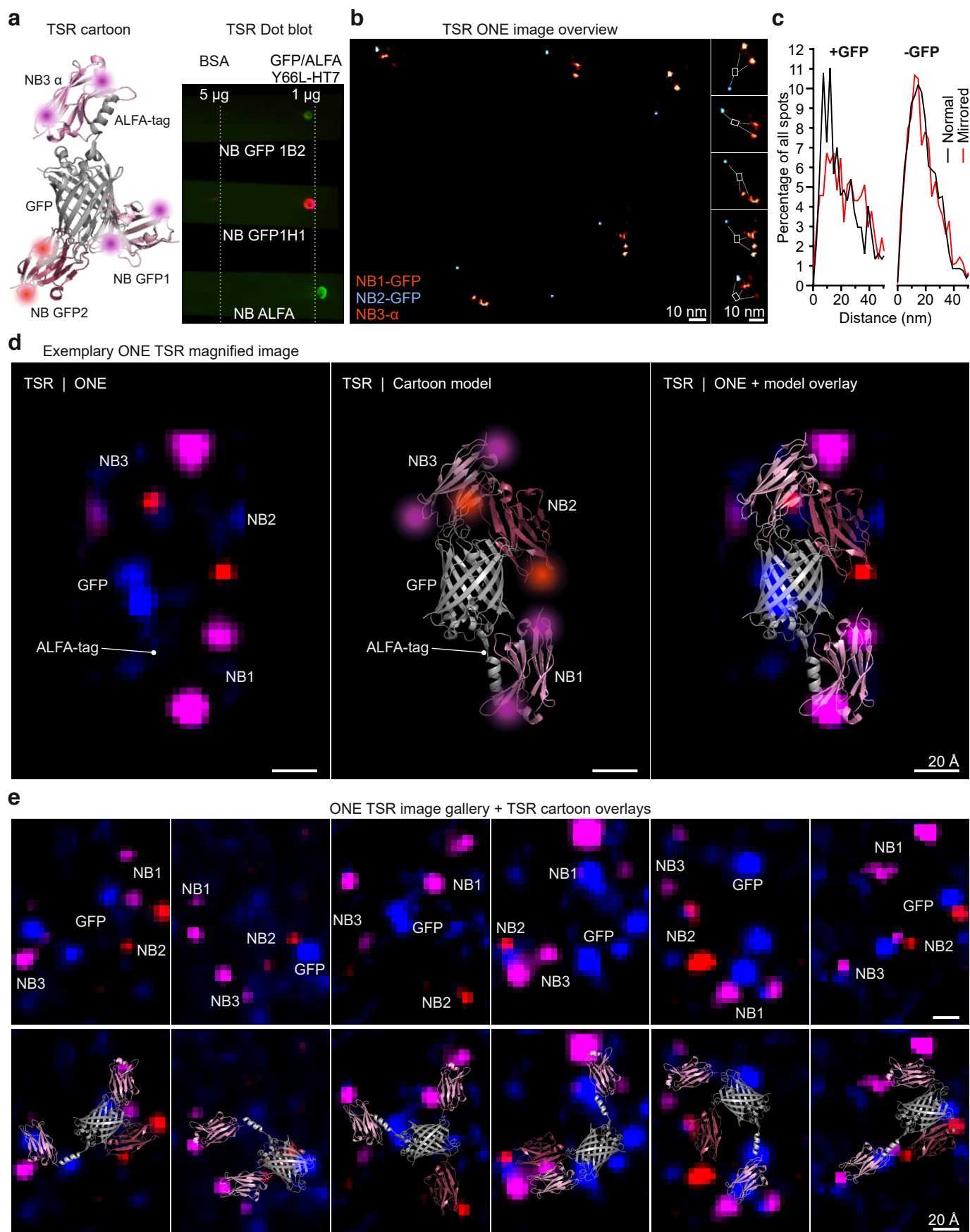


Supplementary Fig. 13. ONE imaging of purified calmodulin. **a**, An overview of calmodulin ONE acquisitions in the presence and absence of calcium. This molecule was expressed and purified as a chimera containing mEGFP. The compact signal associated to the GFP molecule, as observed already in the TSR images in Fig. 1, has a limited contribution to the overall size of the molecule. **b**, Exemplary zoomed calmodulin ONE images. The asterisk denotes the best guess of GFP molecule bound to calmodulin. This is a typical overview from 4 independent gels. **(c)** ONE images of calmodulin in the absence and presence of its ligand calcium after homogenization using autoclaving. **d**, Calmodulin size analysis plotted as violin plot showing the average, the quartiles and all data points. A two-tailed non-parametric Mann-Whitney test was applied ($p = 0.0006$, $N = 155-70$).



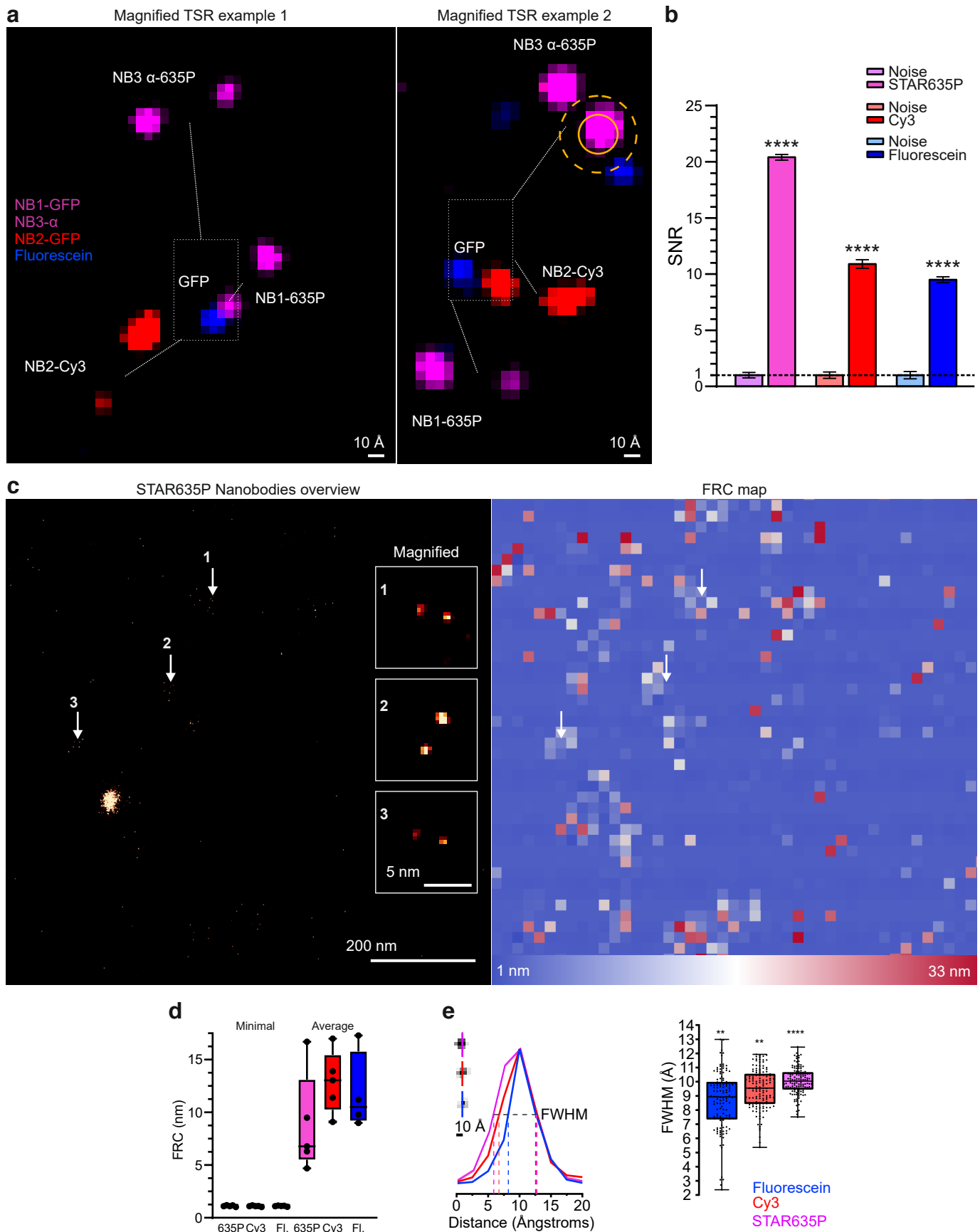
Supplementary Fig. 14. Comparative analysis between ONE microscopy and MINFLUX. **a**, ONE imaging of X2 STAR635P nanobodies. A cartoon showing an ALFA-tag nanobody conjugated to two fluorophores. **b**, An overview of purified X2 STAR635P nanobodies, *in vitro*. **c**, A magnified X2 STAR635P regions that are typically observed from at least 9 independent experiments. The line scans are displayed in **(d)**. **e**, Histograms of measured distances between the two fluorophores from ONE microscopy (left, N = 275 nanobodies) and replotted MINFLUX...

... measurements (right) from Sahl *et al.*, 2023. **f**, ONE imaging of PSD95 immunostained in cultured neurons, using a PSD95-specific nanobody. The exemplary image is followed by a graph that shows the nearest neighbor distances we measured for the PSD95 spots (N = 402 spot distance measurement), in comparison to measurements obtained in similar samples in MINFLUX, from Gürth *et al.*, 2023. **g**, Tubulin immunostainings (relying on primary and secondary antibodies) imaged using STED, without expansion, in confocal ExM and in ONE microscopy, relying on ZOOM ExM (3.5 fold expansion) as observed from 2 independent gels. The graphs depict the line scan indicated by the dashed lines in the tubulin cross-section (top) and a replotted MINFLUX line scan measurement, obtained from Butkevich *et al.*, 2021 (bottom).



