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Molecular coordination of *Staphylococcus aureus* cell division

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Abstract:

The bacterial cell wall is essential for viability, but despite its ability to withstand internal turgor must remain dynamic to permit growth and division. Peptidoglycan is the major cell wall structural polymer, whose synthesis requires multiple interacting components. The human pathogen *Staphylococcus aureus* is a prolate spheroid that divides in three orthogonal planes. Here, we have integrated cellular morphology during division with molecular level resolution imaging of peptidoglycan synthesis and the components responsible. Synthesis occurs across the developing septal surface in a diffuse pattern, a necessity of the observed septal geometry, that is matched by variegated division component distribution. Synthesis continues after septal annulus completion, where the core division component FtsZ remains. The novel molecular level information requires re-evaluation of the growth and division processes leading to a new conceptual model, whereby the cell cycle is expedited by a set of functionally connected but not regularly distributed components.

Impact statement: Morphological constraints dictate division mode in the human pathogen *Staphylococcus aureus*

Competing interests: No competing interests declared

Author contributions:

Simon Foster: Conceptualization; Formal analysis; Supervision; Funding acquisition; Investigation; Project administration; Writing—review and editing Victoria Lund: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing—original draft; Writing—review and editing Katarzyna Wacnik: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing—original draft; Writing—review and editing Robert Turner: Conceptualization; Data curation; Software; Formal analysis; Validation; Investigation; Methodology; Writing—original draft; Writing—review and editing Bryony Cotterell: Resources; Formal analysis; Investigation; Methodology; Writing—review and editing Christa Walther: Formal analysis; Investigation; Methodology; Writing—review and editing Samuel Fenn: Data curation; Methodology Fabian Grein: Resources; Data curation; Methodology Adam Wollman: Formal analysis; Methodology; Writing—review and editing Mark Leake: Conceptualization; Supervision; Funding acquisition; Project administration; Writing—review and editing Nicolas Olivier: Methodology; Writing—review and editing Ashley Cadby: Supervision; Methodology; Writing—review and editing Stephane Mesnage: Formal analysis; Methodology; Writing—review and editing Simon Jones: Formal analysis; Supervision; Funding acquisition; Project administration; Writing—review and editing

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1 Molecular coordination of *Staphylococcus aureus* cell division

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18 Abstract

19 The bacterial cell wall is essential for viability, but despite its ability to withstand internal turgor it must
20 remain dynamic to permit growth and division. In most bacteria peptidoglycan is the major cell wall
21 structural polymer, for which the advent of super resolution microscopy approaches has begun to reveal
22 a complex architecture, whose synthesis requires multiple interacting components. The human pathogen
23 *Staphylococcus aureus* is a prolate spheroid that divides in three orthogonal planes, requiring intricate
24 spatio-temporal process control to complete the cell cycle with fidelity. Here, we have integrated cellular
25 morphology during division with molecular level resolution imaging of peptidoglycan synthesis and the
26 components responsible. Synthesis occurs across the developing septal surface in a diffuse pattern, a
27 necessity of the observed septal geometry, that is matched by a variegated division component
28 distribution. Synthesis continues after septal annulus completion, where the core division component FtsZ
29 remains. The combination of molecular level information requires a re-evaluation of the growth and division
30 processes leading to the development of a new conceptual model, whereby the cell cycle is expedited by
31 a set of functionally connected but not regularly distributed components.

32 Significance Statement

33 Bacterial cell wall peptidoglycan is responsible for maintaining viability, acting as a physical “exoskeleton”
34 and its synthesis is the target of some of the most important antibiotics such as penicillin and vancomycin.
35 Despite this we understand little of how this essential polymer is made and the organisation of the complex
36 set of components required during growth and division. We have used molecular level resolution
37 microscopy to map both peptidoglycan production and the major proteins involved, in the important human

38 pathogen, *Staphylococcus aureus*. This has revealed unprecedented detail and an unexpected diffuse
39 pattern of peptidoglycan synthesis during division, matched by the localisation of the components required.
40 This has led to a new division model driven by cellular morphological constraints.

41 Introduction

42 In order to grow and divide, bacteria must make new cell wall, the major structural component of which is
43 peptidoglycan (1). Bacteria generally have two groups of proteins that co-ordinate peptidoglycan insertion,
44 one involved with elongation (elongasome), the other with division (divisome) (2). *S. aureus* lacks an
45 apparent elongasome machinery, but nonetheless new peptidoglycan is inserted all over the cell surface,
46 throughout the cell cycle, not just during cell division (3, 4). Addition of peptidoglycan, along with its
47 hydrolysis (5), is what enables *S. aureus* cells to get bigger – volume increases at a constant rate (4).

48 The *S. aureus* divisome contains both enzymes that catalyse addition of new monomers to the
49 peptidoglycan envelope (Penicillin Binding Proteins, PBPs), and proteins that co-ordinate this activity.
50 Chief amongst these is FtsZ - an essential protein in almost all bacteria that directs cell division, which has
51 recently been shown to form dynamic filaments that “treadmill” in *Escherichia coli* and *Bacillus subtilis*,
52 giving a framework to assemble other division proteins resulting in cell wall biosynthesis and septum
53 formation (6, 7). FtsZ assembly into the Z-ring is regulated by other cell division components including
54 EzrA (8, 9), a membrane protein crucial for cell division in *S. aureus* (10). It has been shown to interact
55 with both cytoplasmic proteins and those with periplasmic domains and it is therefore proposed to act as
56 an interface between FtsZ and PBPs forming a scaffold for other cell division components (10).

57 Previously, FtsZ and EzrA in *S. aureus* have been imaged using fluorescent fusions (11, 12) and sites of
58 peptidoglycan insertion using fluorescent D-amino acids (3, 13). Here we have applied single molecule
59 localisation microscopy, a technique that provides unprecedented detail compared with other approaches.
60 This has revealed an unexpected arrangement of division proteins and associated peptidoglycan insertion
61 pattern. This defies the conventional view of division in *S. aureus* and has prompted a new model of
62 division that encompasses the morphological idiosyncrasies of this important pathogen.

63 Results

64 **Distribution of divisome components during septation**

65 In order to visualise division machines, we localised the cytoplasmic initiator of division FtsZ and the crucial
66 membrane protein EzrA (10).

67 Four fusions of EzrA with different fluorophores were created. These had wild-type growth rates and the
68 previously observed septal EzrA localization pattern (10, 14) by diffraction limited microscopy (Fig. 1 -
69 supplement 1). Localisation microscopy and 3D Structured Illumination Microscopy (3D-SIM) were used
70 to address the distribution and juxtaposition of the cell division components at super-resolution.
71 3D-SIM revealed that EzrA exhibited punctate distribution at the division site (Fig. 1 – supplement 2a) (11).
72 Unfortunately, the “honeycomb” artefact (which introduces foci in images due to incomplete noise filtering
73 (15)) could not be removed by raising the Wiener filter parameter in reconstructions. Thus, localisation
74 microscopy was employed as a superior approach.

75 eYFP was selected as a blinking fluorescent protein tag (16). Multiple 2D images of septa in the plane of
76 focus were obtained for EzrA-eYFP (Fig. 1a), FtsZ-eYFP (Fig. 1b) and EzrA-meYFP (Fig. 1 - supplement
77 2b). The mean localisation precision of YFP was calculated using two different formulas: the “Thompson
78 Equation” (17) by the ThunderSTORM ImageJ plugin yielded 24 (s.d. 8.5) nm while a using a modified
79 version of this equation (18) yielded 27 (s.d. 8.7) nm. We also measured it experimentally using Nearest
80 Neighbour in Adjacent Frames (NeNA) analysis (19): NeNA analysis determines localisation precision
81 based on spatial proximity of blinks that occur at similar times and is part of a family of clustering-based
82 tools for assessing the quality of localisation microscopy data (20). This method gave us a mean
83 localization precision of 16.23 nm. Many of the septa appeared to be somewhat elliptical. This is likely due
84 to the cells being tilted relative to the plane of focus leading to circular septa appearing elliptical. We
85 therefore fitted ellipses to the septal localisations and calculated the expected tilt of the cells. The results
86 were that all of the localisations included in our analysis are within a 400 nm optical section, within a range
87 to ensure good data (21).

88 To analyse the distributions and address issues of sampling and resolution in our microscopy, a number
89 of simple simulations were carried out where representative numbers of localisations were distributed at
90 random in rings of similar radius to those observed, with a random error applied (Fig. 1c). A circle was
91 fitted to the data points and all the distributions (experimental and simulated) were parameterised with
92 respect to angle and distance from the centre of the circle, generating histograms of localisations (Fig. 1d,
93 e). The autocorrelations of the angular distributions were then averaged to show that the localisations in
94 the experimental data were neither completely randomly, or regularly, distributed around the ring (Fig. 1f).
95 Distributions of distance from the centre of the circle were compared with simulated distributions of a fixed
96 circle radius where different levels of localisation precision error were applied (Fig. 1g). Even with the most

97 conservative assumptions (including simulated localisation precisions worse than we had calculated for
98 our measured data), the localisations were spread out over a sufficiently wide range of distances to
99 indicate that both FtsZ or EzrA do not form a very thin ring at the leading edge of the septum in *S. aureus*.
100 Instead both proteins appear in a non-uniform distribution within the septal annulus. Within the annulus
101 the proteins show no discernible pattern within or across cells. FtsZ distributions were consistent with FtsZ
102 remaining in the division plane after septal fusion were also observed (Fig. 2a).

