

Multicolor single particle reconstruction of protein complexes

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Abstract

Single-particle reconstruction (SPR) from electron microscopy images is widely used in structural biology, but lacks direct information on protein identity. To address this limitation, we developed a computational and analytical framework that reconstructs and co-aligns multiple proteins from 2D super-resolution fluorescence images. We demonstrate our method by generating multi-color 3D reconstructions of several proteins within the human centriole and procentriole, revealing their relative locations, dimensions and orientations.

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26 Macromolecular complexes within cells usually contain multiple protein species, whose precise
27 arrangement is required to generate properly functioning molecular machines. Single particle
28 analysis of electron microscopy (EM) images has been used to build 3D reconstructions of such
29 complexes, recently with near-atomic resolution^{1,2}. To deduce the spatial organization of specific
30 proteins, computational methods have been used to dock structures deciphered from X-ray
31 crystallography or NMR within 3D reconstructed particles^{1,3}. Alternatively, immunogold or
32 nanobody labelling can reveal the location and conformational state of target proteins^{4,5},
33 whereas electron density map differences can provide information on the position of mutated or
34 missing proteins⁶. Nevertheless, it remains challenging to locate native proteins within 3D
35 reconstructions, which is essential for deciphering the architecture underlying the assembly
36 mechanisms and functional modules of macromolecular complexes.

37 Fluorescence-based single-molecule localization microscopy (SMLM) can help to
38 address this challenge, as demonstrated for the nuclear pore complex using 2D averaging⁷.
39 Extending to 3D, a recent implementation of single-particle reconstruction (SPR) from 2D SMLM
40 images demonstrated isotropic reconstruction on DNA origami and simulated data⁸. However,
41 multi-color particle reconstruction of actual macromolecular complexes requires generating
42 large image libraries of multiple proteins and solving the complex problem of 3D multi-channel
43 alignment. Here, we developed a systematic framework that addresses both of these
44 challenges. We used a dedicated high-throughput SMLM setup⁹ to generate the large multi-
45 color particle data sets required for SPR, which we then processed using a semi-automated
46 computational workflow to reconstruct and align multiple proteins onto a single 3D particle. We
47 applied our method to the human centriole, and adapted it to accommodate off-axis
48 structures, such as those important during procentriole formation. Centrioles are evolutionarily
49 conserved sub-diffraction limited cylindrical organelles that seed the formation of cilia, flagella
50 and centrosomes¹⁰. The mature human centriole comprises nine-fold symmetrically arranged
51 microtubule triplets and contains >100 different proteins organized into distinct
52 substructures¹¹. For instance, distal appendages harbor the protein Cep164 and are key for
53 cilium and flagellum formation. Moreover, a torus encircling the proximal part of the mature

54 centriole and comprising the proteins Cep57/Cep63/Cep152 acts as a nucleation site for the
55 emerging procentriole, whose assembly relies on the self-organization of the HsSAS-6 protein
56 into a cartwheel^{12,13}. The exact dimensions of components within the Cep57/Cep63/Cep152
57 torus and the position of the procentriole with respect to this torus remain unclear.