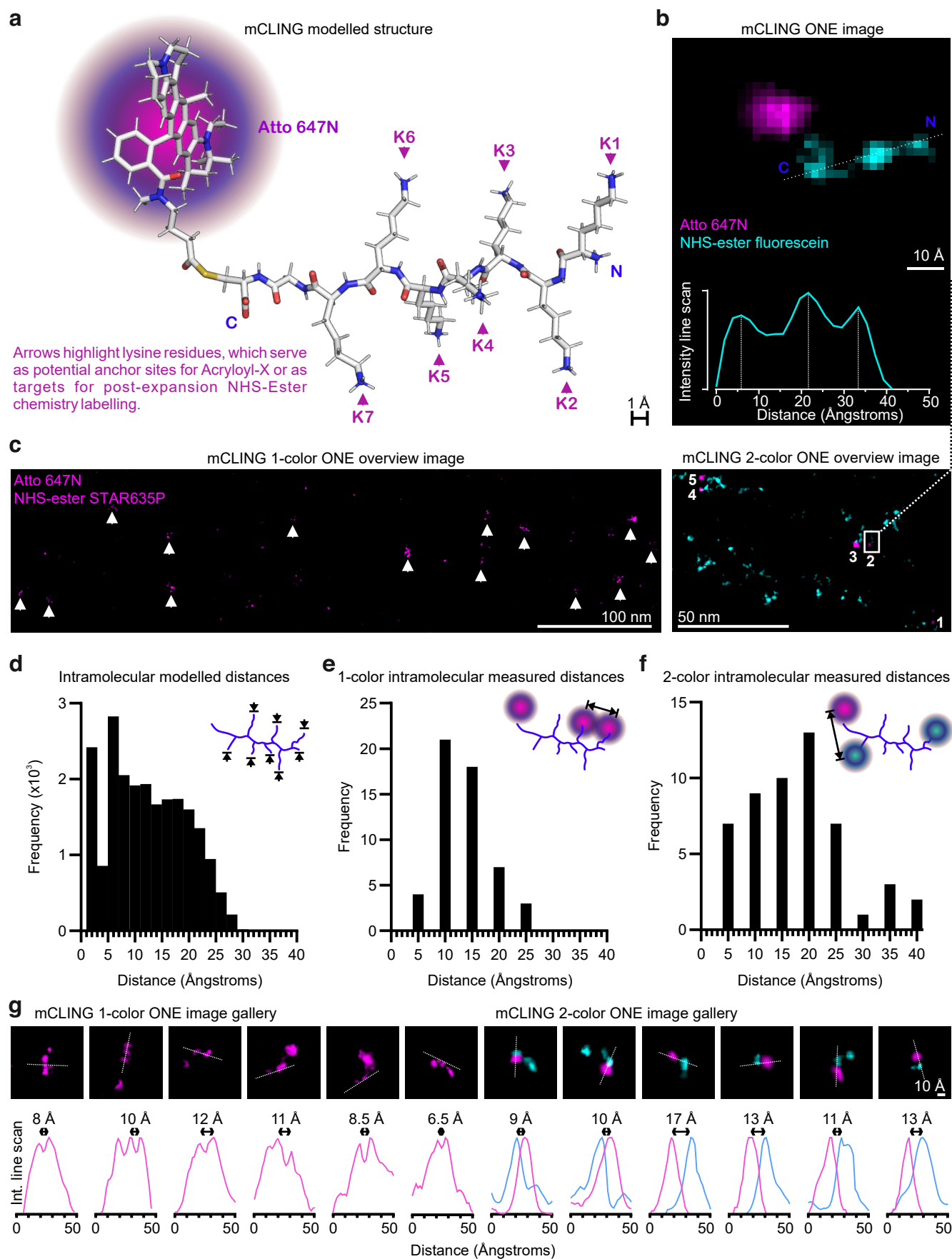
Supplementary Fig. 15. Analysis of GFP-nanobody complexes. **a**, TSR cartoon followed by a one dot blots experiment as a proof of principle that each nanobody was binding specifically the TSR individually, before carrying out ONE experiment. Nitrocellulose membranes were spotted with TSRs and bovine serum albumin, as control, and the spots were revealed with the respective nanobodies, using a fluorescence scanner (GE-Healthcare AI 600)...

... **b**, An overview of an image showcasing two-color nanobodies bound to their GFP target as observed from 3 independent gels. **c**, An analysis of distances from STAR635P to Cy3 nanobodies, in normal images or after mirroring one of the fluorescence channels, as a negative control. The close-distance interval is largely removed by mirroring. $N = 40$ -40 TSRs from at least 3 independent gels. Performing this in samples lacking the GFP, in which the nanobodies are randomly distributed, results in no differences between the normal and mirrored distributions. $N = 40/40$ images. **d**, The first panel shows a magnified TSR using only two-color nanobody labelling. The TSR is also labelled with NHS-ester fluorescein, and a small pixel size (0.48 nm) is used, to enable the optimal visualization. The middle panel shows a cartoon model that fits the TSR. The third panel shows an overlay of the ONE image and the model. **e**, A gallery of TSR ONE images and their respective cartoon overlays.



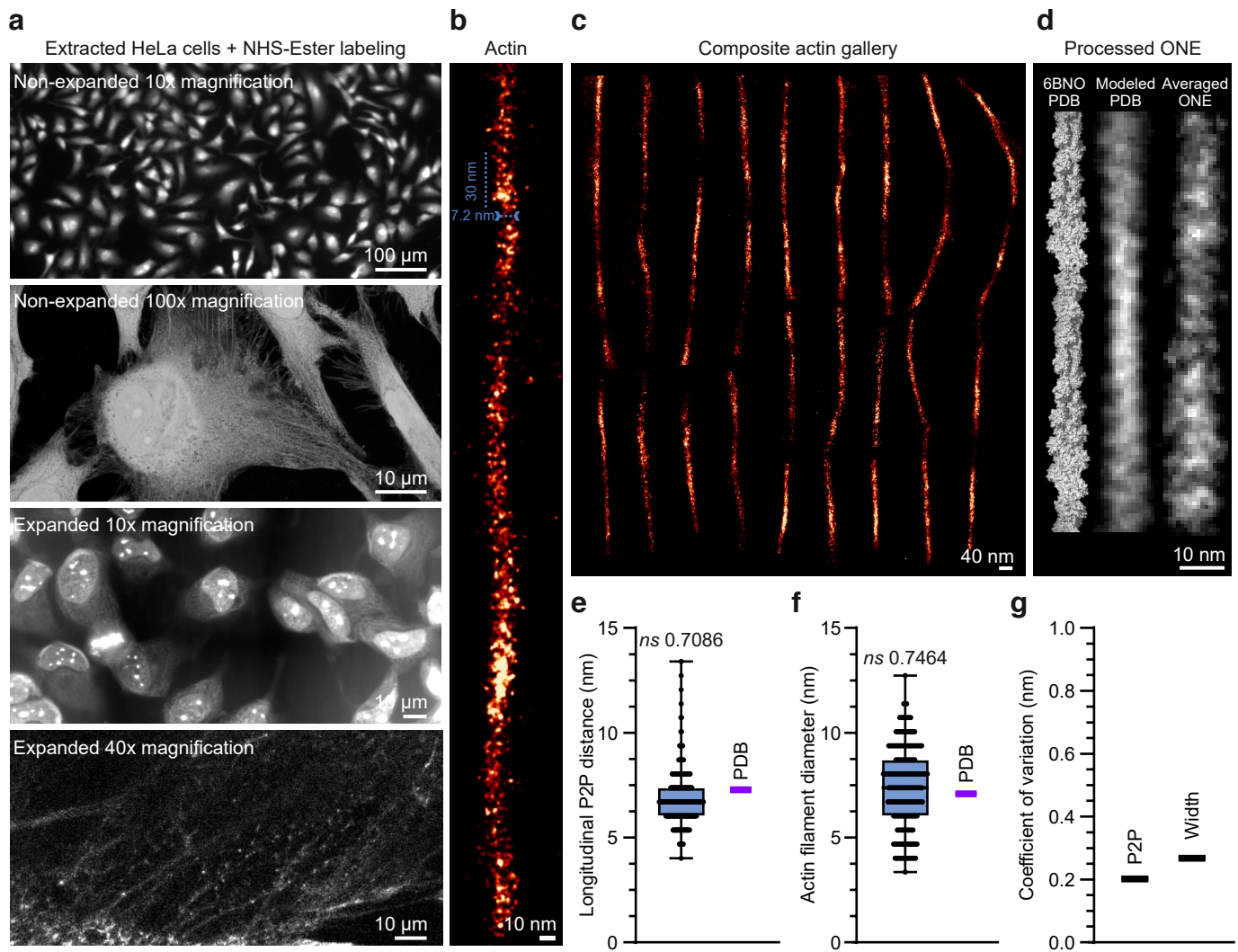
Supplementary Fig. 16. In-depth analysis of GFP-nanobody complexes. **a & b**, A bar graph of the average analysis with the standard error of mean of the signal-to-noise ratio of the TSRs, obtained by measuring the noise levels in the vicinity of the nanobodies. The noise levels are normalized to 1, implying that the normalized signal of the respective nanobodies now provides directly the signal-to-noise ratio. $N = 20-18$, $12-14$, and $17-11$ measurements from the fluorophore and noise signals from nanobodies with STAR635P, Cy3 and fluorescein, $p < 0.0001$, 0.0006 and < 0.0001 , respectively. A two-tailed Mann-Whitney test was applied. **c**, An exemplary display of Fourier Ring Correlation (FRC) analysis of nanobody images.

d, The best and average FRC analyses obtained per image, in the different color channels (N = 4 to 5 analyses for each acquired from different gels). **e**, To approximate the apparent resolution of the system, we drew line scans across spots and measured the full width at half maximum (FWHM) in curve fits executed on the line scans. The graph plots the FWHM of 129, 135, and 132 fluorescein, Cy3 and STAR635P line scans. The values are significantly different between the color channels. $p = 0.0024$, $p < 0.0001$ and $p = 0.0096$ for fluorescein vs. Cy3, fluorescein vs STAR635P and Cy3 vs. STAR635P, respectively. A Kruskal-Wallis test with Dunn's multiple comparisons test were applied. The box plot shows the median, 25th percentile and the range of values.

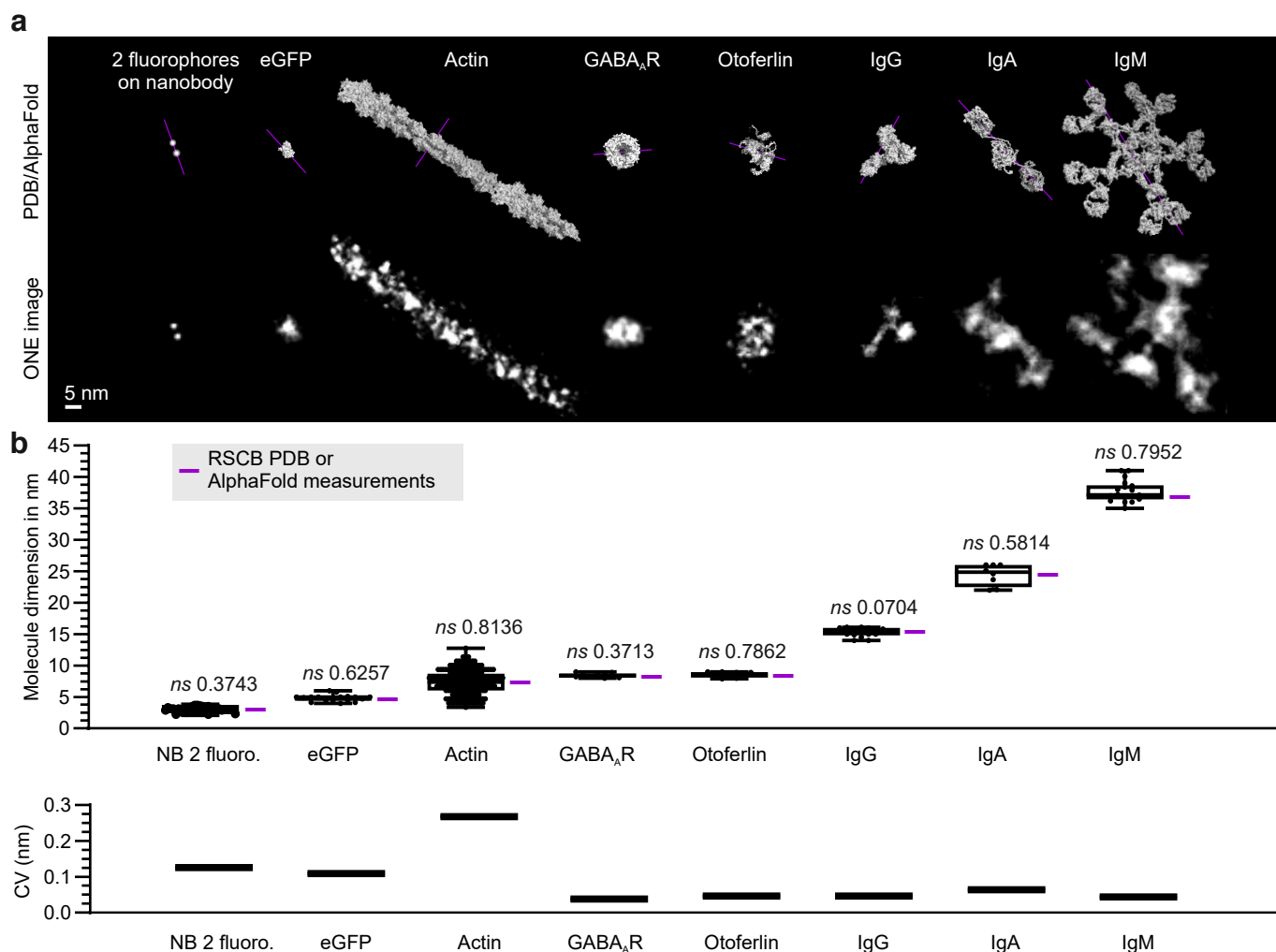


Supplementary Fig. 17. ONE Microscopy multi-color fluorophore imaging of a small peptide. **a**, A modelled mCLING structure, using the AMBER99 force field-GROMACS simulation package (please see Methods sections for more details). In mCLING, seven lysine residues are positioned at a varying distance from the C-terminal end, with separation intervals...