103 To further investigate whether the apparent elliptical shape of the rings had an influence on our
104 interpretation, we also analysed the data using an elliptical, rather than a circular fit. Comparing our results
105 to simulated data (Fig. 1 – supplement 3) corroborated our previous findings.

106 To place these findings in the context of cell wall shape, two colour localisation microscopy was performed
107 where the cell wall was labelled with an Alexa Fluor 647 NHS ester (Fig. 2b, c), which labels all amine
108 groups in the cell wall (4). This confirmed that EzrA and FtsZ were at the expected septal positions in the
109 cell.

110 To analyse rapid molecular dynamics of EzrA, single-molecule Slimfield microscopy (22) was performed
111 on EzrA-meYFP labelled *S. aureus*, SH4604 (*ezrA-meyfp ΔezrA*) optimized to enable blur-free tracking
112 of single fluorescent protein fusion constructs in live cells over a millisecond timescale (23, 24). Analysis
113 of the mobility of tracked EzrA-meYFP foci enabled quantification of their microdiffusion coefficient (D),
114 indicating a mixture of three different mobility components: an apparent immobile population in addition
115 to an intermediate and a rapid mobility population (Fig. 1 – supplement 4a, b). In total, ~600 EzrA foci
116 tracks were analysed in the septum region, whose overall mean D value, which captures both the
117 immobile and two mobile populations, was $0.20 \pm 0.01 \mu\text{m}^2 \text{s}^{-1}$. Whereas, 140 foci tracks were detected
118 outside the septum region, which showed an increased overall mean D of $0.28 \pm 0.03 \mu\text{m}^2 \text{s}^{-1}$. This
119 greater average mobility was principally due to an increase in the proportion of EzrA foci present in the
120 most mobile component (going from $33 \pm 3\%$ of the total to $42 \pm 4\%$).

121 These relatively slow mobility values for EzrA, compared to many freely diffusing bacterial membrane
122 integrated proteins (25), do not preclude putative rotational/treadmilling motions of EzrA (which have been
123 observed in previous studies of FtsZ mobility in *E. coli* and *B. subtilis* (6, 7)) over a longer time scale. For
124 example, the mean speed of putative FtsZ treadmilling estimated from *B. subtilis* recently (7) is only
125 ~30nm/s, which we estimate would be sufficiently slow to appear predominantly in the immobile
126 component over the typical time scales of our Slimfield tracking experiments here, and so putative

127 treadmilling of EzrA at this equivalent mean speed, if present in *S. aureus*, would most likely appear in this
128 apparent immobile fraction. However, in the three component mobility model, which fits the observed
129 distribution of D values well, the intermediate mobility fraction has been interpreted previously in other
130 cellular systems as indicating transient dynamic interactions (26), and so we cannot entirely exclude the
131 possibility that this may be due to transient association of EzrA with FtsZ. Deconvolution analysis (27) of
132 whole cell images obtained using Slimfield microscopy indicated a mean total copy number of 305 ± 23
133 EzrA molecules per cell measured across a population (Fig. 1 – supplement 4c). Estimating the proportion
134 of the most mobile fraction of EzrA foci therefore indicates that at least ~100 EzrA molecules per cell are
135 not likely to be treadmilling in tight association with FtsZ. In other words, we cannot account for the
136 observed mobility of EzrA by a simple treadmilling model alone in which all EzrA is tightly associated to
137 FtsZ, rather the real cellular behaviour is more complex than this.

138 **Peptidoglycan synthesis in *S. aureus* does not occur in discrete foci**

139 We used established metabolic labelling with fluorescent D-amino acids or dipeptides (3, 13) and adapted
140 this for localisation microscopy in order to visualise peptidoglycan insertion with this higher resolution
141 imaging technique. We confirmed that HADA (7-hydroxycoumarin-3-carboxylic acid-amino-D-alanine),
142 ADA (azido D-alanine) and ADA-DA (azido-D-alanyl-D-alanine) mark regions of new peptidoglycan
143 insertion by microscopy and Liquid Chromatography-Mass Spectrometry (LC-MS) (Fig. 3 – supplement
144 1).

145 Cells were pulse labelled with DAAs (D-amino acids) from <15 s to 5 minutes. Even at the very shortest
146 labelling time (<15 s) peptidoglycan synthesis was observed both at the septum and cell periphery but
147 without discrete foci (Fig. 3 – supplement 2a, b). Localisation microscopy of 15 s ADA and ADA-DA labelled
148 cells revealed labelling occurs dispersed across the whole septum as well as the off-septal cell wall (Fig.
149 3a, Fig. 3 – supplement 2d). This was not due to non-specific labelling (Fig. 3 – supplement 2c). XY
150 localisation precision (estimated by the Nikon NSTORM software) was 9.9 (s.d. 3.5) nm or 7.5 nm by
151 NeNA (19). A similar pattern of peptidoglycan synthesis was seen with up to 5 min labelling with ADA or
152 ADA-DA as a zone across the developing septum as well as throughout the off-septal cell wall (Fig. 3b, c,
153 d). Previously PBP4 has been implicated in the presence of off-septal incorporation (3, 28), we therefore
154 carried out DAA labelling and localisation microscopy in a PBP4 null background (SH4425) (Fig. 3 –
155 supplement 3). Cell growth and GlcNAc incorporation were found to be the same as WT, however DAA
156 labelling was reduced in SH4425 (Fig. 3 – supplement 3b-d). The proportion of off-septal labelling was

157 calculated in both SH1000 and SH4425 when labelled with ADA-DA, however no significant difference
158 was observed (Fig. 3 – supplement 3e). Localisation microscopy of both 15s and 5 min labelled SH4425
159 showed peptidoglycan synthesis both at the septal and peripheral cell wall. Discrete foci of insertion were
160 not observed (Fig. 3 – supplement 3f-g). Comparison of autocorrelations (as calculated for EzrA and FtsZ,
161 using elliptical fits) for SH1000 and SH4425 revealed no substantial differences (Fig. 3 supplement 3h).
162 In cells with an incomplete septum, there was a “gap” in peptidoglycan synthesis at the mother cell wall-
163 septum interface (Fig. 3c-i, arrows). In order to investigate the properties of the observed “gap” we used
164 a counter stain to determine if it is filled with peptidoglycan. Fluorescent vancomycin has been used
165 extensively to label peptidoglycan (29). Thus, we synthesised a version of this molecule with a Cy3B
166 fluorophore so it could be used in two colour localisation microscopy with Alexa Fluor 647 click tagged
167 amino acids. Vancomycin binds D-alanyl-D-alanine motifs in peptidoglycan and as these are highly
168 prevalent in *S. aureus* the majority of peptidoglycan is fluorescently labelled. Our two colour images show
169 that the “gap” regions that do not contain ADA-DA (5 minutes labelling), are nonetheless bound by
170 vancomycin and thus are filled with peptidoglycan (Fig. 3e).

171 Also, cells with a filled septal annulus showed continued insertion that could be resolved into 2 distinct
172 zones, one for each daughter (Fig. 3c-ii). These features were not observable by SIM, being smaller than
173 its theoretical resolution.

174 **Inhibition of cell division leads to co-mislocalization of the cell division components and** 175 **peptidoglycan synthesis**

176 The FtsZ inhibitor PC190723 prevents depolymerisation of FtsZ and consequently inhibits cell division,
177 also leading to swollen *S. aureus* cells (30). It has previously been shown by diffraction limited fluorescence
178 microscopy that PC190723 causes mislocalisations of FtsZ and PBP2 (31). We sought to determine the
179 dynamics of this process, and the molecular pattern of associated peptidoglycan insertion. PC190723
180 treatment led to delocalization of peptidoglycan biosynthesis, EzrA and FtsZ even before substantial cell
181 swelling (Figure 4 – supplement 1). Incorporation of HADA does not cause mislocalisation of FtsZ or EzrA
182 (data not shown). Peptidoglycan synthesis was observed around the cell periphery and in distinct foci in
183 the same place as EzrA and FtsZ. This non-uniform peptidoglycan insertion results in misshapen cells
184 with irregular thickening of the cell wall (Fig. 4a). After 60 min treatment, patches of FtsZ, EzrA and
185 peptidoglycan synthesis can be seen (Figure 4 – supplement 1a). Localisation microscopy of

186 peptidoglycan synthesis shows cell shape and the off-septal synthesis with patches of increased synthesis
187 more clearly (Fig. 4b). Thus peptidoglycan synthesis follows localization of FtsZ and EzrA.