58 To demonstrate multicolor 3D SMLM reconstruction, we imaged protein species within
59 centrioles and procentrioles (see experimental workflow in Supplementary Fig. 1). Centrosomes
60 were isolated from human KE37 cells arrested in S phase, concentrated onto coverslips by
61 centrifugation, followed by immunolabeling and dual-color fluorescent staining (Supplementary
62 Note 1). Thereafter, we used high-throughput SMLM⁹ to image on average 150 centrioles per field
63 of view (Supplementary Fig. 2). Localizations belonging to centrioles were segmented using a
64 mask generated through automated OTSU thresholding of the widefield images. A density-based
65 filter (DBSCAN¹⁴) was then applied to separate adjacent centrioles (Supplementary Fig. 3). Only
66 densely labelled centrioles (typically 10-20% of the initial dataset) were rendered and used to
67 populate the particle dataset. Further particle processing was performed as follows using EM
68 routines integrated into Scipion¹⁵ (Supplementary Note 2). Dual-color particles (Supplementary Fig.
69 4) were classified and class-averaged using template-free maximum-likelihood multi-reference
70 refinement (ML2D)¹⁶. Due to the high degree of radial symmetry within centrioles¹⁰, a low number
71 of classes (typically 8-15) were chosen, thereby reducing computational complexity. Nevertheless,
72 this approach is also applicable to particles of unknown symmetry, as verified *in silico*
73 (Supplementary Fig. 5). The class averages best resembling the input particles (Supplementary
74 Note 2) were then used to compute an initial 3D model followed by structural refinement based on
75 matching its 2D projections to the input particles. In this manner, we reconstructed the torus protein
76 Cep152, which we found to exhibit a ~260 nm diameter (Fig. 1a), consistent with the ~242 nm
77 value measured for SNAP-Cep152 by STED microscopy¹⁷. In addition, our 3D reconstruction
78 enabled us to determine for the first time that the height of the torus is ~190 nm. Following the
79 same procedure, we reconstructed the well-known bacteriophage T4 (Supplementary Fig. 6), thus
80 demonstrating the flexibility of this 3D SMLM reconstruction pipeline.

81 To achieve multi-color reconstruction, we first considered the case of proteins sharing a
82 principal symmetry axis. We collected dual-color images of Cep152/Cep164, as well as of
83 Cep152/Cep57, keeping Cep152 as a reference to combine both datasets into a single 3D map.
84 This reduced the problem of alignment to only two 3D volumes at once. We divided the alignment
85 process into two steps: i) co-orient both particles and reconstruct their volumes; ii) co-align the
86 volumes along the symmetry axis (Fig. 1b). Since both proteins are integrated into the same
87 structure, the corresponding particles share the same orientation. Therefore, it is sufficient to find
88 the orientation of particles in one channel (i.e. the reference), then conserve and assign the
89 alignment parameters to the second channel. Given the challenge of imaging two proteins with
90 high resolution, which is typically limited by protein abundance and/or labeling efficiency, this
91 procedure offers the great advantage that only the imaged reference protein needs to contain
92 enough information to be oriented. Following this procedure results in two co-oriented volumes.

93 To co-align protein volumes on a shared symmetry axis, one only needs to align their top
94 (xy) and side (xz) view projections (Fig. 1b, Supplementary Fig. 7). We streamlined this process by
95 using supervised machine learning (MATLAB, Classification Learner) to identify top and side view
96 projections from a combination of 12 calculated shape descriptors (Supplementary Note 3). The
97 models were trained on ~10% of particles and successfully identified ~85 % of side view
98 projections. This method offers the advantage that after having been trained for one reference
99 protein (e.g. Cep152), the model can readily be applied to other datasets exploiting the same
100 reference, greatly facilitating axial alignment. We typically extracted 50–100 side view particles
101 per imaged protein pair. To overlay the particles, we implemented an additional 2D averaging step
102 comprising particle rotation in 1° increments followed by translational alignment and cross-
103 correlation to find optimal overlap between particle pairs. Again, the 2D alignment parameters for
104 the reference protein can be applied to the other imaged protein. This approach automates the
105 computation of Δz while enabling a more precise estimate of particle dimensions (Supplementary
106 Fig. 8).

107 Importantly, this streamlined workflow allowed us to reconstruct and co-align Cep57,
108 Cep152 and Cep164 in a three-color volumetric map of the human mature centriole (Fig. 1d). This

109 reconstruction revealed that whereas the Cep57 torus is aligned axially with that formed by
110 Cep152, as expected from their known association in cells¹⁷, it has smaller dimensions (~230 nm
111 in diameter and ~130 nm in height), placing it close to the outer microtubule wall. Interestingly, we
112 also discovered a previously unidentified nine-fold radially symmetric distribution of Cep57
113 (Supplementary Fig. 8), further suggesting association with the nine-fold symmetrical outer
114 microtubule wall of the centriole, perhaps via its microtubule binding domain¹⁸. We also confirmed
115 Cep164's previously observed nine-fold symmetric organization, while locating its N-terminus
116 more proximally and closer to the centriolar wall than previously reported¹⁹ (see also
117 Supplementary Fig. 9).