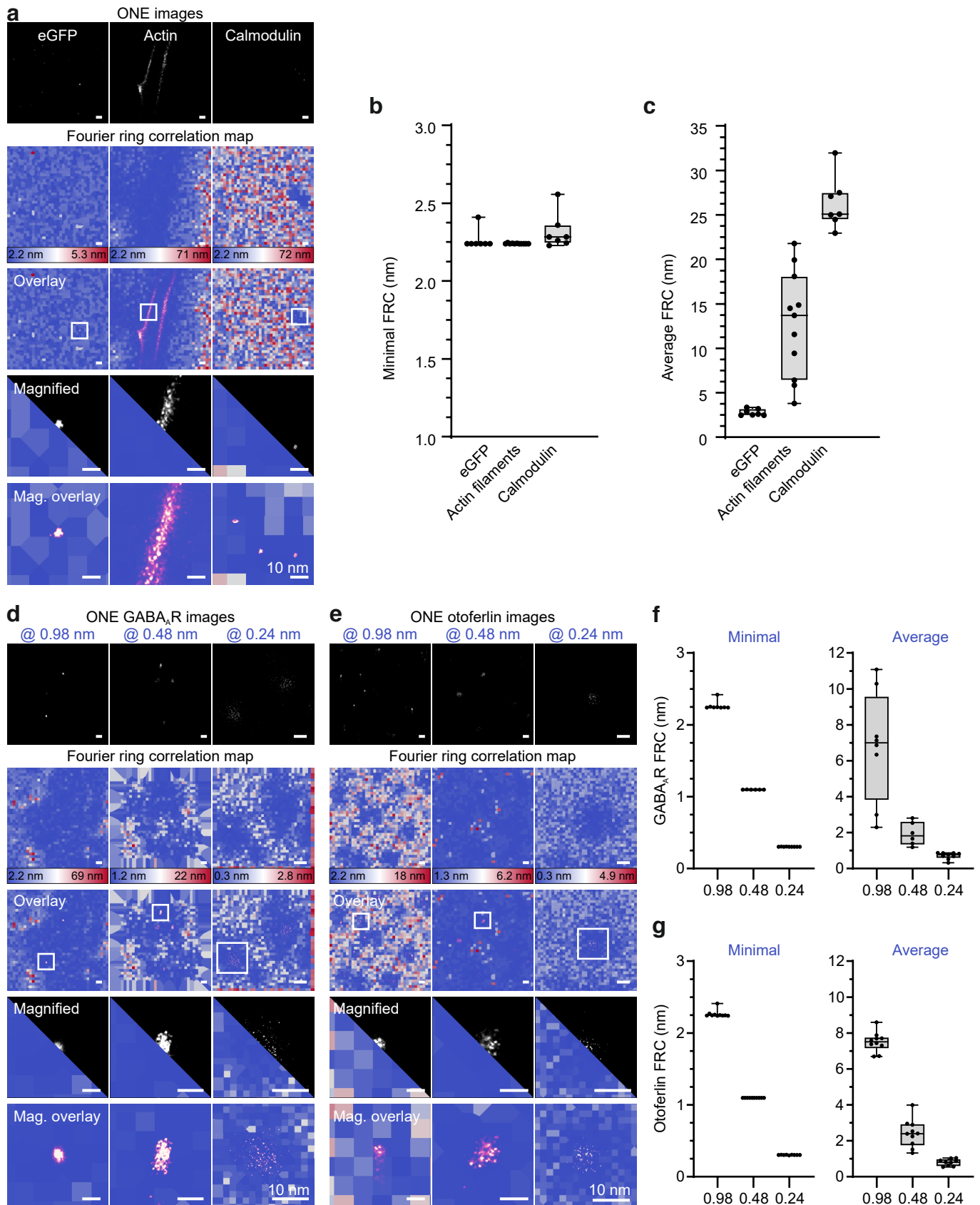
... spanning from a modelled proximal 3.627 Å to a more distal 27.954 Å. In our experiments, mCLING carries an Atto647N fluorophore. **b**, A 2-color image of mCLING-Atto 647N, labeled with NHS-ester fluorescein after expansion. The indicated intensity line scan is displayed below the image. **c**, A 1-color ONE overview image of mCLING-Atto 647N, labeled with NHS-ester STAR635P after expansion (left side), and a 2-color ONE overview of mCLING-Atto 647N, labeled with NHS-ester fluorescein. **d**, A frequency graph showing intramolecular distances between the C terminus and the lysin residues. **e**, A binned frequency graph of intramolecular measured distances from 1-color and **(f)** 2-color mCLING ONE images. Three independent experiments were carried out, and N1-color = 53 and N2-color = 52 measurements were performed. All mCLING experiments were independently repeated 3 times with 2 gels each. **g**, Exemplary 1- and 2-color mCLING ONE images, with line scans drawn over the exemplary pairs of spots. The intensity line scans are displayed below each panel.



Supplementary Fig. 18. ONE imaging of cytoskeletal components, in NHS-ester labelled cells. **a**, HeLa cells were subjected to extraction according to a previously published protocol (Svitkina *et al.*, 2016). Panels from top to bottom show an overview of non-expanded, extracted HeLa cells labelled with NHS-ester chemistry (10x objective), followed by a higher magnification view and by two views of cells expanded after extraction. **b**, An exemplary image of a cytoskeletal filament. **c**, Composite actin gallery. **d**, A PDB cartoon, followed by a modeled averaged PDB, depicting a best-case scenario for ONE microscopy of actin fibrils under ideal conditions, and the achieved ONE image reconstructed from 47 different actin regions from 2 independent experiments from 4 gels. **e**, **f**, & **g**, A set of measurements that were applied over actin filaments, quantifying the longitudinal peak-to-peak distance along the filament (distance between actin monomers), the actin filament width, and the coefficient of variance of these two measurements, respectively. These values resemble well the known dimensions from actin PDBs (magenta lines). N = 198 and 130 actin measurement for the first graph and second graph, respectively; 2 independent experiments. Data in **e** and **f** are plotted as box plots with all data points and whiskers showing minimum and maximum values. An unpaired nonparametric two-tailed t-test was applied.

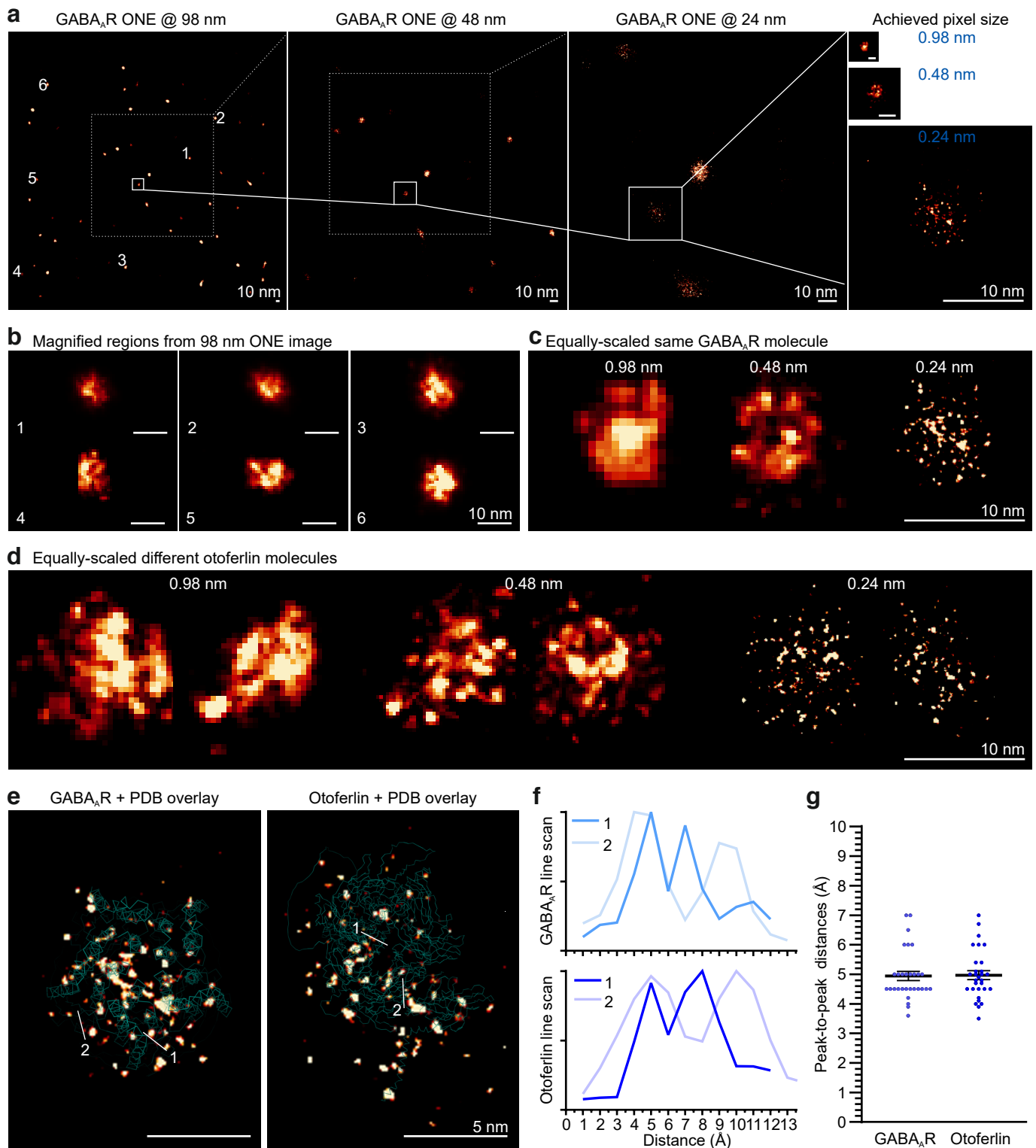


Supplementary Fig. 19. Expansion precision evaluation. **a**, A direct comparison between single-molecule ONE images and their respective PDB/AlphaFold models. The purple line indicates the line scan used to measure the molecule dimension indicated in the first graph. **b**, The upper graph shows measurements of molecule dimensions, in nm displayed as box plots with averages and whiskers showing minimum and maximum values. The horizontal purple line indicates the expected value, obtained from measurements of PDB structures (for all molecules except otoferlin), or AlphaFold predictions (for otoferlin). The lower graph shows the variability of these measurements, in the form of the coefficient of variance. N = 34, 17, 192, 10, 10, 14, 8, and 18 size measurement for NB, eGFP, actin, GABAAR, otoferlin, IgG, IgA, and IgM; at least 2 experimental replicates were carried for all experiments. Paired t tests were carried out to determine whether the measured values are different from the values predicted by the PDBs; the respective *p* values are reported above the plots. A two-tailed Mann-Whitney tests were applied.