188 **Morphology of the *Staphylococcus aureus* septum**

189 It has been shown that the incomplete *S. aureus* septum is thinner at the leading than at the lagging edge
190 (32, 33). However, the significance of this has remained unknown. We observed sections of cells from
191 different stages in the cell cycle and measured septal geometry using thin section Transmission Electron
192 Microscopy (TEM). The septum of *S. aureus* is thinner at the leading edge and progressively thicker
193 towards the lagging edge until it fuses, at which point it is thinner at the centre and progressively thicker
194 towards the lagging edge until ultimately uniform thickness is established (Fig. 5a, b). This dictates that
195 peptidoglycan insertion cannot be confined to the leading edge of the septum and gives a morphological
196 explanation for the observed peptidoglycan insertion pattern.

197 The surface area available for peptidoglycan insertion in the nascent septum was modelled resulting in
198 the following expression for septal surface area prior to fusion (Fig. 5c):

$$199 \quad A = \pi(2r - s)\sqrt{s^2 + d^2}$$

200 Where d is half the thickness of the septum, r is the radius of the cell in the plane of septation and s is the
201 distance from the leading to the lagging edge of the septum (measured from the inner surface of the cell
202 wall).

203 The surface area of a septum with consistently uniform thickness is that of the leading edge of that septum:

$$204 \quad A = 4\pi(r - s)d$$

205 Not only is the available surface area always larger for the morphology we observe, but it increases as the
206 septum closes (whereas with a uniformly thick septum, it decreases). This provides a framework for septal
207 synthesis in an organism in which the septum comprises a substantial proportion of the cell wall.

208 **Discussion**

209 The non-standard cross section of the septum in *S. aureus* distinguishes it from other model organisms
210 (Fig. 5a, b, c) and indicates that not all peptidoglycan insertion occurs at the leading edge of the septum
211 in this species prompting the development of a new model for how peptidoglycan is synthesised during
212 the cell cycle (Fig. 5d). This is likely advantageous to the bacteria, enabling more biosynthetic enzymes to
213 work on the cell wall without steric hindrance. We sought to explain this phenomenon by analysing the
214 distribution of peptidoglycan insertion and investigating key cell division components. Our novel application

215 of localisation microscopy to DAAs revealed that even at the shortest timescales and with considerably
216 more precision than previous studies (3, 4, 34), there were no foci of peptidoglycan insertion – the diffuse
217 pattern throughout the septum and periphery of the cell was ever-present. This surprising finding was
218 corroborated by the distribution of core cell division components in *S. aureus*. Localisation microscopy of
219 FtsZ and EzrA in the septal ring showed, like the distribution of peptidoglycan insertion, that they occurred
220 in a zone, and were not limited to the leading edge of the septum. Also, FtsZ remained at the septum after
221 the annulus had fused. When FtsZ depolymerisation was inhibited, peptidoglycan insertion was found to
222 occur in areas with large amounts of FtsZ, resulting in local thickening of the cell wall, suggesting all
223 synthesis may depend on FtsZ. This is a different scenario to *E. coli* and *B. subtilis*, where division-
224 associated foci of peptidoglycan synthesis have been identified (albeit without the precision of localisation
225 microscopy) and associated with cell division components driven by treadmilling FtsZ filaments (6, 7).
226 The divisome has been proposed to be a multi-component machine, present within a ring, based on
227 diffraction-limited microscopy and interaction studies (10, 35). Previous localisation microscopy studies
228 have begun to reveal intricate structural and spatial relationships between division components (36-38).
229 Our data shows that divisome components are not placed exclusively at the leading edge of the septum,
230 and that some individual proteins move more rapidly than others. There may, therefore, be a number of
231 essentially identical machines executing peptidoglycan insertion within a region of the septum, with
232 exchange of machine components with a more mobile population of molecules. It could also be the case
233 that the machines are very non-uniform and can execute their tasks with a subset of the complete list of
234 divisome proteins and with more or less of an individual protein. Alternatively, stable, stoichiometric
235 complexes are not present and the interactions between proteins required to make new peptidoglycan are
236 highly transient.

237 Materials and Methods

238 **Bacterial Growth Conditions**

239 Strains used in this study are listed in Appendix I Table 1, while plasmids and oligonucleotide sequences
240 are shown in Appendix I Table 2 and Appendix I Table 3. *S. aureus* was grown in Brain Heart Infusion
241 (BHI) broth at 37°C with aeration at 250 rpm, except for Slimfield microscopy and ¹⁴C-GlcNAc incorporation
242 experiments (and associated growth curves) which were carried out using Chemically Defined Media
243 (CDM) (39). For solid media 1.5% (w/v) agar was added. Where required, antibiotics were added at the
244 following concentrations; erythromycin (5 µg ml⁻¹), lincomycin (25 µg ml⁻¹), kanamycin (50 µg ml⁻¹), and

245 tetracycline (5 $\mu\text{g ml}^{-1}$). To induce protein production strains carrying gene fusions under the control of the
246 Pspac promoter were grown in the presence of 50 μM isopropyl β -D-thiogalactopyranoside (IPTG).

247 **Construction of *S. aureus* mutants**

248 All vectors were constructed in *E. coli* NEB5 α (New England Biolabs) following previously described
249 methods (40, 41). The resulting constructs were passed through a restriction-deficient *S. aureus* RN4220
250 before being transduced into a final *S. aureus* SH1000 strain. Transformation and phage transduction of
251 *S. aureus* were carried out as described previously (42, 43).

252 **SH4388** (*ezrA-eyfp Δ ezrA*): The EzrA-eYFP fusion was created by EcoRI and BamHI digestion of pGM074
253 and insertion of *eyfp* amplified by PCR from SU492(44) using primer pair eYFP-F and eYFP-R. pGM074
254 is pKASBAR-kan(35) containing *ezrA* under the control of its own promoter with the C-terminal *psmorange*
255 (flanked by Ascl and NotI restriction sites). In the resulting plasmid pKASBAR-EzrA-eYFP the translational
256 fusion of *ezrA-eyfp* is linked by linker A (see below). pKASBAR-EzrA-eYFP was electroporated into
257 CYL316(45) and its integration at the *geh* locus was confirmed by disruption of lipase production on Baird-
258 Parker medium. The chromosomal fragment containing the integrated plasmid was moved into *S. aureus*
259 SH1000 by phage transduction, creating SH4384 (*ezrA-eyfp*).

260 To delete native *ezrA*, an *ezrA* deletion vector was constructed. Fragments encompassing ~ 1.5 kb regions
261 flanking *ezrA* were PCR amplified from *S. aureus* SH1000 genomic DNA using pOB-*ezrA*-up-F/-R and
262 pOB-*ezrA*-down-F/-R. A 2.1 kb fragment encoding a tetracycline resistance cassette (TetR) was amplified
263 from pAISH by PCR using pOB-TetR-F/-R primers. The three PCR products were ligated with HindIII and
264 EcoRI cut pOB(46) by Gibson assembly, creating a deletion vector pOB- Δ *ezrA*. The plasmid pOB- Δ *ezrA*
265 was electroporated into RN4220. The plasmid integrated into the chromosome through a single cross-over
266 event and the DNA fragment containing the deletion cassette was transduced into SH4386 (*ezrA-eyfp*).
267 Tetracycline-resistant/erythromycin-sensitive colonies were selected. In the resulting strain, SH4388
268 (*ezrA-eyfp Δ ezrA*), *ezrA-eyfp* was the only copy of the *ezrA* gene. Replacement of *ezrA* for TetR was
269 confirmed by PCR and Southern blot.

270 **SH4640** (*ezrA-gfp Δ ezrA*): To construct an EzrA-GFP translational fusion linked by linker A, *gfp* was PCR
271 amplified from JGL227(10) using GFP-F/-R primers and ligated into Ascl and EcoRI cut pGM074, creating
272 pKASBAR-EzrA-GFP. The resulting plasmid was electroporated into CYL316. pKASBAR-EzrA-GFP
273 integration at the *geh* locus was confirmed by disruption of lipase production on Baird-Parker medium. The
274 chromosomal region containing the plasmid integrated within *geh* was moved to SH1000 creating SH4639

275 (*ezrA-gfp*). To delete native *ezrA*, SH4639 was transduced with a phage lysate from SH4388 (*ezrA-eyfp*
276 Δ *ezrA*), creating SH4640 (*ezrA-gfp* Δ *ezrA*). Replacement of *ezrA* for TetR was confirmed by PCR and
277 Southern blot.

278 **SH4642** (*ezrA-snap* Δ *ezrA*): A translational fusion of EzrA linked by linker A to the SNAP tag was
279 constructed by PCR amplification of *snap* from pSNAP-tag (T7)-2 (New England Biolabs) using SNAP-F/-
280 R primers. The PCR product was ligated into pGM074 using *Ascl* and *EcoRI* cut sites to create pKASBAR-
281 EzrA-SNAP. The resulting plasmid was electroporated into CYL316 and its integration at the *geh* locus
282 was confirmed by disruption of lipase production on Baird-Parker medium. The chromosomal fragment
283 containing integrated pKASBAR-EzrA-SNAP was transduced into SH1000, resulting in SH4641 (*ezrA-*
284 *snap*). Native *ezrA* was replaced by TetR by transducing SH4641 with the phage lysate from SH4388
285 (*ezrA-eyfp* Δ *ezrA*), creating SH4642 (*ezrA-snap* Δ *ezrA*). Replacement of *ezrA* for TetR was confirmed by
286 PCR and Southern blot.