118 The above approach works well for proteins positioned on the same principal symmetry
119 axis, but there are important cases for which this does not hold. To exemplify this point, we
120 extended our method to the procentriole, marked by the protein HsSAS-6, which emerges from a
121 single focus on the torus containing Cep57/Cep63/Cep152 during organelle assembly²⁰. We
122 collected dual-color images of Cep152/HsSAS-6, and generated average top and side views
123 following the procedure described above (Supplementary Fig. 7). In this case, the orientation of
124 Cep152 was insufficient to define that of HsSAS-6, since the two proteins do not share a symmetry
125 axis (Fig. 2a). One solution would be to combine the images from both proteins into a single
126 channel and perform class averaging and alignment on the resulting dataset. However, the signal
127 from the larger Cep152 structure would dominate and prevent alignment of the smaller HsSAS-6
128 volume. Instead, we combined the information from the two channels by fusing both rendered
129 images in a weighted sum, where the HsSAS-6 signal was given twice the weight of Cep152. We
130 then used the fused particles for structural refinement of the initial Cep152 volume without any
131 symmetry constraint (Fig. 2b), and fit the individually reconstructed protein volumes into the
132 asymmetric global structure to achieve two-color volumetric reconstruction of the growing
133 procentriole in the context of its centriole (Fig. 2c). Interestingly, we found that the combined
134 reconstruction displays a lower resolution than the individually resolved structures (Fig. 2c),
135 indicative of a flexible relative positioning of the two entities. Indeed, we found the angle θ between
136 the two measured from individual side views (Fig. 2a) to be variable, with an average value of 15.5

137 ± 2.7 (SEM, n=25), in agreement with our 3D reconstruction. Together, these findings indicate a
138 loosely defined orientation between the torus and the emerging procentriole, consistent with
139 suggestions from some EM analysis²¹.

140 In conclusion, we developed a novel framework that generates multi-component 3D
141 volumes from dual-color 2D SMSM datasets, and demonstrated it to construct a three-component
142 3D model of the human centriole and procentriole, thus revealing novel features of their
143 architecture. This method is a flexible workflow that can easily be adapted to other multiprotein
144 complexes and imaging modalities. Combining information from 3D SMLM reconstructions with EM
145 particle reconstructions will likely prove invaluable in the future and so will improvements in
146 labelling to permit higher fidelity of fluorescence to the underlying protein structure.

147

148 **Methods**

149 **Materials and Sample Preparation**

150 Samples were imaged on gold-embedded fiducial cover slips (custom 18 mm, Hestzig).
151 Imaging buffer components were purchased from Sigma. Additional gold fiducials were obtained
152 from CorpuScular (C-Au-0.1) and diluted (1:5) in 0.1 % poly-L-lysine (Sigma) before application.
153 To create a bead sample for two-channel registration, glass cover slips (1.5, Menzel, 25 mm) were
154 plasma cleaned, coated with 0.1 % poly-L-lysine (Sigma) for 30 min and incubated with
155 FluoroSpheres (Dark Red, F8789, Life Technologies) diluted (1:50,000) in water for 10 min.
156 Human centrioles were purified from KE37 incubated for 24h with thymidine following a standard
157 protocol^{22,23} and spun (10 min at 10,000 g in Corex tubes, JS13.1 Beckman swinging rotor) in 10
158 mM K-Pipes on gold-embedded fiducial cover slips (custom 18 mm, Hestzig) using a custom
159 centrifuge concentrator, followed by methanol fixation (5 min at -20°C). Samples were then
160 immunostained by overnight incubation at 4° C with primary antibodies (Supplementary Table 1),
161 diluted 1:500 (in PBS supplemented with 1% BSA and 0.1% Tween 20), then washed three times
162 15 min in PBS and incubated with secondary antibodies coupled with Alexa 647 or DyLight 755 for
163 1h at room temperature. Finally, the samples were washed again three times for 15 min and
164 stored in the dark at 4 °C until further use.