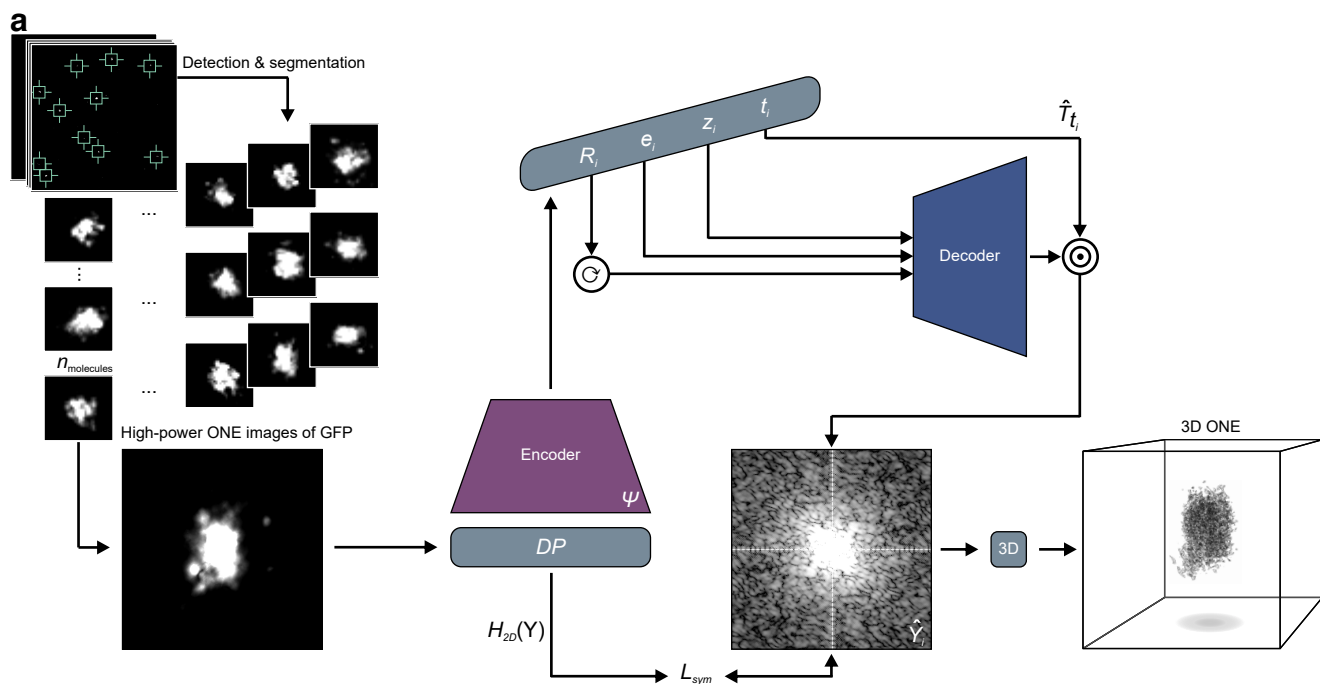


Supplementary Fig. 20. Detailed analysis of Fourier ring correlation. **a**, FRC analysis of ONE images collected with a pixel size of 0.98 nm. The first panel row shows ONE images of the different specimens. The second row shows the corresponding FRC maps. The third row shows ONE images overlaid over FRC maps, using a screen-blend mode. The fourth and fifth rows show magnified views. These data are as typically observed from 3, 2, and 4 independent gels for eGFP, actin and calmodulin, respectively. **b**, A graph plotting the minimal FRCs in nm. **c**, A graph plotting the average FRCs in nm. Please note that all the labelled targets reside in the “bluest” regions of the map, indicating minimal FRCs that correspond to high resolution. N = 7, 12, and 7 FRC measurement for eGFP, actin, and calmodulin, respectively. **d** & **e**, FRC analysis of ONE...

... images achieved with a pixel size at 0.98, 0.48, and 0.24 nm for GABAAR and otoferlin, respectively, as observed from at least 4 independent experiments for each. **f**, The graphs shows minimal and average FRCs in nm for GABAAR ONE images. N = 8, 6, and 9 for 0.98, 0.48, and 0.24 nm images, respectively. **g**, The graphs shows minimal and average FRCs in nm for otoferlin ONE images N = 10, 10, and 9 FRC measurement for 0.98, 0.48, and 0.24 nm images, respectively. All experimental sets were performed with at least 2 replicates. Data in **b**, **c**, **f** and **g** are plotted as box plots with the average and whiskers showing minimum and maximum values.

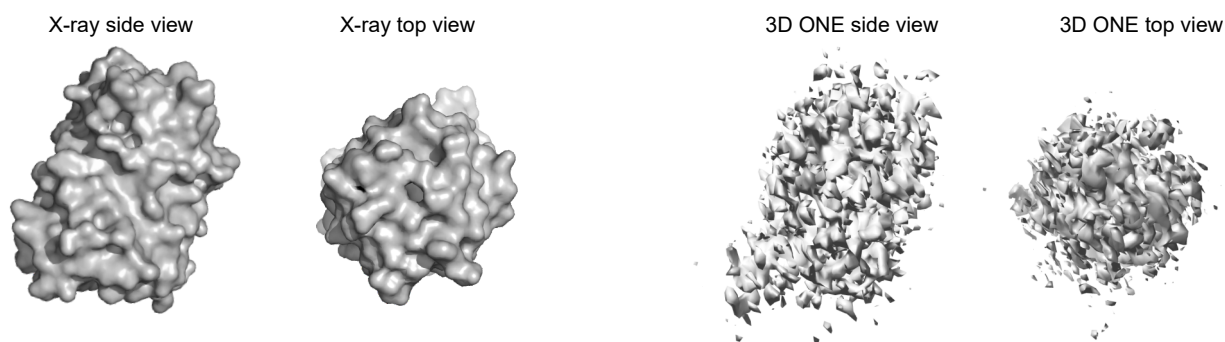


Supplementary Fig. 21. Intra-molecular measurements within a single protein molecule. **a**, GABA_AR ONE images acquired with 0.98, 0.48, and 0.24 nm pixel sizes, for the same region. **b**, GABA_AR magnified examples from the first image in the panel above. **c**, One particular GABA_AR molecule displayed at different resolutions. **d**, Equally-scaled otoferlin molecules acquired at different resolutions. **e**, ONE images of GABA_AR and otoferlin at 0.24 nm overlaid with their respective PDBs. **f**, The graphs show 2 exemplary line scans for peptide segments in GABA_AR and otoferlin. **g**, A dot plot graph with the average and standard error of mean showing peak-to-peak distances in Ångström. N = 30 line scans for GABA_AR, and 30 line scans for otoferlin, 10 independent experiments for GABA_AR and 4 independent experiments for otoferlin, of which representative images are shown in panels **a-e**.