287 **SH4604** (*ezrA-meyfp* Δ *ezrA*): To create a C-terminal fusion of EzrA with monomeric eYFP (meYFP) the
288 whole pKASBAR-EzrA-eYFP plasmid was PCR amplified using meYFP-F/-R primers. The meYFP-F
289 primer introduced an A207K substitution(47) into the *eyfp* gene. The PCR product was digested with *DpnI*
290 to remove methylated DNA, the 5' ends of DNA were phosphorylated with T4 polynucleotide kinase (New
291 England Biolabs) and DNA was circularized using Quick-Stick ligase (Bioline), resulting in pKASBAR-
292 EzrA-meYFP. The resulting plasmid was electroporated into CYL316. The chromosomal fragment
293 containing the integrated plasmid in the *geh* locus was moved into *S. aureus* SH1000 by phage
294 transduction, creating SH4603 (*ezrA-meyfp*). To delete native *ezrA*, SH4603 was transduced with a phage
295 lysate from SH4388 (*ezrA-eyfp* Δ *ezrA*), creating SH4604 (*ezrA-meyfp* Δ *ezrA*). Replacement of *ezrA* for
296 TetR was confirmed by PCR and Southern blot.

297 **SH4652** (*ezrA-eyfp* Δ *ezrA* pCQ11-FtsZ-SNAP): In order to construct a strain simultaneously producing
298 EzrA-eYFP and FtsZ-SNAP, a plasmid encoding a translational *ftsZ-snap* fusion placed under the control
299 of the Pspac promoter was constructed. The *ftsZ* gene was PCR amplified from *S. aureus* N315 genomic
300 DNA using FGFtsZXhoI-F and FGFtsZEcoRI-R primers and cloned into *EcoRI* and *XhoI* cut pSS26b
301 (Covalys), resulting in pSS26bFtsZ-C. The fragment encoding *ftsZ-snap* was PCR amplified from
302 pSS26bFtsZ-C using FGFtsZNheI-F and FGFtsZAscl-R and inserted into pCQ11(48) using *NheI* and
303 *Ascl* cut sites, creating pCQ11-FtsZ-SNAP. The plasmid was electroporated into RN4220 and moved to

304 SH4388 (*ezrA-eyfp ΔezrA*) by phage transduction, resulting in SH4652 (*ezrA-eyfp ΔezrA* pCQ11-FtsZ-
305 SNAP).

306 **SH4665** (pCQ11-FtsZ-eYFP): To construct a translational fusion of FtsZ with eYFP, an insert containing
307 a fragment of linker B (see below) followed by a full length *eyfp* gene was synthesized by the GeneArt
308 Gene Synthesis service, PCR amplified using *ftsZ-eyfp-F/-R* primers and cloned into *NcoI* and *Ascl* cut
309 pCQ11-FtsZ-SNAP, creating pCQ11-FtsZ-eYFP. The plasmid was electroporated to RN4220 and moved
310 to SH1000 by phage transduction, resulting in SH4665 (pCQ11-FtsZ-eYFP).

311 **SH4425** (*pbp4*): NE679 (*pbp4*) containing a transposon insertion within the *pbp4* gene was obtained from
312 NARSA library (49). SH1000 was transduced with a phage lysate from NE679. Insertion of the transposon
313 within *pbp4* in resulting SH4425 (*pbp4*) was confirmed by PCR and sequencing.

314

315 Sequences of genes encoding fluorescent proteins, tags and linkers

316 ***eyfp*** in pKASBAR-EzrA-eYFP, pMAD-eYFP-PBP2, pCQ11-eYFP-PBP2
317 ATGGTGAGCAAGGGCGAGGAGCTGTTACACGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCG
318 ACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGGCGAGGGCGATGCCACCTACGGCAAGCT
319 GACCCTGAAGTTCATCTGCACCACGGCAAGCTGCCCGTGCCCTGGCCACCCTCGTGACCACCT
320 TCGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCAGACTTCTTCAAGT
321 CCGCCATGCCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAG
322 ACCCGCGCCGAGGTGAAGTTCGAGGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCG
323 ACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTACAACAGCCACAACGTCT
324 ATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGG
325 GCGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGGCGACGGCCCCGTGCT
326 GCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCG
327 ATCACATGGTCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTAC
328 AAG

329 ***eyfp*** in pCQ11-FtsZ-eYFP
330 ATGGTTTCAAAGGTGAAGAATTATTCACAGGTGTTGTTCCAATTTTGGTTGAATTAGATGGTGATGT
331 TAATGGTCATAAATTCTCAGTTTCAGGTGAAGGTGAAGGTGATGCAACATATGGTAAATTAACATTA
332 AATTTATTTGTACAACAGGTAAATTACCAGTTCCTTGGCCAACATTAGTTACAACATTCGGTTATGGT
333 TTACAATGTTTTGCACGTTATCCAGATCATATGAAACAACATGATTTTTTCAAATCAGCAATGCCTGA
334 AGGTTATGTTCAAGAACGTACAATTTTCTTTAAAGATGATGGTAATTACAAAACACGTGCTGAAGTGA
335 AATTTGAAGGTGATACATTAGTTAATCGTATTGAATTAAGGTATTGATTTTAAAGAAGATGGAAATA
336 TTTTAGGTCATAAATTAGAATATAATTATAATTCACATAATGTTTATATTATGGCAGATAAACAAAAA
337 TGGTATTAAAGTTAATTTCAAATTCGTCATAATATTGAAGGTGGTTCAGTTCATTAGCAGATCATT
338 TCAACAAAATACACCTATTGGTGATGGTCCAGTTTTATTACCAGATAATCATTATTTATCATATCAATC
339 AGCATTATCAAAGATCCAAATGAAAACGTGATCATATGGTTTTATTAGAATTTGTTACAGCAGCAG
340 GTATTACATTAGGTATGGATGAATTATATAAATAA

341 ***gfp*** in pKASBAR-EzrA-GFP
342 ATGGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATG
343 TTAATGGGCACAAATTTTCTGTGAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCC
344 TTAATTTATTTGCACTACTGGAAAACCTGTTCCATGGCCAACACTTGTCACTACTTTGACCTAT
345 GGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGC
346 CCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACACTACAAGACGCGTGCTG

347 AAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGAT
348 GGAAACATTCTCGGACACAACTCGAGTACAACATACTCACACAATGTATACATCACGGCAGACA
349 AACAAAAGAATGGAATCAAAGCTAACTTCAAATTCGCCACAACATTGAAGATGGATCCGTTCAACT
350 AGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCTTTTACCAGACAACCATTAC
351 CTGTGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGT
352 TTGTAAGTCTGCTGGGATTACACATGGCATGGATGAGCTCTACAAATAA

353 **snap** in pSNAP-tag (T7)-2 and pKASBAR-EzrA-SNAP

354 ATGGACAAAGACTGCGAAATGAAGCGCACCCCTGGATAGCCCTCTGGGCAAGCTGGAAGTGTG
355 TGGGTGCGAACAGGGCCTGCACCGTATCATCTTCTGGGCAAAGGAACATCTGCCGCCGACGCCG
356 TGGAAGTGCCTGCCCCAGCCGCCGTGCTGGGCGGACCAGAGCCACTGATGCAGGCCACCGCCTG
357 GCTCAACGCCTACTTTACCAGCCTGAGGCCATCGAGGAGTTCCTGTGCCAGCCCTGCACCACC
358 CAGTGTTCAGCAGGAGAGCTTTACCCGCCAGGTGCTGTGGAAACTGCTGAAAGTGGTGAAGTTC
359 GGAGAGGTCATCAGCTACAGCCACCTGGCCGCCCTGGCCGGCAATCCCGCCGCCACCGCCGCCG
360 TGAACCGCCCTGAGCGGAAATCCCGTGCCATTCTGATCCCTGCCACCGGGTGGTGCAGGGC
361 GACCTGGACGTGGGGGGCTACGAGGGCGGGCTCGCCGTGAAAGAGTGGCTGCTGGCCCACGAG
362 GGCCACAGACTGGGCAAGCCTGGGCTGGGT

363 **snap** in pSS26b, pSS26bFtsZ-C and pCQ11-FtsZ-SNAP

364 ATGGACAAAGATTGCGAAATGAAACGTACCACCCTGGATAGCCCGCTGGGCAAAGTGGAACTGAGC
365 GGCTGCGAACAGGGCCTGCATGAAATTAAGTGGTAAAGGCACCAGCGCGCCGATGCGGT
366 TGAAGTTCGGCCCCGGCCGCCGTGCTGGGTGGTCCGGAACCGCTGATGCAGGCGACCGCGTGG
367 CTGAACGCGTATTTTCATCAGCCGGAAGCGATTGAAGAATTTCCGGTTCGGCGCTGCATCATCCG
368 GTGTTTCAGCAGGAGAGCTTTACCCGTCAGGTGCTGTGGAAACTGCTGAAAGTGGTTAAATTTGGC
369 GAAGTGATTAGCTATCAGCAGCTGGCGGCCCTGGCGGGTAATCCGGCGGCCACCGCCGCCGTTAA
370 AACCGCGCTGAGCGGTAACCCGGTGCCGATTCTGATTCCGTGCCATCGTGTGGTTAGCTCTAGCG
371 GTGCGGTTGGCGGTTATGAAGGTGGTCTGGCGGTGAAAGAGTGGCTGCTGGCCCATGAAGGTCAT
372 CGTCTGGGTAAACCGGGTCTGGGATGA