165 Bacteriophage T4 was grown and purified following established procedures²⁴. To
166 characterize the purified sample, phages were spotted on mica and imaged using atomic force
167 microscopy (JPK Nanowizard). To achieve all-protein labelling, phages were incubated with Alexa
168 647 NHS-Ester (Life Technologies) (final concentration 10 μM in phosphate buffer, pH 8) overnight
169 at 4° C. The labelled phages were separated from unbound dye using a NAP-5 (GE Healthcare)
170 size exclusion column and stored in the dark at 4 °C until further use. Before SMLM imaging,
171 phages were adsorbed on plasma-cleaned glass cover slips (1.5, Menzel, 25 mm) after coating
172 with 0.1 % poly-L-lysine (Sigma) for 30 min.

173

174 **High-throughput SMLM**

175 Two-color SMLM imaging was performed using a flat-field epi illumination microscope⁹.
176 Briefly, two lasers with wavelengths of 642 nm (2RU-VFL-P-2000-642-B1R, MPB
177 Communications) and 750 nm (2RU-VFL-P-500-750-B1R, MPB Communications) were used to
178 switch off fluorophores in the sample, while a 405 nm laser (OBIS, Coherent) controlled the return
179 rate of the fluorophores to the fluorescence-emitting state. A custom dichroic
180 (ZT405/561/642/750/850rpc, Chroma) reflected the laser light and transmitted fluorescence
181 emission before and after passing through the objective (CFI60 PlanApo Lambda $\dot{\text{A}}$ -60/NA 1.4,
182 Nikon). After passing the respective emission filter (ET700/75M, Chroma or ET810/90m, Chroma),
183 emitted light from the sample was imaged onto an sCMOS camera (Prime, Photometrics). The
184 sample was excited with laser output power of 1200 mW (642 nm) and 500 mW (750 nm). The 405
185 nm laser was operated with laser output power 1-10 mW. Axial sample position was controlled
186 using the pgFocus open hardware autofocus module
187 (<http://big.umassmed.edu/wiki/index.php/PgFocus>). Typically, 30-60k frames at 10 ms exposure
188 time were recorded for each field of view using Micromanager²⁵. Single- and dual-color SMLM
189 imaging was performed using an optimized SMLM buffer as described previously²⁶. See
190 Supplementary Note 1 for more details on the choice of the fluorophores and buffer generation.

191

192 **Single-Fluorophore Localization, Channel registration, Drift Correction**

193 Image stacks were analyzed using a custom CMOS-adapted analysis routine (adapted
194 from²⁷). Alignment of the Alexa 647 and DyLight 755 data sets was carried out in three steps. We
195 first calculated an affine transformation function from images of fluorescent beads (see **Materials**
196 **and Sample preparation** section above) acquired in both channels. This first step corrects for
197 differences and aberrations (rotation, magnification) of the emission path between both detection
198 channels. During the next step, both datasets were independently drift-corrected using gold
199 fiducials visible in both channels. For each field of view, we selected 3–6 fiducial markers and used
200 their average trajectory for drift correction. We further extracted bead trajectories from both
201 channels to use them for the third correction step, during which the centroids of the fiducial
202 markers in both channels were matched, resulting in a final lateral translation. Steps one and three

203 were only calculated for and applied to the DyLight 755 data set. Our channel correction procedure
204 resulted in a residual error of on average 25 nm per field of view of typically 80 x 80 μm . Gold
205 fiducial-based drift correction was performed using the open source data management and
206 analysis environment B-Store²⁸. All other processing steps were performed in MATLAB 2016a
207 (Mathworks) and are available as part of the supplementary software package.