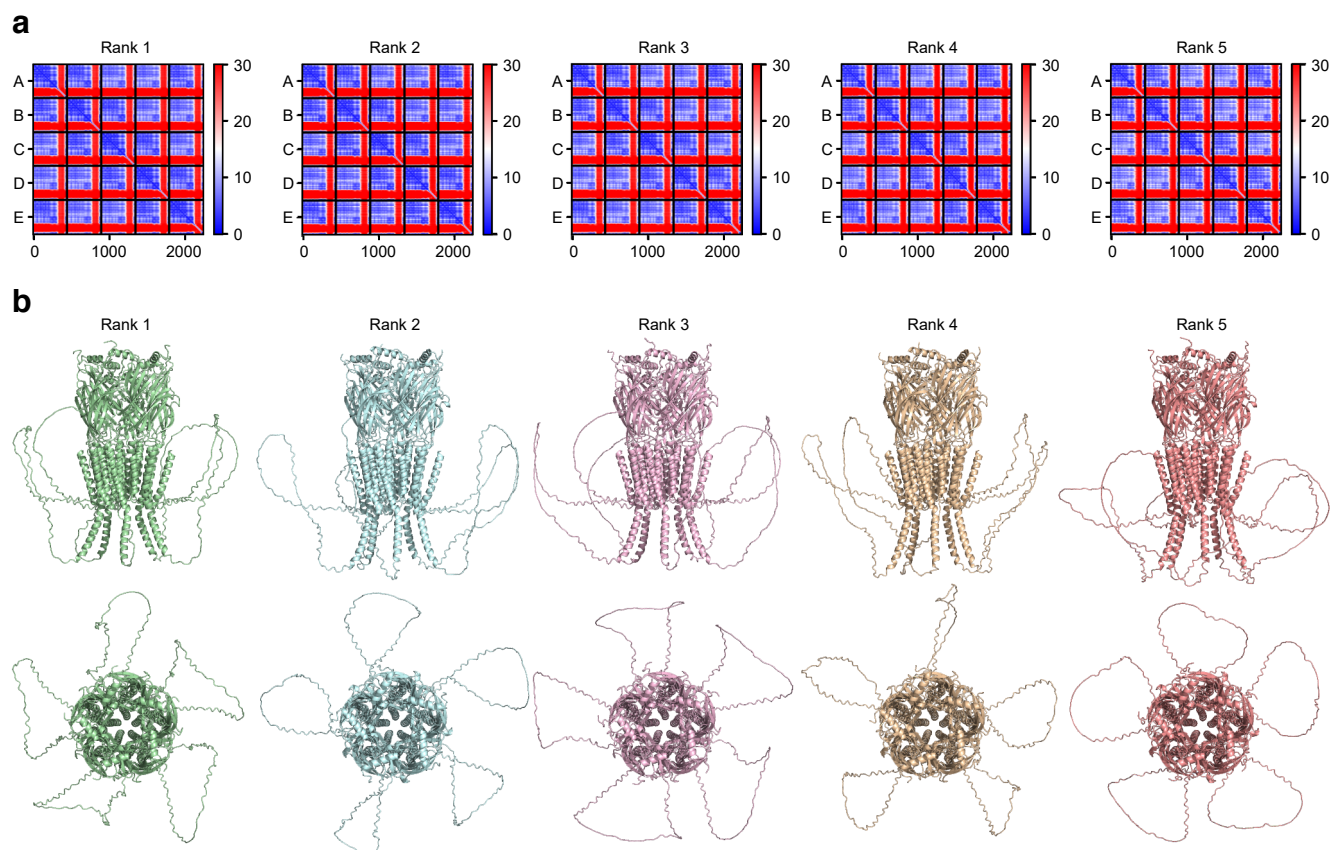


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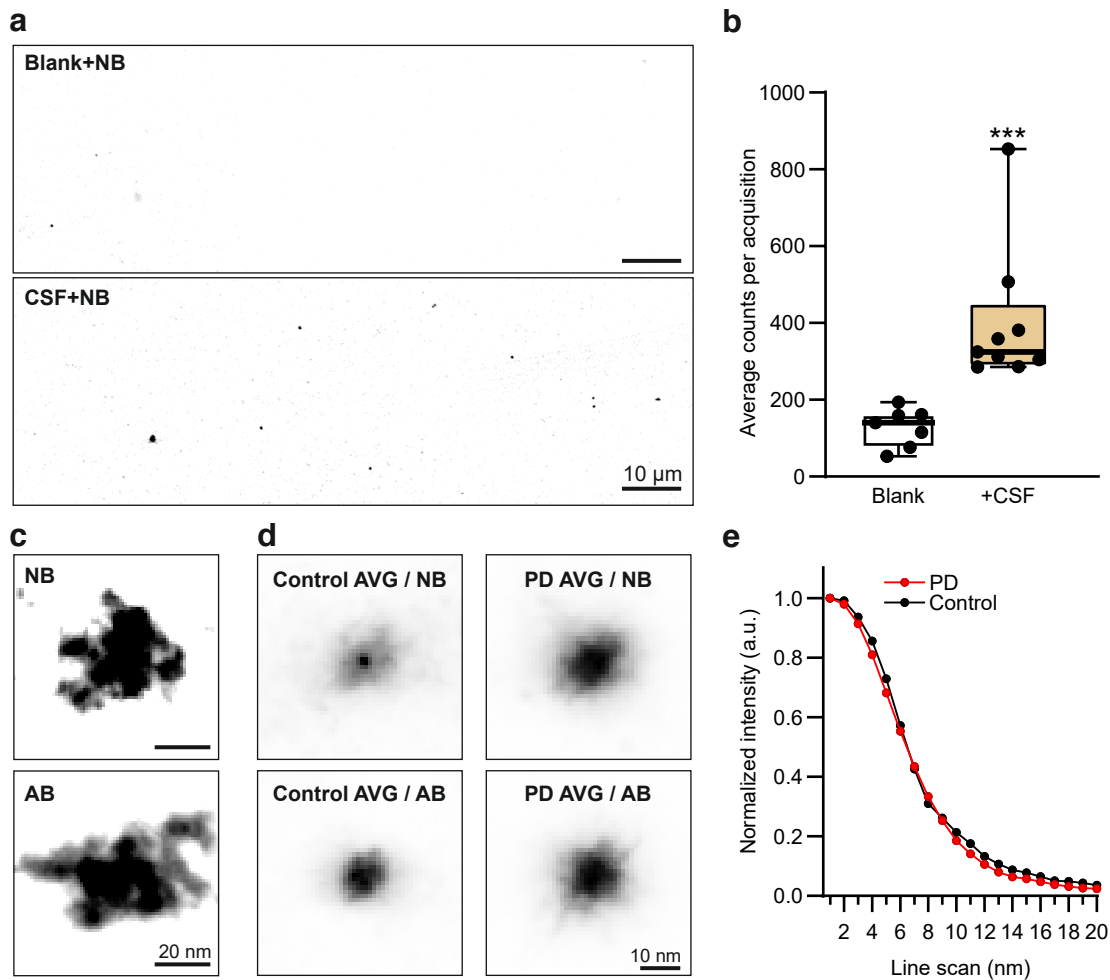
3D ONE GFP reconstruction



Supplementary Fig. 22. 3D ONE reconstruction via unsupervised *ab initio* artificial intelligence architecture. **a**, Molecules of interest are automatically detected and segmented from 2D ONE images. Fluorescence images are converted to density points *DP*. *DP* images are transferred into a modified cryoFIRE network (Levy *et al.*, 2022, arXiv:2210.07387v1) that performs heterogeneous reconstructions in an amortized interference framework of images with unknown positions in the 3D space. The encoder predicts, for each molecular image, a set of features that includes a linear scale of intensity D_i , the latent variables rotation R_i , expansion factor correction e_i , molecule conformation Z_i , and the translation t_i . The decoder deciphers the encrypted features in the Hartley domain, followed by performing elementwise multiplication with t_i to form the predicted image $\hat{Y}_i$. The network backpropagates using the symmetric loss function L_{sym} with the $H_{2D}(Y)$, and the decoded information is stacked in perpendicular slices, allowing the generation of 3D reconstructed files that can be viewed using UCSF ChimeraX. **b**, 3D reconstruction of GFP from 885 molecules. For comparison, GFP side and top views are shown from X-ray 4KW4 PDB and from our reconstruction. Fourier shell correlation analysis indicated that the 3D ONE structure is generated at a resolution of 18 Å.

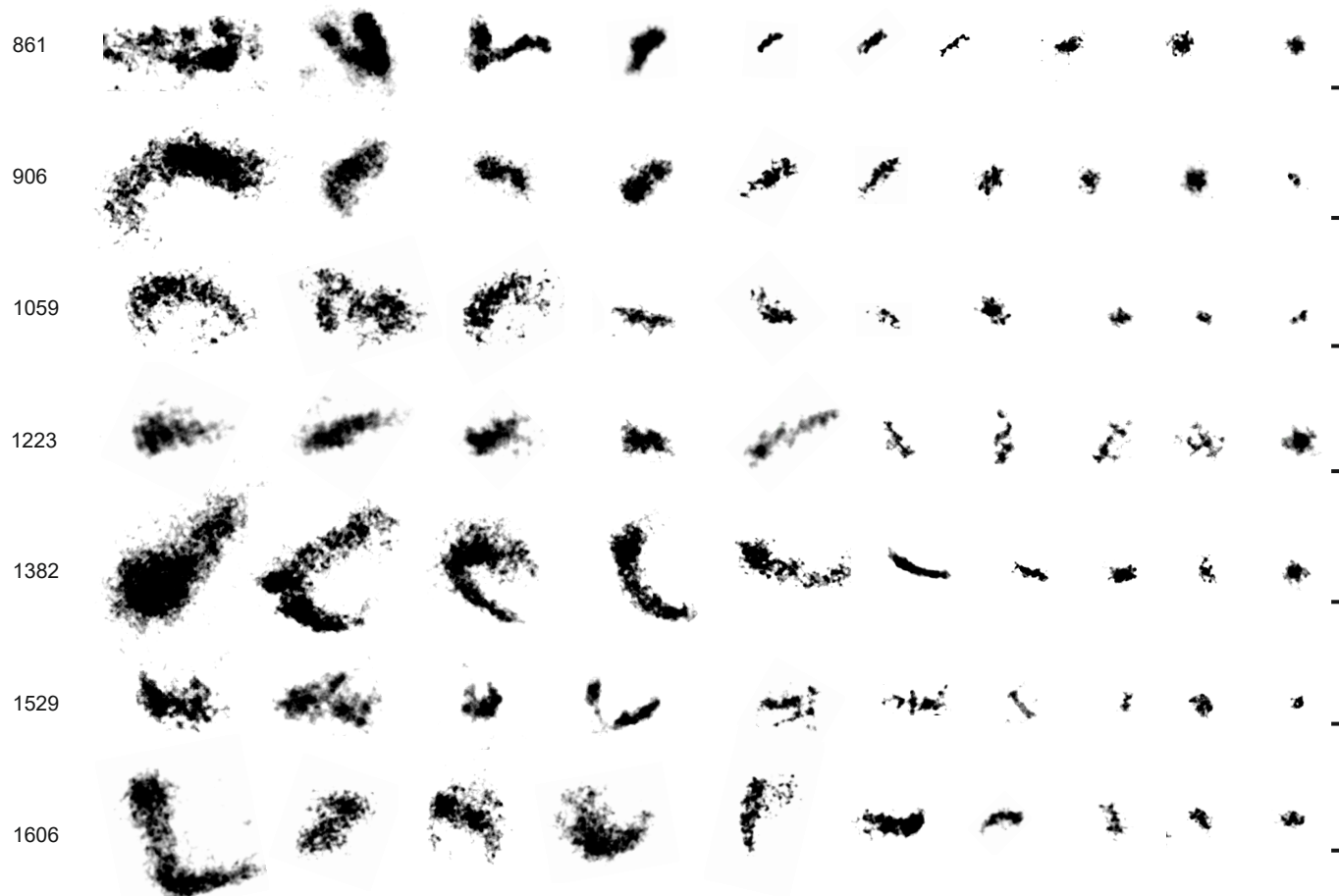


Supplementary Fig. 23. AlphaFold multimer prediction of a homopentameric GABAA beta 3 receptor. a, Matrices of predicted aligned errors (PAE) for the top 5 ranked models. **b,** Top five GABAA beta3 receptor predictions. For more information, see Supplementary Material.

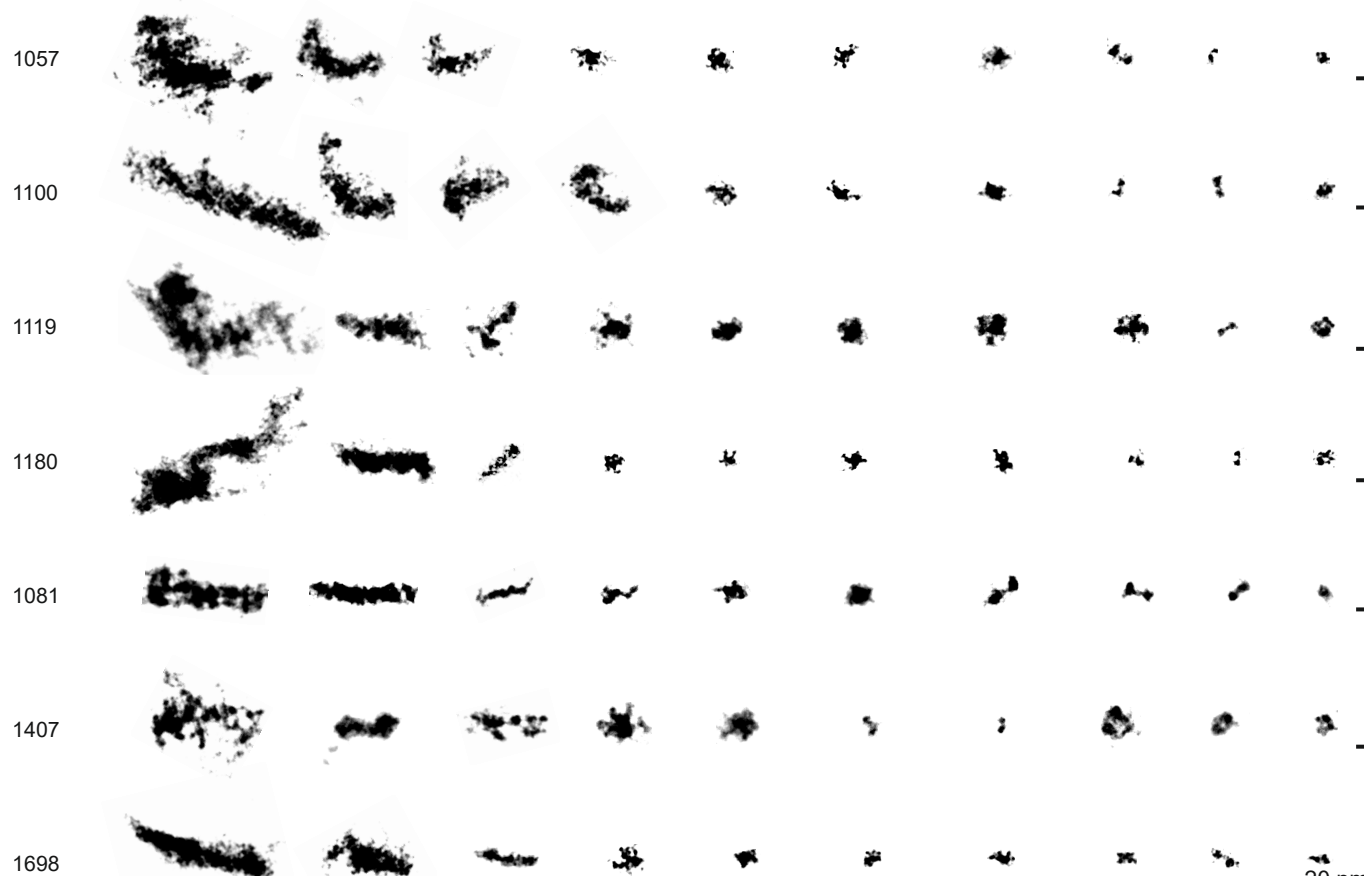


Supplementary Fig. 24. The nanobody imaging of ASYN objects is specific and is not easily reproduced by antibodies. **a**, Low-resolution images of CSF-containing samples, or blanks (clean, BS-coated coverslips). Only a few dim spots, presumably representing single nanobodies, are seen in the blanks. **b**, Quantification of the signal intensity, as a sum across all image pixels plotted as a box plot with average and whiskers showing the minimum and maximum values. $N = 7$ and 9 independent region measurements for blank and CSF; two-tailed non-parametric Mann-Whitney test, $P = 0.0002$. **c**, Individual examples of oligomers immunolabelled with nanobodies (top) or antibodies (bottom) as observed in 986 and 66 acquired images with nanobody or antibody labeling from multiple gels. **d**, Averages of SYN objects from individual patients, immunolabeled with nanobodies or antibodies. **e**, An analysis of the average object size in antibody-labelled samples, as in Fig. 4. $N = 2$ patients for each condition. Nanobodies reveal differences between patients, at object sizes of only a few nm. Antibodies have difficulties in this direction, as their large size causes a lower-fidelity labelling, and as their sizes obscure the actual sizes of small objects.