373 **Linker A**

374 TCAGGTTTCAGGTTTCAGGTGGGCGCGCCTCAGGTTTCAGGTTTCAGGT

375 **Linker B**

376 GAATTCATCCCATGGGTTTCAGGTGGTGGTGGTTCA

377 **Labelling *S. aureus* with DAAs**

378 DAAs were prepared by published methods (9-11) or by modified procedures described in Appendix II.
379 ADA was obtained from Iris Biotech. These were incubated with mid-exponential phase ($OD_{600} \sim 0.3$ to
380 0.4) *S. aureus* at 500 μ M (1 mM for ADA-DA) and incubated on a rotary shaker at 37°C for the required
381 labelling time. Samples were imaged using widefield microscopy, 3D-SIM or localisation microscopy as
382 required. For 15 s labelling DAAs were used at 10 mM, 1ml samples were mixed briefly by vortexing and
383 fixed by addition of 500 μ l 8% (w/v) ice-cold paraformaldehyde immediately after vortexing.

384 **Click Chemistry**

385 DAAs containing an azide functional group (ADA & ADA-DA) required chemical attachment of a
386 fluorophore via the Click reaction (copper (I)-catalysed alkyne-azide cycloaddition). This was carried out
387 using the Click-iT® Cell Reaction Buffer Kit (ThermoFisher) as per the manufacturers protocol. Alkyne
388 dyes were added at 5 μ g ml⁻¹.

389

390 **Labelling *S. aureus* with Fluorescent Vancomycin**

391 Fixed cells were resuspended in PBS containing fluorescent vancomycin at 2 μM (prepared using
392 succinimidyl ester of Amersham Cy3B (GE Healthcare) as previously described(29). Samples were
393 protected from light and incubated at room temperature for 30 minutes then washed by centrifugation and
394 resuspension in water. For dual labelled samples, cells were labelled with required DAA as described
395 above and fixed with 4% (w/v) paraformaldehyde prior to labelling with fluorescent vancomycin.

396 **Labelling *S. aureus* with NHS ester**

397 *S. aureus* grown to mid-exponential phase ($\text{OD}_{600} \sim 0.5$) were resuspended in PBS containing Alexa Fluor
398 647 NHS ester (Invitrogen) at 8 $\mu\text{g ml}^{-1}$ and incubated at room temperature for 5 min. Cells were then
399 washed by centrifugation and resuspension in PBS.

400 **Labelling *S. aureus* with SNAP-Cell TMR-Star**

401 *S. aureus* grown to mid-exponential phase ($\text{OD}_{600} \sim 0.5$) were incubated with SNAP-Cell TMR-Star (New
402 England Biolabs) at 500 nM for widefield microscopy or 3 μM for SIM at 37°C for 15 min. Cells were
403 washed by centrifugation and resuspension in PBS.

404 **Fixing**

405 With the exception of Slimfield microscopy which involved no fixation and 15 s DAA labelling which used
406 8% (w/v) ice-cold paraformaldehyde, all samples were fixed with 4% (w/v) paraformaldehyde prior to
407 imaging.

408 **Widefield Epifluorescence Microscopy**

409 Fixed cells were mounted on poly-L-Lysine coated slides and imaged on a Nikon Ti Inverted
410 microscope fitted with a Lumencor Spectra X light engine. Images were taken using a 100x PlanApo (1.4
411 NA) oil objective using 1.518 RI oil and detected by an Andor Zyla sCMOS camera.

412 **OMX Microscopy**

413 Coverslips (High-precision, No.1.5H, 22x22mm, $170 \pm 5 \mu\text{m}$, Marienfeld) were sonicated for 15 min in 1 M
414 KOH, washed with water and incubated in poly-L-Lysine solution for 30 minutes. Coverslips were then
415 further washed and dried with nitrogen. Fixed cells were then dried onto the coverslips with nitrogen and
416 mounted on slides with $\sim 5 \mu\text{l}$ Slow Fade Diamond (Invitrogen).

417 Structured Illumination Microscopy was carried out using a v4 DeltaVision OMX 3D-SIM system fitted with
418 a Blaze module (Applied Precision, GE Healthcare, Issaquah, USA). Samples were illuminated using laser

419 illumination. For each z slice, samples were imaged in 5 phase shifts and 3 angles, z-steps were 0.125
420 nm. Reconstructions were performed with the Softworx software (GE Healthcare) using OTFs optimised
421 for the specific wavelength and oil used. The same software was used for deconvolution.

422 **Sample Preparation for Localisation Microscopy**

423 For all samples coverslips were prepared as for 3D-SIM Microscopy. All samples except for eYFP/meYFP
424 and were mounted on slides with 5 μ l GLOX buffer (0.5 mg ml⁻¹ glucose oxidase, 40 μ g ml⁻¹ catalase, 10%
425 (w/v) glucose in 50 mM Tris-HCl containing 10 mM NaCl (pH 8.0) containing either 10 or 100 mM
426 mercaptoethylamine (MEA).

427 For eYFP/meYFP imaging (single colour) samples were mounted in 5 μ l PLOX buffer (5 U ml⁻¹ pyranose
428 oxidase, 40 μ g ml⁻¹ catalase, 10% (w/v) glucose in 50 mM Tris-HCl, 10 mM NaCl (pH 8.0) prepared in
429 heavy water(50)).

430 For eYFP/Alexa Fluor 647 imaging (two-colour) samples were mounted in 5 μ l PLOX containing 50 mM
431 MEA. Where required, coverslips were sparsely coated with TetraSpeck beads (0.1 μ m, Molecular Probes)
432 prior to the application of cells.

433 **Bespoke Localisation Microscope**

434 Localisation microscopy was carried out as previously described(51, 52), but using OBIS 405 (50 mW)
435 and OBIS 647 (120 mW) lasers, a 662 nm dichroic and a 676 (29) nm emission filter. Calibration data for
436 3D reconstructions was obtained by recording images of fiducial particles while stepping the objective
437 piezo.

438 **Nikon N-STORM Localisation Microscope**

439 Localisation microscopy was carried out using a Nikon Ti-NS N-STORM version 1 with 3D capability in
440 continuous mode. Objective used was a SR Apo TIRF 100x NA 1.49 and images detected using EMCCD
441 camera (Andor DU-897) using the 17 MHz 16 bit mode with an EM Multiplier Gain of 300 and a conversion
442 gain of 3. Calibration data for 3D reconstructions was obtained by recording images of fiducial particles
443 using the calibration mode. Custom-made filter cubes were used for eYFP/meYFP (no excitation filter, 488
444 nm dichroic, 525/50 nm emission) and two-colour imaging (red/far red; no excitation filter, multi-band
445 dichroic with transmission at 410-480 nm, 500-550 nm, 570-630 nm and above 650 nm, multi-band
446 emission with transmission at 570-620 nm and above 660 nm) imaging and the N-STORM cube for single
447 colour Alexa Fluor 647 imaging. Imaging was done under oblique illumination but not full TIRF. Two colour

448 eYFP and Alexa Fluor 647 imaging was performed using separate filter cubes whereas two colour imaging
449 using Cy3B and Alexa Fluor 647 was performed using a single cube, as specified.

450 **Image Reconstruction**

451 Images were reconstructed as previously described(53) using either custom Matlab scripts, the
452 ThunderSTORM ImageJ/Fiji plugin(54) or Nikon elements software. All of these methods identify the
453 locations of molecules by fitting Gaussian functions to regions of source data, and all yielded similar
454 results.

455 Two colour data (where using a single multi-band filter cube) was reconstructed and aligned (registered)
456 using Nikon elements. In summary, alignment is achieved by obtaining calibration images of the same
457 fluorescent beads in both channels. The software then determines the way in which localisations in one
458 channel must be offset to align with the other, based on the offsets in the apparent positions of the beads.
459 For two colour eYFP/Alexa Fluor 647 NHS ester imaging, using two filter cubes, the average position of a
460 TetraSpeck fiducial was determined in both channels and a translational offset calculated for each image.
461 This was applied to the Alexa Fluor 647 channel to approximately align the data. Whilst more sophisticated
462 co-alignment methods exist, this was sufficient for us to draw the qualitative conclusions necessary for this
463 part of our study.

464 **Image Rendering**

465 Images were rendered as 2D histograms using the ThunderSTORM ImageJ/Fiji plugin(54). Unless
466 otherwise stated images were projected onto a single plane and the reconstructed pixel size was 10 nm.
467 Semi-quantitative Matlab contour plots were used in some instances for ease of visualisation of key
468 features in 3D reconstructions both on screen and in print. eYFP and eYFP/Alexa Fluor 647 NHS ester
469 dual colour images were reconstructed with a pixel size of 5 nm with a Gaussian blur of 20 nm applied to
470 make them easier to see.