208

209 **Particle Extraction and 3D Reconstruction.**

210 Following channel registration, the two localization data sets were ready for particle
211 extraction. The localization maps for each field of view were loaded into MATLAB together with the
212 corresponding wide-field images taken prior to the SMLM stack acquisition. One of the two
213 widefield (WF) images was used for automatic OTSU segmentation to identify the location of
214 individual particles within each field of view. Each identified segment was expanded into a slightly
215 larger rectangle to account for any mismatch between WF image and localization data. The size of
216 the bounding rectangle was chosen dependent on the radius of the segmented region. For
217 diameter < 3 pixel, we expanded the region by a factor of 3, for diameter between 3 and 5 pixel by
218 a factor of 1.5, and for diameter > 5 pixel, we did not expand the region any further. The
219 localizations from both channels were extracted for each segmented particle. Particles were filtered
220 for a minimum number of localizations (typically >100) to ensure good dual particle labelling. At this
221 point, we also applied an upper cut-off to reject too large particle clusters. During the next step,
222 adjacent particles within the same segment were separated using density-based clustering
223 (DBSCAN¹⁴). An example is shown in Supplementary Fig. 3. We then calculated a number of
224 particle quality and shape descriptors (Supplementary Note 3), as well as the resolution (using
225 Fourier ring correlation²⁹) for each particle, which allowed for efficient particle filtering and
226 classification. Finally, particles from both channels were rendered into a pixel image using a 2D
227 histogram function with a bin size of 10 nm and blurred using a Gaussian filter with σ
228 corresponding to the measured localization precision. The final image approximates the probability
229 density distribution of the fluorescent labels on the underlying structure and is a widely-used
230 approach to visualize SMLM data. The particle images were stitched together using the Montage

231 function in ImageJ (Miji for MATLAB) resulting in the final input image for the 3D reconstruction
232 (example shown in Supplementary Fig. 4).

233 Single particle reconstruction was performed using Scipion, a freely available software
234 package that integrates several widely-distributed and well-developed 3D EM particle
235 reconstruction routines¹⁵. A brief tutorial of the required steps is provided in Supplementary Note 2.
236 The particle montage images were first imported into Scipion. Depending on the size of the
237 dataset, each montage contained only ~500 particles, which consequently required the generation
238 and import of multiple montages. Since the particles were well aligned, they could be picked and
239 extracted automatically. The particles were aligned again using CL2D (Xmipp3) as the picked
240 region was usually not exactly centered over each particle. The particles were classified using
241 template-free multi reference maximum likelihood (ML2D, Xmipp3) or 2D clustering (CL2D,
242 Xmipp3) classification. Class averages that resembled well the input particles were used (see
243 Supplementary Note 2) to generate the initial model. For symmetric centriolar reconstructions
244 (Cep164, Cep57, Cep152), we selected between 8-12 classes, applying rotational or nine-fold
245 symmetry (Supplementary Note 2). Likewise, for bacteriophage T4, we selected 10 class averages
246 and calculated the initial model using rotational six-fold symmetry (c6). Initial models were
247 calculated using Xmipp2 or Eman2 providing similar results. Finally, the best initial model was
248 refined using particle back projection (Xmipp3). Fourier shell correlation (FSC) was calculated as
249 an output function within the last refinement step (particle back projection, Xmipp3). For the
250 reconstruction of two proteins, we first reconstructed the reference protein using the steps
251 described above, then apply the final alignment to the extracted particles of the protein of interest
252 (function alignment assign), from where the final 3D model can be generated. Please see
253 Supplementary Note 2 for a detailed description. The asymmetric reconstruction was performed
254 using an adapted workflow in Scipion. We first reconstructed both proteins separately. The
255 symmetric volume of the reference (Cep152) was then refined using the weighted sum of the input
256 particle images (Cep152+2*HsSAS-6) without applying a symmetry constraint. Into the resulting
257 asymmetric joint volume, we could fit the individual volumes to obtain a high-resolution dual color
258 model. The volume fitting was performed using Chimera³⁰.

259 **2D particle averaging and volume alignment**

260 Since the centriole has a principal rotational symmetry, we can collapse one of the spatial
261 axes (x/y or z) to then identify the spatial translation parameters required to align two volumes.
262 Specifically, we used particle projections of centriolar top (xy) or side (xz) views. In order to
263 efficiently identify these specific orientations from a large number of individual particles, we
264 calculated 12 parameters whose values could be used as a characteristic signature for top (xy) and
265 side (xz) view projections. Next, a subset of 200 particles was selected and manually classified into
266 top, side or intermediate views (i.e. the response). The shape descriptors and the manual
267 classification were copied into a data table that can be used as a training dataset to generate
268 models using supervised machine learning. We used MATLAB's Classification Learner to identify
269 the best model able to predict the classified outcome (response) based on the shape parameters.
270 The best model was subsequently saved and could later be applied to other data sets. The model's
271 accuracy in predicting a certain shape worked in general better for top (xy) orientations, requiring
272 little manual selection/filtering. Importantly, only one of the two imaged centriolar proteins (i.e. the
273 reference) needs to be classified onto top/side view.