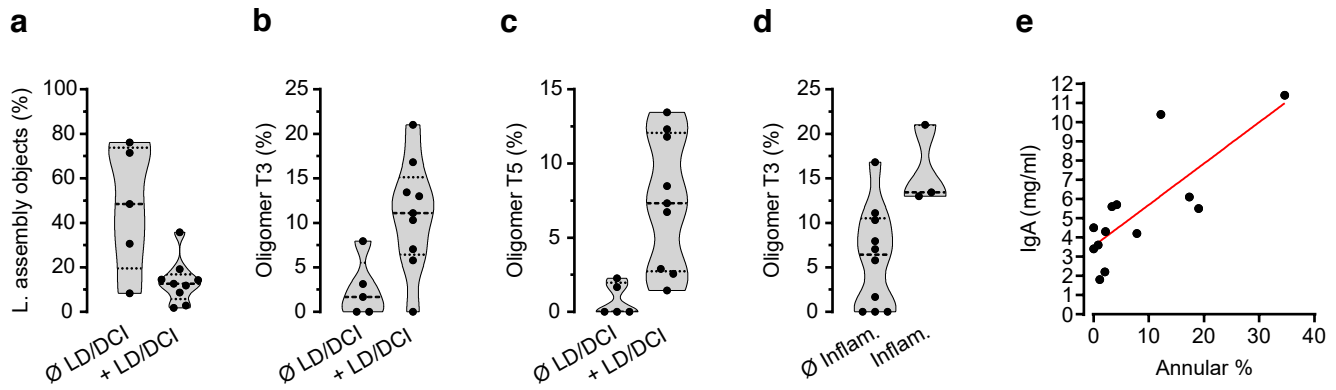
Control



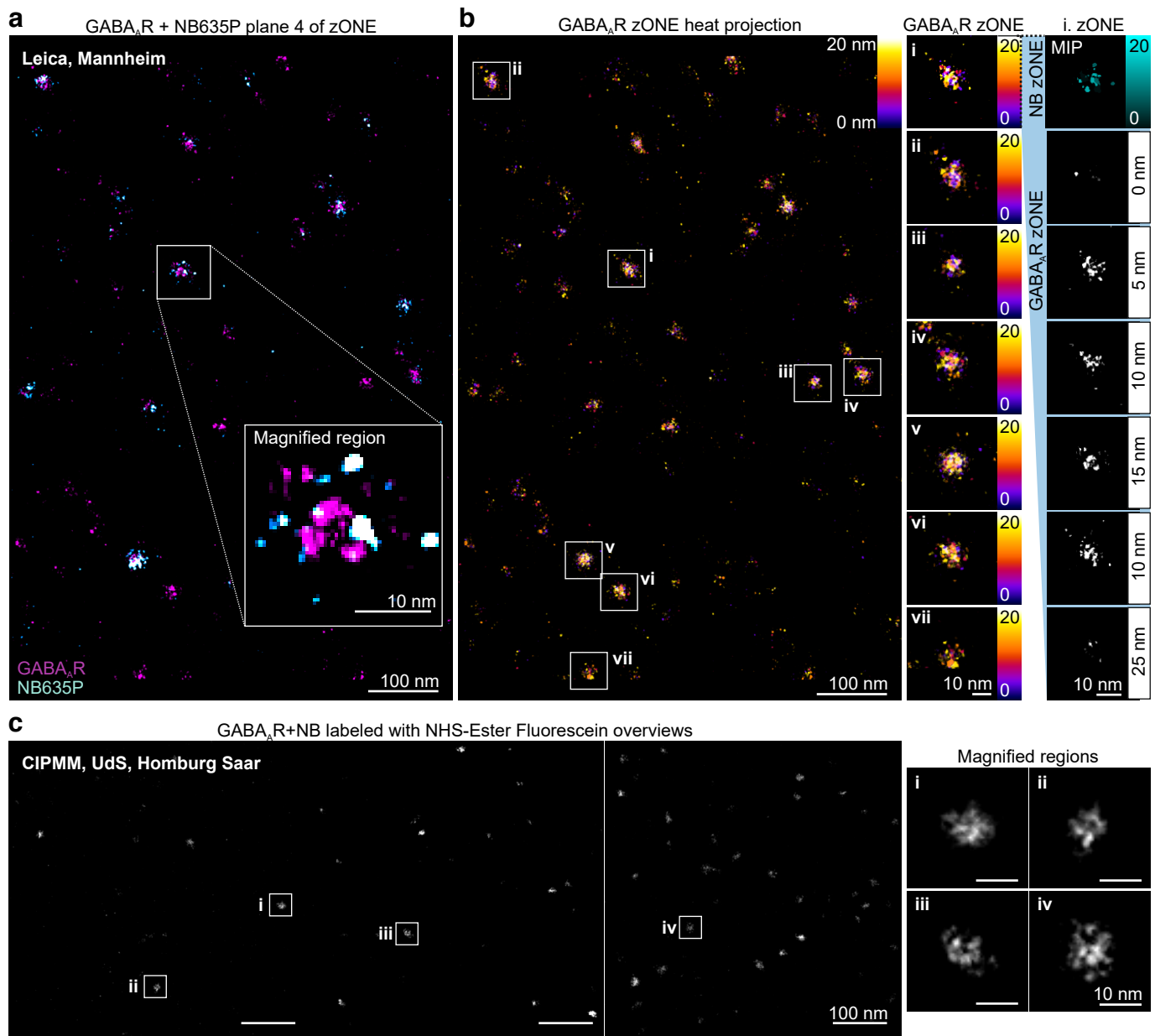
PD



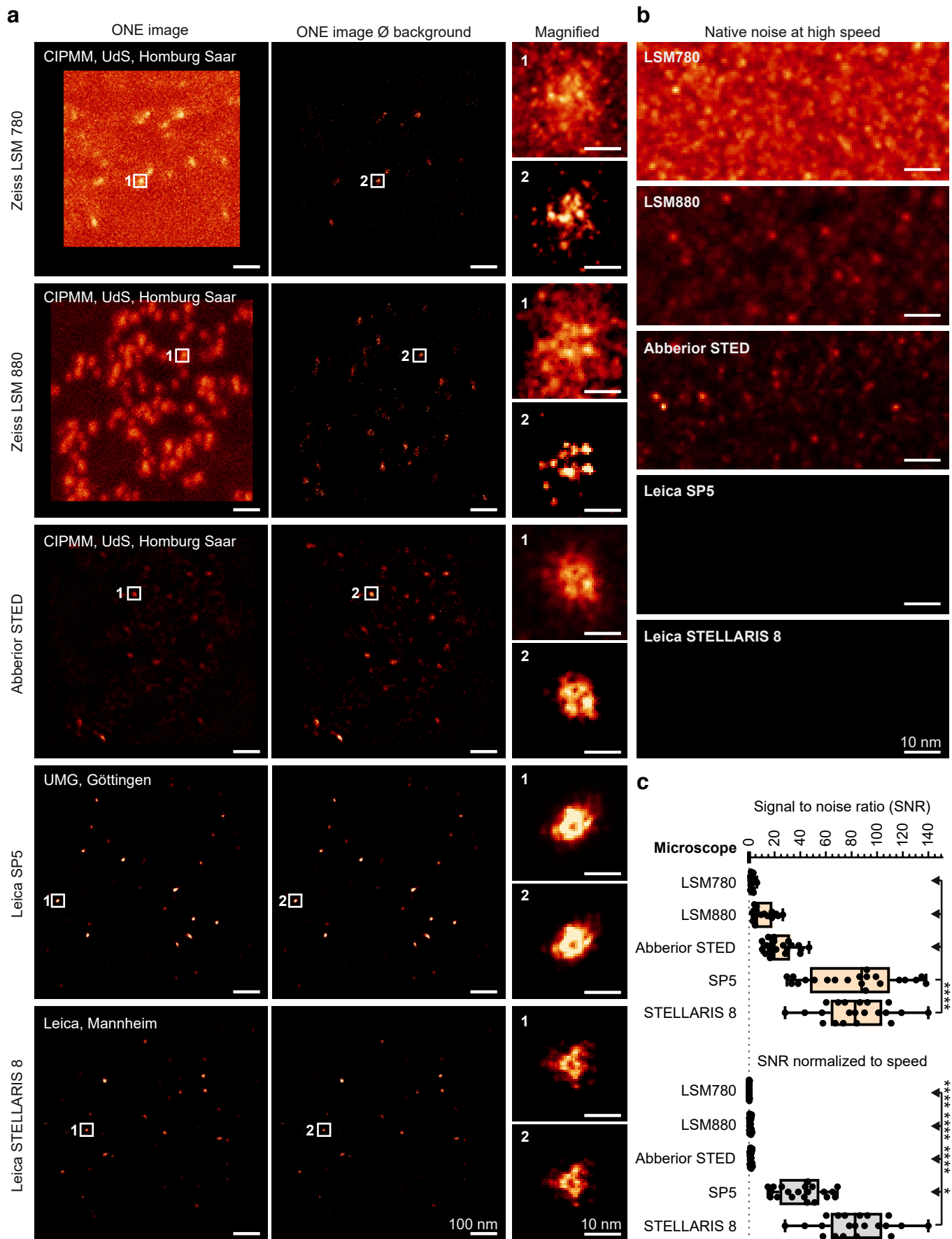
Supplementary Fig. 25. A gallery of ASYN object images from 7 PD patients and 7 controls. The images were obtained following the procedure indicated in Fig. 4a. See Supp. Table 1 for details on the respective patients. 20 nm