471 **Analysis of Localisation Microscopy Data**

472 Ring-like groups of localisations were manually selected from fields. The centre and radius of a circle that
473 best fit the points was then determined allowing the localisations to be represented using polar co-
474 ordinates. Histograms of localisations with respect to angle (2° bin size) and distance from the centre of
475 the circle (10 nm bin size) were then generated. The angular histograms were auto-correlated to test for
476 the presence of similarly sized large groups of molecules which would create peaks or a very slow decay

477 from 0° in the resulting graph. The distance histograms were plotted and compared with those resulting
478 from simulations.

479 An additional, similar, analysis was carried modelling the septal shape as an ellipse (Fig. 1 - supplement
480 3).

481 **Simulation of Localisation Microscopy Data**

482 We used the simplest possible methods to simulate data to compare with that acquired on the microscope.
483 Localisations were randomly distributed by angle on circles of a fixed radius. Localisation error comes from
484 several physical sources, but was simulated by adding offsets in x and y taken independently and at
485 random from a normal distribution of a defined standard deviation.

486 **Slimfield Microscopy: Microscope Setup**

487 A bespoke single-molecule microscope was used, constructed around the body of a Zeiss inverted
488 microscope with a 100x 1.49 numerical aperture oil immersion total internal reflection fluorescence (TIRF)
489 objective lens (Olympus) and an xyz nano positioning stage (Nanodrive, Mad City Labs). A 20 mW Obis
490 514 nm laser expanded to 10 µm full width at half maximum was used to excite meYFP fluorescence
491 combined with a dual pass CFP/YFP dichroic mirror with 20 nm transmission windows centred on 440 nm
492 and 514 nm. A high speed camera (Andor iXon DV860-BI) was used to image at 5 ms/frame with
493 magnification at 50 nm/pixel. Data was acquired using custom LabView software.

494 **Slimfield Microscopy: Sample preparation and imaging**

495 *S. aureus* SH4604 (*ezrA-meyfp ΔezrA*) cells were imaged by immobilising them on an agarose pad
496 suffused with media. These were constructed by placing a gene frame (Life Technologies) on a BK7 glass
497 microscope slide (Fisher) and filling with ~500 µl 1% (w/v) agarose containing media. Once set, 5 µl of cell
498 culture was spotted over the agarose and covered with a plasma cleaned coverslip.

499 **Slimfield Microscopy: Image analysis**

500 Cell bodies and apparent EzrA rings were segmented as outlined previously(55). In brief, the cell body
501 was found by segmenting both a 5 frame average EzrA-meYFP fluorescence and brightfield image using
502 a threshold set by the background peak in the pixel intensity distribution. The brightfield segmentation was
503 used as seeds for watershedding the segmented fluorescence image to identify individual cells. Further
504 thresholding within cell pixels yields a mask for the EzrA ring.

505 Diffraction-limited fluorescent foci were tracked using custom Matlab software as described previously(56).
506 In brief, in each frame, candidate foci are identified by thresholding top-hat transformed images using

507 Otsu's method. The spot centre is determined to sub-pixel precision using iterative Gaussian masking(57)
508 and accepted if its signal-to-noise ratio, as defined by the foci intensity, the background-corrected
509 integrated pixel intensity within a 5 pixel radius circular region of interest centred of the foci intensity
510 centroid, divided by the standard deviation of the background pixels, is greater than 0.4. Foci are linked
511 into the same track between image frames if they are within a distance of 1 optical resolution width
512 (approximately 5 pixels), generating single particle tracks to a typical localization precision of ~40 nm(58).
513 The mean squared displacement of each track over its first 4 time interval points was used to calculate its
514 microdiffusion coefficient, D, using a linear fit(59). These were binned into 0.01 $\mu\text{m}^2 \text{s}^{-1}$ bins and fitted with
515 1-3 gamma functions(26), with 3 gammas generating the lowest reduced chi².
516 Copy number values were calculated using a deconvolution method called CoPro(27) which utilised the
517 symmetrical geometry of *S. aureus* cells and the *in vivo* characteristic intensity of single meYFP
518 molecules(60). Detection of single meYFP was confirmed by observation of single, distinct photobleach
519 steps. This characteristic brightness value corresponding to a single meYFP molecule was determined as
520 the peak of the intensity distribution of fluorescent foci found after 200 ms of photobleaching, and was
521 equivalent to 2000±500 counts on our EMCCD camera detector.

522 **Transmission Electron Microscopy**

523 Samples were prepared for electron microscopy as previously described (35) .

524 **Cell Volume Calculation**

525 Cell volumes calculations were carried out as previously described(4), specifically, the long and short axis
526 of cells were measured using Fiji. The volume was then calculated based on a prolate spheroid shape
527 with volume $V = \frac{4}{3}\pi ab^2$, where a and b are the dimensions of the long and short axis respectively.

528 **Gel-based analysis of SNAP tagged proteins**

529 SNAP-Cell TMR-Star (New England Biolabs) was added to a 1 ml aliquot of mid-exponential phase (OD₆₀₀
530 ~1) grown culture at a concentration of 500 nM and incubated at 37°C for 1 h. Cells were washed three
531 times by resuspension and centrifugation in PBS, resuspended in PBS supplemented with 200 $\mu\text{g ml}^{-1}$
532 lysostaphin and 20 U ml^{-1} DNase I and lysed at 37°C for 30 min. Cell extracts were resolved in SDS-PAGE,
533 the gel was rinsed with dH₂O and scanned using ChemiDoc MP System (Bio-Rad).

534 **Incorporation of ¹⁴C-GlcNAc into cell wall Peptidoglycan**

535 *S. aureus* strains were grown overnight in CDM and used to inoculate fresh CDM to an OD₆₀₀ of 0.05 and
536 grown to OD₆₀₀ ~0.2. At this point 5 μM ¹⁴C-GlcNAc was added to cultures. At 30 minute intervals samples

537 were collected and prepared for analysis of ^{14}C -GlcNAc incorporation via Liquid Scintillation as previously
538 described(61).
539

540 **Fluorescence Intensity Measurements**

541 Fluorescence intensity was measured using Image J/Fiji and calculated as counts/pixel. To determine the
542 % off-septal fluorescence the fluorescence intensity for both the septum and the whole cell was measured
543 and the percentage of non-septal fluorescence calculated.

544

545 **Peptidoglycan purification and Mass-Spectrometry Analysis**

546 *S. aureus* peptidoglycan was purified as previously described(34). Specifically, 1L cultures of *S. aureus*
547 SH1000 and *S. aureus* SH1000 containing 1 mM ADA were grown for 4 hours before peptidoglycan was
548 extracted and purified. Peptidoglycan was solubilized by digestion with 50 µg Cellosyl per mg
549 peptidoglycan (dry weight) overnight at 37°C. Samples were boiled to inactivate the Cellosyl and reduced
550 using sodium borohydride(62). Reduced muropeptides were separated on an Agilent Technologies
551 Accurate Mass Q-TOF LC/MS using a Hypersil Gold aQ column (200 x 42.1 µm, 1.9 µm particle size) with
552 a gradient of 0-30 % (v/v) water/ACN both containing 0.1% (v/v) formic acid over 60 mins.

553

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558 assistance.

559 **Figure Legends**

560
561 **Figure 1. Distribution of cell division components during septation.** a) Examples of EzrA
562 distributions obtained using localisation microscopy of SH4388 (*ezrA-eyfp ΔezrA*). Scale bars 200 nm. b)
563 Examples of FtsZ distributions obtained using localisation microscopy of SH4665 (pCQ11-FtsZ-eYFP)
564 grown with 50µM IPTG. Scale bars 200 nm). c) Simulated distributions of localisations randomly
565 distributed by angle with different radii (r), number of localisations (n) and random error from a normal
566 distribution with standard deviation (σ) [i] r=440 nm, n=1118, σ=20 nm, [ii] r=440 nm, n=1118, σ=40 nm,
567 [iii] r=440 nm, n=1118, σ=80 nm, [iv] r=440 nm, n=145, σ=20 nm, [v] r=440 nm, n=2010, σ=20 nm. Scale
568 bars 200 nm. d) An enlarged example of EzrA-eYFP distribution. Scale bar 200 nm. e) The distribution
569 from 'd' plotted as a scatter graph, and as histograms of number of localisations with respect to angle

570 and distance from centre. f) Mean angular autocorrelations of 14 EzrA, 19 FtsZ and 15 simulated
571 distributions. Autocorrelation drops less quickly for EzrA and FtsZ than for simulations where angle is
572 randomised. This shows that neither EzrA or FtsZ are randomly distributed by angle. g) Histograms of
573 localisations with respect to distance from the centre of a fitted circle with varying localisation precision.
574 Data for EzrA and FtsZ is spread more widely than simulated data with poor localisation precision.