274 All of the following operations are then performed on both channel datasets. The identified
275 particles were aligned to the center of mass of the reference protein and rendered using a pixel
276 size of 10 nm. We next performed a rotational alignment using an extended version of efficient
277 subpixel registration by cross-correlation³¹. Specifically, during the original procedure, we rotated
278 each image stepwise from 1 – 359° by 1° at each iteration, resulting in 360 cross-correlations, from
279 which we picked the orientation with the maximum root mean square (RMS) error, giving the
280 optimal angle of rotation. The alignment was performed over ten iterations. The sum of all images
281 was used as a reference for the first iteration. For all following iterations, we used the sum of all
282 aligned particles from the previous iteration as the reference. The translation between both
283 channels along the z axis was determined using a line profile measurement of the two-color
284 reconstruction (Supplementary Fig. 9). To generate a final multi-color volume representation, the
285 co-oriented volumes were loaded into Chimera³⁰ and centered on top of each other. The Δz axial
286 transformation was applied using the transform coordinates tool (Tools > Movement > Transform

287 coordinates). The volume obtained from the lower resolution SMLM channel (i.e. DyLight755
288 channel, Supplementary Fig. 4) was then replaced by a higher resolution volume of the same
289 structure. To this end, the high resolution volume was loaded into Chimera and aligned to the low
290 resolution volume using the 'Fit in Map' tool (Tools > Volume Data > Fit in Map) and then
291 transformed as described above.

292

293 **SMLM Simulations**

294 In order to evaluate the contribution of labelling noise and efficiency as well as to test the
295 particle processing workflow, we developed a particle simulator that generates localization maps
296 from ground truth models. To define a starting structure, we generated ground truth models of
297 expected fluorophore positions using geometric dimensions of the complex as obtained from
298 SMLM. The starting structure was then randomly rotated and projected onto the XY plane. A
299 random number of molecules were chosen according to the labeling efficiency and a defined
300 number of noise molecules placed at random positions around each particle. Localizations (single
301 frame appearance of a blinking event) originating from each fluorophore were simulated according
302 to measured distributions for photon count, localization precision, as well as on- and off-time. The
303 measurement distributions were obtained from single molecule calibrations for Alexa 647
304 performed under experimental conditions. The resulting simulated particles were analyzed as
305 described for experimental SMLM data sets. All simulations were performed using custom-written
306 Matlab code supplied as part of the supplementary software package.

307

308 **Code availability**

309 All developed code is provided as Supplementary Software. Updates will be available from GitHub
310 (<https://github.com/christian-7/MultiColorSPR>). Test data sets are available via Zenodo
311 (<https://doi.org/10.5281/zenodo.1127010>).

312

313

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325

326 **Author Contributions**

327 C.S, N.B, P.G, S.M. conceived and designed the project. C.S and S.M supervised the project. C.S.
328 and N.B. performed all experiments and data analysis. C.S and K.M.D wrote analysis code. All
329 authors wrote and revised the final manuscript.

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403

404 **Figure Legends**

405

406 **Figure 1: Multi-color single particle reconstruction.** (a) Purified human centrosomes were
407 immunolabelled against Cep152 and Cep164 and imaged using high-throughput SMLM to collect
408 2306 single dual-color particles. Shown are examples of input raw particles (Cep152 channel) and
409 the corresponding class averages (1, 2). A 3D volume model (top right) was reconstructed at a
410 resolution of 52 nm as assessed by Fourier shell correlation (FSC) (lower right). (b) The workflow
411 for multi-volume alignment from two-color (2C) SMLM particles is divided into two steps: (i)
412 orientation and (ii) position alignment. (i) The two imaged proteins belong to the same structure
413 and are thus in the same orientation, so we identified the orientation of only one (reference
414 protein, magenta) and assigned it to the protein of interest (cyan). (ii) To correctly position the two
415 resulting co-oriented volumes with respect to each other, reference protein particles were
416 identified in side view. By collapsing one axis (x or y), we could determine the axial translation (Δz)
417 between the two imaged proteins owing to the symmetry of the centriole. (c) (i) Orientational
418 alignment of the two centriolar proteins Cep57 (cyan)/Cep152 (magenta) results in two co-
419 oriented volumes shown in top (xy) and side (xz) views. (ii) Translational alignment of Cep152
420 (magenta)/Cep164 (green) and Cep152 (magenta)/Cep57 (cyan) results in two 2-color
421 reconstructions. (d) The resulting three-color volumetric map is shown in bottom (xy) and side (xz)
422 views, with a volume of the known dimensions of the microtubule outer wall (grey) as reference.