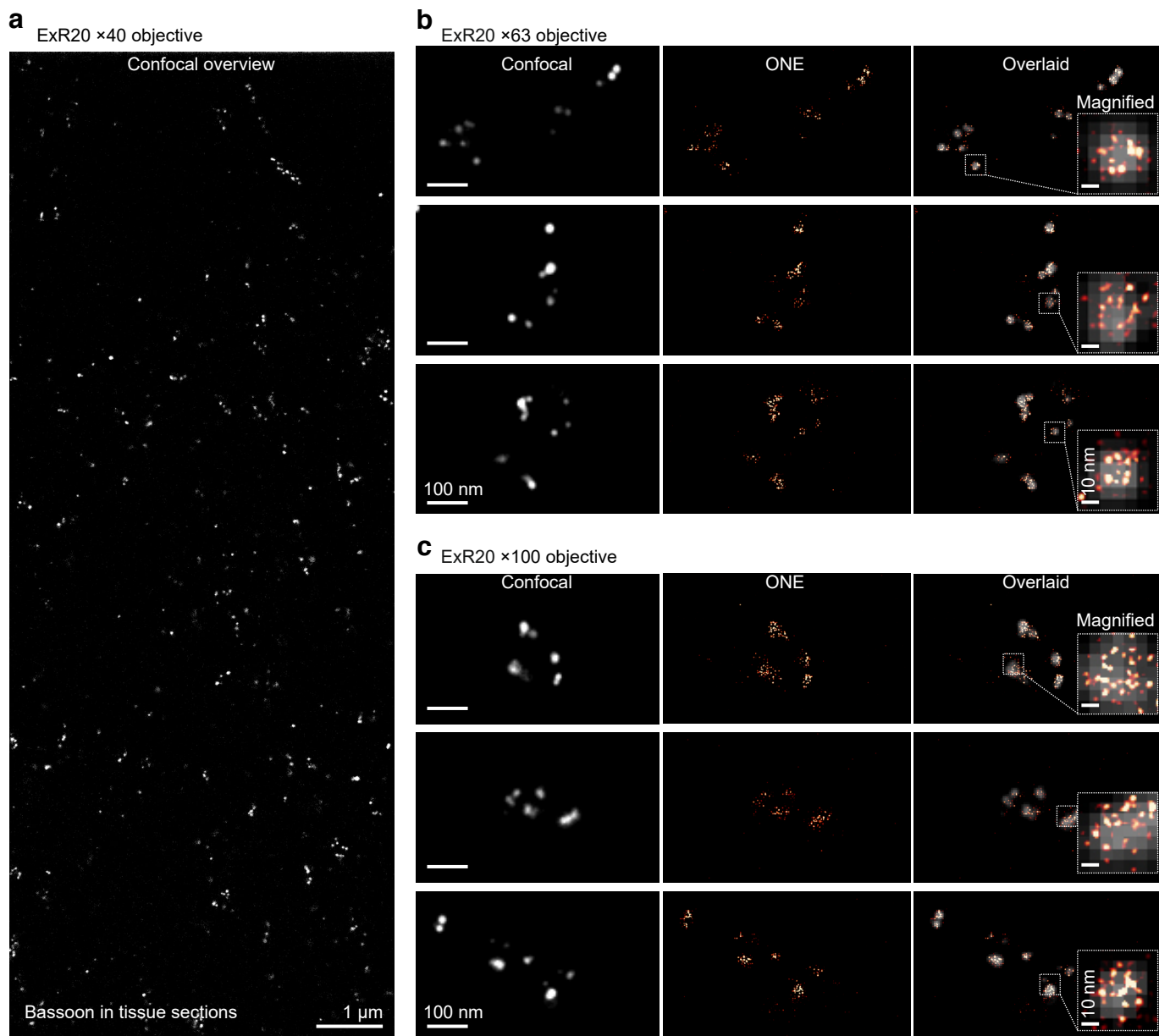
Supplementary Fig. 26. Analysis of ASYN species in relevance to medication status and clinical features. The violin plots (**a**, **b**, & **c**) show large assembly objects, Oligomer T3, and Oligomer T5 in relation to the presence of absence of Levo-DOPA treatment. **d**, Oligomer T3 percentage in the presence or absence of inflammation. **e**, The relation between IgA and Annular ASYN species. Large assemblies correlate inversely with Levo-DOPA and decarboxylase inhibitor (LD/DCI) treatments; oligomer types 3 and 5 correlate positively with these treatments (two-tailed t-tests followed by a Benjamini-Hochberg multiple testing correction with FDR of 5%; $p = 0.0066$ for panel **a**; $p = 0.0166$ for panel **b**; $p = 0.0075$ for panel **c**). Type 3 also correlates significantly with brain inflammation ($p = 0.021$). Annular oligomers correlate well with IgA levels in CSF ($p = 0.0015$, Pearson correlation test; the Pearson's correlation test was calculated using the corr2 function in MATLAB).



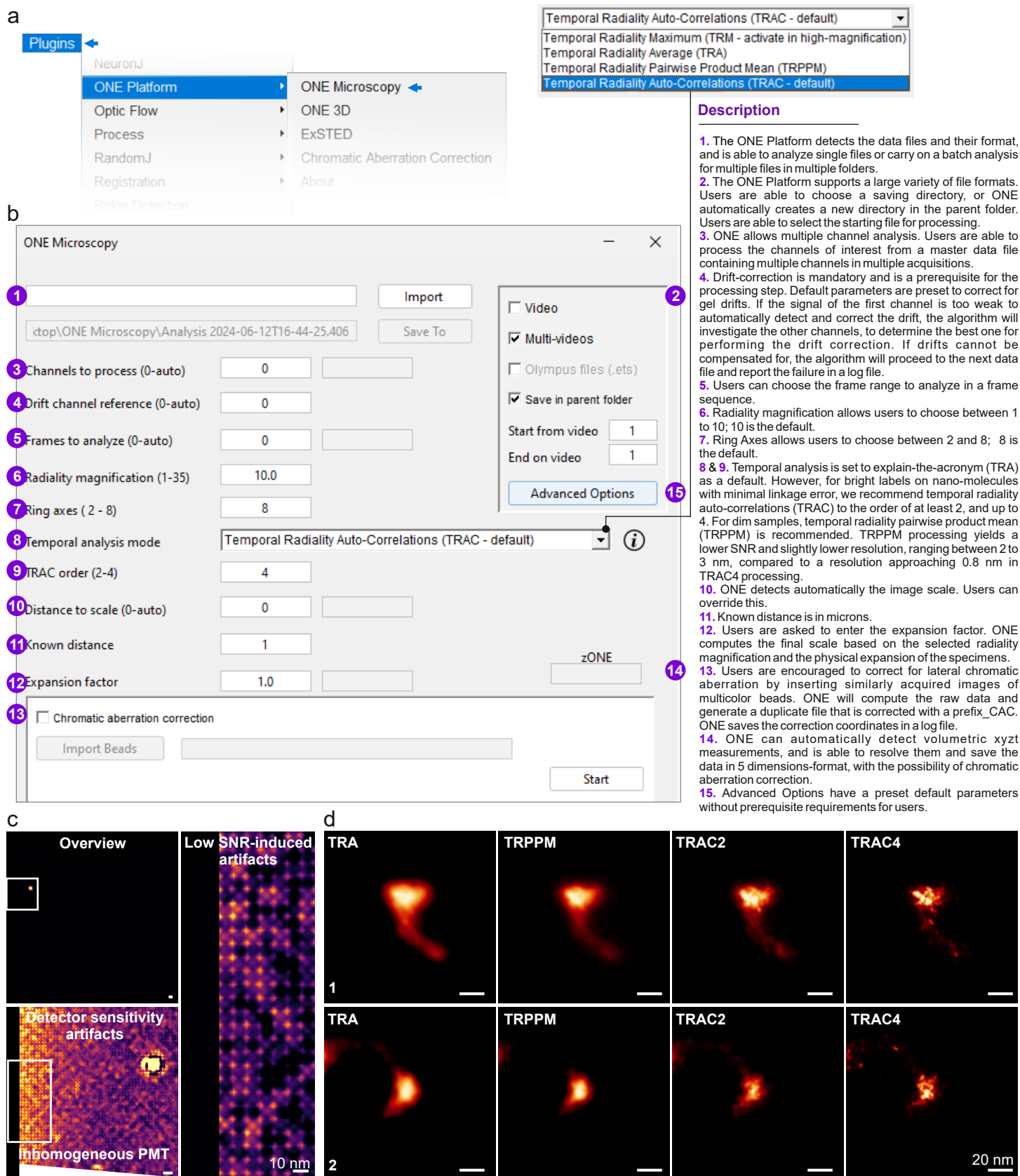
Supplementary Fig. 27. ONE microscopy applied at the confocal headquarters of Leica Microsystems and at the Center for Integrative Physiology and Molecular Medicine (CIPMM) of the Saarland University (UdS). As GABA_AR were systematically investigated in this study, we chose them as a reference to evaluate the applicability of ONE technique at different laboratories using different systems. **a**, Using a STELLARIS 8 microscope at Leica Microsystems, we present a snapshot of a plane from a 5-dimension x,y,z,c,t image of GABA_AR+NB. **b**, The first panel shows a depth projection of a zONE stack. The second panel shows a set of GABA_AR that were magnified. The optical sectioning of the first example is displayed in the rightmost panel. Three independent gels were used for this experiment. **c**, GABA_AR were also successfully imaged at CIPMM from at least 4 independent gels, Saarland University (UdS), as shown in full 3 full-scale overviews and in their respective magnified regions. Several microscopes were used at the CIPMM, which are presented in the next figure.



... to their speed limit, background noise was substantially higher on older models. The second panel shows ONE images with background subtraction. The third panel shows a magnified receptor example. **b**, Magnified regions showing the noise readings of each of the used microscopes. **c**, A box plot graph with averages and whiskers displaying the minimum and maximum values showing the achievable signal-to-noise ratio (SNR), as well as the SNR normalized to acquisition speed. Not surprisingly, higher SNRs yielded better ONE images. N = 22, 22, 22, 23, and 23 full region measurement for LSM780, LSM880, Abberior STED, SP5, and STELLAIRS 8, respectively, with each from at least two independent gels. Kruskal-Wallis test was applied. SNR graph *p* values equal to < 0.0001, < 0.0001, 0.0004, and 0.9838 for STELLAIRS 8 vs. LSM780, LSM880, Abberior STED and SP5, respectively. SNR normalized to speed graph *p* values equal to < 0.0001, < 0.0001, < 0.0001 and 0.0705 for STELLAIRS 8 vs. LSM780, LSM880, Abberior STED and SP5, respectively.



Supplementary Fig. 29. ONE microscopy applied at the MIT, Cambridge, USA. Post-expansion bassoon labeling ONE images from tissue sections that were expanded and then labelled against bassoon following the expansion revealing (ExR) protocol (Sarkar *et al.*, 2022) at the MIT, Cambridge, US. **a**, An ExR20 (X20 expansion) confocal overview imaged with a $\times 40$ objective. **b**, Three different exemplary ONE images of bassoon using a $\times 63$ objective. The first image is a resonant scan MIP of 20 frames, followed by a ONE image, and an overlay with its respective confocal image. The white square indicates the magnified region to the right. **c**, Similar to **b**, but using a $\times 100$ objective. Images were acquired from two independent gels.



Supplementary Fig. 30. ONE analysis and examples. **a & b**, Several views of the starting interface of the ONE software package. The examples show the intuitive software choices. See also the “Readme/Help” file of the software package. **c**, Examples of different potential artifacts that should be avoided in ONE imaging. **d**, Different potential choices in how to resolve ONE images. We suggest using the temporal radiality pairwise product mean (TRPPM) procedure for dim samples. This reduces the obtainable resolution, but follows much better the potential sample shape. For brightly labelled samples with direct labeling, the temporal radiality auto-correlation (TRAC4) procedure provides the best resolution and SNR, indicating the positions of the individual fluorophores. This is typically observed in the hundreds of acquired ONE images.

Supplementary Tables

Supplementary Table 1. Patient details.

ID	Sex	Age	Diagnosis
1180	m	74	PD
1407	m	82	PD
1698	m	83	PD
1057	f	74	PD
1081	m	69	PD
1100	m	71	PD
1119	f	84	PD
861	f	60	RLS
906	m	73	CBD
1059	m	70	PSP
1223	f	77	PNP
1382	f	75	PNP
1529	m	84	PNP
1606	f	65	PNP

Average ages: 76.7 ± 2.3 years (PD), 72.0 ± 2.9 years (controls); no significant difference (Mann-Whitney Ranksum test).

PD: Parkinson's disease. CBD: Corticobasal degeneration. PNP: Peripheral neuropathy. PSP: Progressive supranuclear palsy. RLS: Restless legs syndrome.

Supplementary Table 2. Image format and analysis technical information.