575

576 **Figure 2. Relative locations of division components.** a) Localisation microscopy images: of FtsZ-
577 eYFP distributions in bacteria in the late stages of division. Scale bars 500 nm. Ellipses show
578 approximate cell location and orientation. b) Dual colour localisation microscopy image of FtsZ-eYFP
579 and the cell wall (labelled with Alexa Fluor 647 NHS ester, NHS-647). Scale bars 500 nm. c) Dual colour
580 localisation microscopy image of EzrA-eYFP and the cell wall (labelled with NHS-647). Scale bars 500
581 nm.

582

583 **Figure 3. Peptidoglycan insertion.** Localisation microscopy images: a) 15 s labelling of ADA (Azido-D-
584 alanine) clicked to Alexa Fluor 647. Scale bars 0.5 μm . b) 5 min labelling of i) ADA clicked to Alexa Fluor
585 647 and ii) ADA-DA clicked to Alexa Fluor 647. Scale bars 1 μm . c) 3D projections of *S. aureus* labelled
586 for 5 minutes with ADA clicked to Alexa Fluor 647. i) Cells with incomplete septum (yellow arrows show
587 gaps in labelling), ii) cell with annulus complete. Images in black box are z-projections while 3D
588 representations show projections in all 3 planes. Scale bar 0.5 μm . d) Cross sections of incomplete
589 septa. The sketch graph (top row) hypothetically shows labelling exclusively at the leading edge of the
590 septum. This is not the case for the data shown below - labelling is spread throughout the septum. The
591 full width half maximum spread of labelling is ~ 230 nm. Data is plotted with blue dots, fits in red lines. e)
592 Two colour STORM, sample labelled for 5 minutes with ADA-DA clicked to Alexa Fluor 647 (yellow) and
593 vancomycin linked to Amersham Cy3B (magenta). Images are z-projections and in merged images
594 where localisations are in white show labelling by both ADA-DA and vancomycin. Boxed regions show
595 slot in ADA-DA labelling but not vancomycin. Scale bars 1 μm .

596

597 **Figure 4. Effect of FtsZ inhibitor PC190723 on *S. aureus*.** a) TEM of *S. aureus* SH1000 grown in the
598 presence of PC190723 ($10 \mu\text{g ml}^{-1}$) for 60 minutes. Scale bars 200 nm. b) STORM image of *S. aureus*
599 SH1000 pre-treated with PC190723 ($10 \mu\text{g ml}^{-1}$) for 60 minutes labelled with ADA clicked to Alexa Fluor

600 647 for 5 minutes. Scale bar 1 μm . i) & ii) zoomed images of the corresponding area, scale bars 0.25
601 μm .

602
603 **Figure 5. Conceptual model of peptidoglycan insertion during the *S. aureus* cell cycle.**

604 a) Schematic of measurement used in b) measurement of the angle (θ) between a line parallel to the
605 surface of the septum (yellow) and a tangent to the surface of the bacterium in incomplete (blue) and
606 complete (red) septa. c) Surfaces available for peptidoglycan insertion for different septal geometries
607 where d is half the thickness of the septum, r is the cell radius in the septal plane and s is the distance
608 from the leading to the lagging edge of the septum (measured from the inner surface of the cell wall). d)
609 Conceptual model of peptidoglycan insertion in *S. aureus*. i, ii) Cell size increases and aspect ratio
610 changes prior to observation of the start of septum formation by 3D-SIM (3). iii) The septum then starts
611 to form, beginning with the “piecrust” feature (red) observed by AFM (34). The septum is thinner at the
612 leading edge (33). iv) New peptidoglycan is inserted in a zone at the leading edge of the septum, as well
613 as across the rest of the cell surface as visualised here by localisation microscopy. v, vi) After the
614 annulus has fused, peptidoglycan insertion continues in the septum, executed by cell division
615 components, until it is of uniform thickness. vii) ATL (a peptidoglycan hydrolase) is present at the outer
616 surface of the cell in the plane of septation(63). Cracks or splits begin to form at the outer surface in the
617 plane of septation(64), followed by rapid popping apart of the daughter cells (4). vii) “Scars” or “ribs”
618 remain marking the site of division (3, 34) and may provide spatial cues to subsequently enable correct
619 sequentially orthogonal divisions.

620 Supplementary Figure Legends

621 **Figure 1 – figure supplement 1. EzrA fusions are functional.** a) Construction of *S. aureus* strains in
622 which the only copy of *ezrA* is tagged (FL). Integration of pKASBAR-EzrA-FL at *S. aureus* lipase (*geh*)
623 resulted in an ectopic copy of *ezrA-fl* under the control of the native *ezrA* promoter (P). A double-
624 crossover event of pOB- Δ *ezrA* allowed for marked with a tetracycline resistance (*tetR*) gene deletion of
625 *ezrA* from its native chromosomal location. FL represents either eYFP, meYFP, GFP or SNAP. Not to
626 scale. b) Growth rates of *ezrA* fusions. EzrA-eYFP, EzrA-meYFP, EzrA-GFP and EzrA-SNAP
627 complement native *ezrA* knock-out in SH4388 (*ezrA-eyfp* Δ *ezrA*), SH4604 (*ezrA-meyfp* Δ *ezrA*), SH4640
628 (*ezrA-gfp* Δ *ezrA*) and SH4642 (*ezrA-snap* Δ *ezrA*), respectively. The mutant strains (doubling time 24
629 min) showed similar growth to the wild type strain, SH1000 (doubling time 25 min). Growth rates were

630 obtained by fitting an exponential growth equation to the most linear region of growth curves ($R^2 > 0.98$).
631 Bacterial cultures were prepared in triplicate and the error bars represent standard deviation from the
632 mean. c) Epifluorescence microscopy images of EzrA-eYFP in SH4388 (*ezrA-eyfp ΔezrA*), EzrA-meYFP
633 in SH4604 (*ezrA-meyfp ΔezrA*), EzrA-GFP in SH4640 (*ezrA-gfp ΔezrA*) and SNAP-Cell TMR-Star
634 labelled EzrA-SNAP in SH4642 (*ezrA-snap ΔezrA*). Images are maximum intensity fluorescence
635 projections of z stacks. Scale bars 3 μm . d) EzrA-eYFP in SH4388 (*ezrA-eyfp ΔezrA*) and EzrA-meYFP
636 in SH4604 (*ezrA-meyfp ΔezrA*) were detected by Western blot analysis of total protein extracts using
637 anti-GFP antibodies. Whole cell lysate of SH1000 and a recombinant GFP-HisTag protein were used as
638 controls. Bands detected at ~95 kDa (EzrA-eYFP and EzrA-meYFP) and ~28 kDa (GFP-HisTag) are
639 indicated with black arrows. Sizes of a protein ladder are shown in kDa. e) EzrA-GFP in SH4640 (*ezrA-*
640 *gfp ΔezrA*) was detected by immunoblot analysis of total protein extract using anti-GFP antibodies.
641 Whole cell lysate of SH1000 and a recombinant GFP-HisTag protein were used as controls. Bands
642 detected at ~95 kDa (EzrA-GFP) and ~28 kDa (GFP-HisTag) are indicated with black arrows. Sizes of a
643 protein ladder are shown in kDa. f) Whole cell lysate of SNAP-Cell TMR-Star labelled SH4642 (*ezrA-*
644 *snap ΔezrA*) was resolved by 10% (w/v) SDS-PAGE and visualised by fluorescence detection. Whole
645 cell lysate of SNAP-Cell TMR-Star labelled SH1000 and a purified SNAP-Cell TMR-Star labelled HisTag-
646 SNAP protein were used as controls. Bands detected at ~85 kDa (EzrA-SNAP) and ~23 kDa (SNAP-
647 HisTag) are indicated with black arrows. Sizes of a protein ladder are shown in kDa.

648 **Figure 1 – figure supplement 2. STORM and SIM data.** a) EzrA-GFP (i) and SNAP-Cell TMR-Star
649 labelled EzrA-SNAP (ii) localisation in SH4640 (*ezrA-gfp ΔezrA*) and SH4642 (*ezrA-snap ΔezrA*) by
650 3D-SIM, respectively. The images are maximum intensity projections of reconstructed z stacks. Scale
651 bars 1 μm . 3D surface profiles of the circled area show distribution of fluorescence intensity of EzrA-GFP
652 and EzrA-SNAP TMR-Star rings. b) Localisation microscopy of EzrA-meYFP in SH4604 (*ezrA-meyfp*
653 *ΔezrA*).

654 **Figure 1 – figure supplement 3. Quantitative analysis of EzrA and FtsZ distributions from**
655 **localisation microscopy data based on elliptical fits.** a) Example image of EzrA distribution. Scale
656 bar 200 nm. b) EzrA distribution represented as a scatter plot overlaid on an elliptical ring. Yellow points
657 are included in the subsequent analyses, grey ones are not. The elliptical ring is derived from an elliptical
658 fit to all of the points. c) Enlargement of boxed region in b. The elliptical ring is split up into blocks –
659 darker blue blocks contain more localisations than lighter ones. This gives a measure of how the number

660 of localisations varies around the ring. d) Autocorrelations of localisations around the ring for EzrA, FtsZ
661 and simulated data with a random distribution. EzrA and FtsZ distributions are more self-correlated than
662 a random distribution, but have no periodic order. e) Distributions of absolute distances of localisations
663 from the fitted ellipse for EzrA, FtsZ and simulated data. Simulated data had a localisation precision from
664 a normal distribution with a mean of 27 nm and a standard deviation of 8.7 nm – representative of our
665 measured values. The spread and magnitude of distances of EzrA and FtsZ localisations from the fitted
666 ellipse cannot be accounted for by localisation uncertainty alone.