423

424 **Figure 2: Multi-color single particle reconstruction of an asymmetric protein complex. (a)**
425 Top (xy) and side (xz) view averages for HsSAS-6 (cyan) and Cep152 (magenta) display a
426 protruding, off-axis HsSAS-6 density. **(b)** Overview of procedure for three-dimensional
427 reconstruction of an asymmetric assembly. First, both the reference protein (magenta) and the
428 protein of interest (cyan) are reconstructed individually, then the joint density (yellow mesh) is
429 reconstructed using the weighted sum of the individual channels with the reference volume as
430 initial template. The individual volumes are then fit into the joint density map to obtain the final
431 asymmetric two-color reconstruction. **(c)** Reconstruction of Cep152 and HsSAS-6 assemblies.
432 The individual volumes of Cep152 (magenta) and HsSAS-6 (cyan) were fit into their joint density
433 map (yellow mesh). The final 3D arrangement shows a non-orthogonal orientation of HsSAS-6
434 with respect to the toroidal surface of Cep152. Inset: the average orientation of HsSAS-6 arises
435 from a broad distribution of angles measured from 2D aligned side views (red, individual particles;
436 yellow, average angle ($\theta = 15.5 \pm 2.7^\circ$)).
437

Figure 1

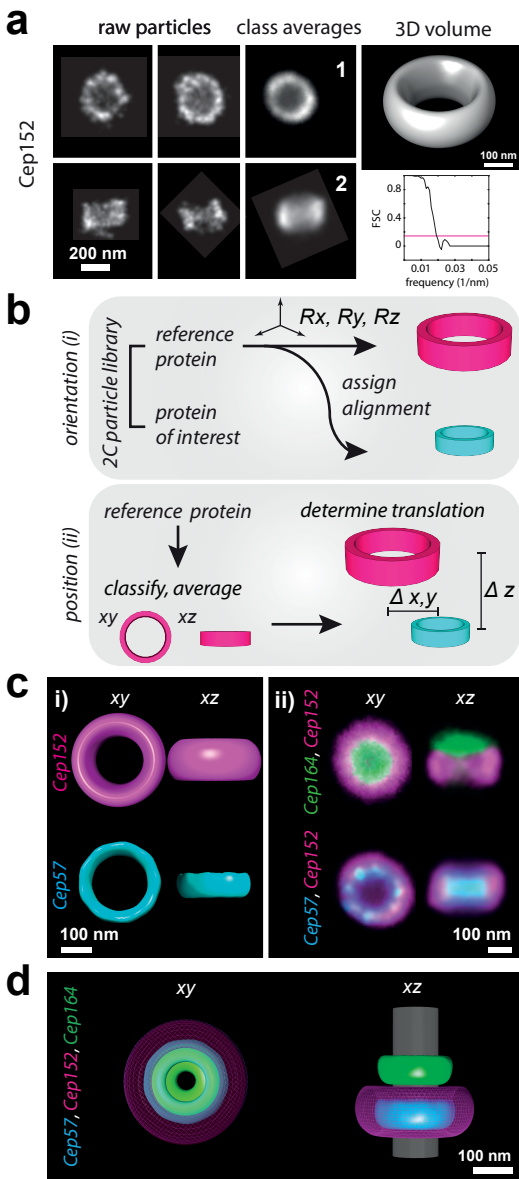


Figure 2