Figure	Panel	Microscope	Objective	Number of frames	Resonant scanner frequency	Pixel size (nm)	SRRF analysis	Radiality mag.	zONE step size**
1	a	TCS SP5 STED	100× 1.4 NA	2000-3000	8 kHz	0.98	TRAC4	R35*	-
	b	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.98	TRAC4	R10	-
2	a	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.73	TRPPM	R35	-
	b	TCS SP5 STED & TCS SP8 Lightning	100× 1.4 NA, 63× 1.41 NA	Up to 3000	8 kHz, 12 kHz, & 24 kHz	0.98, 0.48	TRAC4	R35	-
	c	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R35	-
	d	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R35	-
3	a	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R10	-
	b	TCS SP5 STED	100× 1.4 NA	1500-4000	8 kHz	0.98	TRAC4	R35	-
4	a & b	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.98	TRPPM, TRAC4	R10	-
Sup. figures	Panel	Microscope	Objective	Number of frames	Resonant scanner frequency	Pixel size (nm)	SRRF analysis	Radiality mag.	zONE step size*
1	c	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R10	-
	f	TCS SP5 STED	100× 1.4 NA	200-4000	8 kHz	0.98	TRAC4	R10	-
4	a & b	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4, TRA	R10	-
5	a	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRA	R10	-
	b	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.48	TRA	R10	-
	c	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R10	-
	d	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.48	TRAC4	R10	-
6	a & b	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98, 0.48	TRAC4	R10	-
8	a & b	TCS SP5 STED & TCS SP8 Lightning	100× 1.4 NA	Up to 3000	8 kHz	0.98	TRAC4	R10	-
9	d	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.73	TRAC4	R10	-
10	a, b, c, g & i	TCS SP5 STED	100× 1.4 NA	Up to 2000	8 kHz	0.73	TRPPM	R10	-
11	a-c	TCS SP5 STED & TCS SP8 Lightning	100× 1.4 NA, 63× 1.41 NA	Up to 3000	8 kHz, 12 kHz, & 24 kHz	0.98, 0.48	TRAC4	R10	-
12	a & c	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R10	-
13	a-c	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R10	-
14	b, c, f & g	TCS SP5 STED, & TCS SP8 Lightning	100× 1.4 NA	1500-2000	8 kHz, 12 kHz, & 24 kHz	0.98	TRAC4	R10	-
15	b, d, & e	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.98, 0.48	TRAC4	R10	-
16	a & c	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.98, 0.48	TRAC4	R10	-
17	b, c & g	STELLARIS 8	100× 1.4 NA	2000	12 kHz	0.81	TRAC4	R35	-
18	b & c	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.98	TRAC4	R10	-
19	a	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R10	-
18	b & c	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRAC4	R10	-
20	a, d & e	TCS SP5 STED	100× 1.4 NA	Up to 4000	8 kHz	0.98, 0.73, 0.36	TRPPM, TRAC4	R10	-
21	a-e	TCS SP5 STED	100× 1.4 NA	Up to 4000	8 kHz	0.73, 0.36, 0.18	TRAC4	R10	-
22	a	TCS SP5 STED	100× 1.4 NA	Up to 4000	8 kHz	0.98, 0.48	TRAC4	R10 & R35	-
24	c	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.98, 0.48	TRAC4, TRPPM	R10	-
25	-	TCS SP5 STED	100× 1.4 NA	1500	8 kHz	0.98	TRAC4, TRPPM	R10	-
27	a & b	STELLARIS 8	100× 1.49 NA	Up to 2000	16 kHz	0.73	TRAC4	R10	0.05 µm

	c	Abberior STED	100× 1.4 NA	Up to 2000	1.4 kHz	0.73	TRAC4	R10	-
28	a	LSM780	63× 1.4 NA	Up to 2000	1.2 kHz	0.67	TRAC4	R10	-
	a	LSM880	63× 1.4 NA	Up to 2000	1.2 kHz	0.67	TRAC4	R10	-
	a	Abberior STED	100× 1.4 NA	Up to 2000	1.4 kHz	0.73	TRAC4	R10	-
	a	TCS SP5 STED	100× 1.4 NA	Up to 2000	8 kHz	0.73	TRAC4	R10	-
	a	STELLARIS 8	100× 1.49 NA	Up to 2000	16 kHz	0.73	TRAC4	R10	-
29	b & c	STELLARIS 8	100× 1.4 NA	2000	24 kHz	0.49	TRAC4, TAC2	R10	-
30	d	TCS SP5 STED	100× 1.4 NA	2000	8 kHz	0.98	TRA, TRPPM, TRAC2-4	R10	-

*Referred to in the main text as high radiality magnification ONE images.

** Piezo stage step size (not corrected for expansion factor).

Supplementary Note

Resolution

As presented in the main text, the ONE resolution enhancement relates almost exclusively to the lateral (XY) plane. Resolution along the Z axis depends on the expansion factor of the gel, being equivalent to the axial resolution of the confocal microscope used, divided by the expansion factor. This results in a difference of more than 20-fold between the axial and the lateral resolution, which will have significant effects on the image quality. This situation parallels conventional transmission electron microscopy (TEM), in which the thickness of the specimen limits the axial resolution to a similar 20- to 40-fold above the lateral resolution.

This situation implies that the optimal samples for ONE imaging would have a limited number of objects within the axial imaging volume of 40-60 nm (pre-expansion; volume calculated for a conventional confocal microscope and a 10-15x expansion factor). Denser structures will cause a signal overlap that will confuse the identification of individual structures. The use of purified proteins, which can be diluted to the desired signal density, is an optimal application for ONE microscopy, since the dilution factor avoids the potential issues with axial resolution. We have not encountered any issues relating to the sample density in the lateral (XY) plane: the shape of individual proteins is maintained well, and all measurements we performed provided results within the expected boundaries.

Sample anchoring into the gel

We are currently relying on NHS-ester chemistry to anchor proteins into the gels, using the well-established chemical Acryloyl-X. This molecule reacts to amine groups on lysines and on the N-termini of the proteins in the sample. As lysines make up ~5% of all amino acids in proteins, most proteins should have sufficient anchor points for accurate gel anchoring. The only problem we can envision is the fact that aldehyde fixatives also modify amine groups. If gel anchoring appears faulty in specific samples, possibly due to excessive fixation, we suggest using an epitope retrieval strategy, in which the sample is heated to 95°C in basic buffers (pH 8-9). This strategy should eliminate some of the fixative effects, and should enable accurate gel anchoring. Performing the anchoring in basic buffers, overnight, should also assist with this issue.

Homogenization

The heat-based homogenization is optimal for retaining fluorophores already present in the samples (pre-expansion labeling), since it breaks the proteins, but it does not proceed, in the version we optimized, to the removal of every amino acid. At the same time, it does not rely on the diffusion of an enzyme deep into tissues, so it is optimal for these preparations. In contrast, the proteinase K presumably removes all amino acids that are not anchored into the gel. This approach is optimal for single proteins, since the fluorophore positions become quite precise, being always near the anchor points. However, proteinase K diffusion in thick tissues is poor, and therefore this approach is not suited for tissue slices of over ~10 µm.

SRRF performance

The initial implementation of SRRF resulted in a 50-70 nm resolution¹, leading to the impression that this is the best achievable resolution for this technique, as it is implied by some subsequent works (e.g. ²). This is not the case, as demonstrated in our work on nanorulers (**Supplementary Fig. 4 & 5**). The name SRRF serves as an umbrella term for a number of different analyses, including the temporal radiality average (**TRA**) and temporal radiality auto-correlation (**TRAC**)¹. The latter method is a higher-order statistical analysis (following the procedures initially introduced for SOFI³, whose contrast, accuracy and final resolution are substantially higher than those of the TRA method. The TRA analysis does not consider higher-order temporal correlations, which makes it comfortable to use with limited numbers of frames (e.g. 100-300 frames), thus rendering it a method of choice for live-cell SRRF^{1, 4}. TRA is heavily dependent on the distance between the fluorophores, and performs best when the different fluorescent objects are separated by more than 70% of the full width at half maximum (FWHM) of the point-spread-function (PSF ²). This implies that this procedure is not intended to produce a very high resolution, unlike the TRAC analyses. These analyses do produce better resolutions, but require larger numbers of frames for optimal performance, something that does not seem to be clear in the literature; since all SRRF implementations are often performed with as few as 100 frames. Nevertheless, the optimal resolution obtained with TRAC analyses can be pushed towards 20 nm, and maybe even beyond this value, under ideal imaging conditions (**Supplementary Fig. 4 & 5**). We therefore conclude that SRRF should not be considered to be limited to 50-70 nm resolutions, as explained in the Supplementary Notes of the original SRRF publication¹.

As for most other super-resolution approaches, the pixel size limits the resolution to a value of approximately its double⁵. This limitation can be overcome, as indicated in **Supplementary Fig. 4**, by reducing the initial pixel size. This, however, will result in a lower signal-to-noise ratio (SNR), which is an essential parameter for all fluctuation-based analyses. Even when applied to low numbers of frames, SRRF provided excellent images when the signal-to-noise ratio surpassed 10-15^{1, 2}. Below these values, SRRF will perform more poorly than many other related methods, as MUSICAL or ESI², implying that users should carefully consider the noise levels of their images, as explained in **Supplementary Fig. 6**.

The ONE procedure is designed to alleviate two of the main problems of the TRAC analysis, the fluorophore distance and the SNR. First, the distance between the fluorophores increases in all dimensions, leading to their dilution by the third power of the expansion factor. Second, the SNR increases profoundly (**Supplementary Fig. 3a & b**). This is an important side effect of removing all cellular materials that are not embedded into the gels.

The remaining problem, that of acquiring sufficient frames for optimal performance, depends on 1) sample stability, and 2) fluorophore bleaching. The solutions to these issues come in the form of an improved gel-holding chamber (**Supplementary Fig. 7**) and of rapid resonant scanning, which reduces fluorophore bleaching

(**Supplementary Fig. 3c & d**). The latter effect is known from other super-resolution fields, as STED ⁶ and is probably due to the fact that rapid scanning lowers the light dose received continuously by every fluorophore, thereby reducing the possibility of excessive excitation and damage.

SRRF radiality magnification

The typical implementation of SRRF relies on splitting the original pixels 10x10, or even 5x5 (in the eSRRF environment). Expansion procedures separate the fluorophores, as discussed in the main text, leading to a potential for higher resolution, which is then limited by these pixel-splitting paradigms. By trial and error, we realized that a radiality magnification of 35 (R35, splitting pixels 35x35) provides substantially more information, and is especially useful for 3D reconstructions (**Fig. 3**). For details of radiality magnification used in each dataset, see Supplementary Table 2. The use of R35 magnification requires more computing time, so that lower values are advised for routine observation of 2D images.

References

1. Gustafsson, N. et al. Fast live-cell conventional fluorophore nanoscopy with ImageJ through super-resolution radial fluctuations. *Nat Commun* **7**, 12471 (2016).
2. Torres-Garcia, E. et al. Extending resolution within a single imaging frame. *Nat Commun* **13**, 7452 (2022).
3. Dertinger, T., Colyer, R., Iyer, G., Weiss, S. & Enderlein, J. Fast, background-free, 3D super-resolution optical fluctuation imaging (SOFI). *Proc Natl Acad Sci U S A* **106**, 22287-22292 (2009).
4. Culley, S., Tosheva, K.L., Matos Pereira, P. & Henriques, R. SRRF: Universal live-cell super-resolution microscopy. *Int J Biochem Cell Biol* **101**, 74-79 (2018).
5. Shannon, C.E. Communication in the Presence of Noise. *Proceedings of the IRE* (1949).
6. Wu, Y. et al. Resonant Scanning with Large Field of View Reduces Photobleaching and Enhances Fluorescence Yield in STED Microscopy. *Sci Rep* **5**, 14766 (2015).