667 **Figure 2 – figure supplement 1. Dynamics of EzrA.** Nominal diffusion coefficient (D) distributions of
668 EzrA-meYFP molecules a) inside and b) outside the EzrA “ring” in SH4604 (*ezrA-meyfp ΔezrA*). The
669 distribution of D values could be fitted using a 1-3 component gamma distribution model, as developed
670 for heterogeneous protein mobility observed previously in bacteria (26), with 3 components producing
671 the lowest reduced $\chi^2=0.05$ c) Distribution of number of EzrA-meYFP molecules per cell.

672 **Figure 3 – figure supplement 1. Identification of mechanism of DAA labelling in *S. aureus*.** Cellosyl
673 digested peptidoglycan from *S. aureus* SH1000 grown in the presence (a) or absence (b) of ADA for 4
674 hours were investigated using LC-MS, with total ion chromatogram for acquisition time 20-36 minutes
675 showing all detected ions (i). ii) Extracted ion chromatogram for $m/z [H_+] = 1294.5970$ shows a clear
676 peak in (a) not present in (b). c) The mass-spectrum of this peak shows both the monoisotopic mass of
677 the single-charged ion 1294.5863 and the doubly-charged ion at 647.7968, corresponding to
678 disaccharide-pentapeptide-pentaglycine molecule with ADA replacing one of the D-alanine residues.

679 **Figure 3 – figure supplement 2. 15 second labelling of peptidoglycan insertion with DAAs and**
680 **controls.** a) 15s labelling of ADA clicked to Alexa Fluor 647. Sample imaged by epifluorescence and
681 image is a maximum intensity projection of z stacks. b) 15s labelling with HADA imaged by i)
682 epifluorescence and ii) 3D-SIM. c) Cells labelled with Alexa Fluor 647 by the click reaction in the
683 absence of ADA imaged by i) epifluorescence and ii) STORM. d) localisation microscopy of 15s labelling
684 of ADA-DA (azido-D-alanyl-D-alanine) clicked to Alexa Fluor 647. Scale bars a-c) 5 μ m d) 1 μ m.

685 **Figure 3 – figure supplement 3. DAA labelling of PBP4 null *S. aureus*.** a) SH4425 (SH1000 *pbp4*)
686 labelled for 5 minutes with i) HADA and ii) ADA-DA clicked to Alexa Fluor 647. Scale bars 5 μ m. b)
687 Growth rate of SH1000 and SH4425 in CDM, c) Rate of peptidoglycan synthesis as measured by ^{14}C
688 GlcNAc incorporation. d) DAA incorporation with 5 minutes labelling (HADA & ADA-DA) in SH1000 and
689 SH4425. e) % off-septal labelling in 5 minutes ADA-DA labelling of SH1000 and SH4425. f) Localisation

690 microscopy of 5 minutes FDAA labelling of SH4425 with i) ADA clicked to Alexa Fluor 647 and ii) ADA-
691 DA clicked to Alexa Fluor 647. Scale bars 1 μm . g) Localisation microscopy of 15s labelling of SH4425
692 with i) ADA clicked to Alexa Fluor 647 and ii) ADA-DA clicked to Alexa Fluor 647. Scale bars 1 μm . h)
693 Comparison of autocorrelations of localisations around a fitted elliptical ring for SH1000 and SH4425
694 (SH1000 *pbp4*) labelled for 15 with i) ADA or ii) ADA-DA. n=10 bacteria per group. There is no
695 substantial difference between autocorrelations in either comparison.

696 **Figure 4 – figure supplement 1. Effect of FtsZ inhibitor PC190723 on *S. aureus*.** a) SH4652 (*ezaA-*
697 *eyfp* Δ *ezaA* pCQ11-FtsZ-SNAP) grown in the presence of 50 μM IPTG in the absence (control) or
698 presence of PC190723 (10 $\mu\text{g ml}^{-1}$) for 0, 15, 30 and 60 minutes, labelled with SNAP-Cell TMR-Star was
699 incubated with HADA for 5 min. Images are average intensity projections of z stacks. Scale bars 3 μm .
700 Arrows indicate localisation defects. b) Cell volume of *S. aureus* SH1000 during treatment with
701 PC190723 (10 $\mu\text{g ml}^{-1}$). Data is expressed as mean \pm standard deviation.

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865 Appendix I

866 Appendix I -Table 1. Strains used in this study.

Strain	Relevant Genotype/markers	Source
SH1000	Functional <i>rsbU</i> derivative of 8325-4	(65)
RN4220	Restriction deficient transformation recipient	(66)
CYL316	<i>S. aureus</i> RN4220 pCL112Δ19 (cm)	(45)
JGL227	<i>S. aureus</i> SH1000 <i>ezrA-gfp+</i> (ery)	(10)
SH4386	<i>S. aureus</i> SH1000 <i>ezrA-eyfp</i> (kan)	This study
SH4388	<i>S. aureus</i> SH1000 <i>ezrA-eyfp ΔezrA</i> (kan, tet)	This study
SH4603	<i>S. aureus</i> SH1000 <i>ezrA-meyfp</i> (kan)	This study
SH4604	<i>S. aureus</i> SH1000 <i>ezrA-meyfp ΔezrA</i> (kan, tet)	This study

SH4639	<i>S. aureus</i> SH1000 <i>ezrA-gfp</i> (kan)	This study
SH4640	<i>S. aureus</i> SH1000 <i>ezrA-gfp</i> Δ <i>ezrA</i> (kan, tet)	This study
SH4641	<i>S. aureus</i> SH1000 <i>ezrA-snap</i> (kan)	This study
SH4642	<i>S. aureus</i> SH1000 <i>ezrA-snap</i> Δ <i>ezrA</i> (kan, tet)	This study
SH4652	<i>S. aureus</i> SH1000 <i>ezrA-eyfp</i> Δ <i>ezrA</i> pCQ11-FtsZ-SNAP (kan, tet, ery)	This study
SH4665	<i>S. aureus</i> SH1000 pCQ11-FtsZ-eYFP (ery)	This study
NE679	<i>S. aureus</i> JE2 with transposon insertion in <i>pbp4</i> (ery)	(49)
SH4425	<i>S. aureus</i> SH1000 <i>pbp4</i> (ery)	This study
N315	Methicillin-resistant <i>S. aureus</i>	(67)
SU492	<i>B. subtilis</i> SU5 P _{<i>xyI</i>} -ftsZ- <i>yfp</i> (spec)	(44)

867 Appendix I -Table 2. Plasmids used in this study

Plasmid	Relevant Genotype/markers	Source
pGM074	pKASBAR-kan(35) carrying <i>ezrA-psmorange</i> under the putative <i>ezrA</i> promoter (amp, kan)	G. McVicker
pSNAP-tag (T7)-2	<i>E. coli</i> expression plasmid carrying the <i>snap</i> gene under the control of the T7 promoter (amp)	New England Biolabs
pOB	pGEM3Zf(+) cloning vector containing the erythromycin resistance cassette (amp, ery)	(46)
pAISH	TetR derivative of pMUTIN4	(68)
pKASBAR-EzrA-eYFP	pKASBAR-kan containing <i>ezrA-eyfp</i> under the putative <i>ezrA</i> promoter (amp, kan)	This study
pKASBAR-EzrA-meYFP	pKASBAR-kan containing <i>ezrA-meyfp</i> under the putative <i>ezrA</i> promoter (amp, kan)	This study
pKASBAR-EzrA-GFP	pKASBAR-kan containing <i>ezrA-gfp</i> under the putative <i>ezrA</i> promoter (amp, kan)	This study
pKASBAR-EzrA-SNAP	pKASBAR-kan containing <i>ezrA-snap</i> under the putative <i>ezrA</i> promoter (amp, kan)	This study
pOB- Δ <i>ezrA</i>	pOB containing the <i>ezrA</i> deletion cassette consisting of a 1.5 kb fragment of the upstream region of <i>S. aureus ezrA</i> , the tetracycline resistance cassette from pAISH and a 1.5 kb fragment of the downstream region of <i>S. aureus ezrA</i> (amp, ery, tet)	This study
pSS26b	pUC19 encoding <i>snap</i> (amp)	Covalys
pSS26bFtsZ-C	pSS26b containing <i>ftsZ-snap</i> (amp)	This study

pCQ11	<i>E. coli-S. aureus</i> shuttle vector containing <i>lacI</i> , Pspac and <i>gfp</i> (amp, ery)	(48)
pCQ11-FtsZ-SNAP	pCQ11 derivative containing <i>ftsZ-snap</i> under Pspac (amp, ery)	This study
pCQ11-FtsZ-eYFP	pCQ11-FtsZ-SNAP with <i>eyfp</i> replacement of <i>snap</i> (amp, ery)	This study

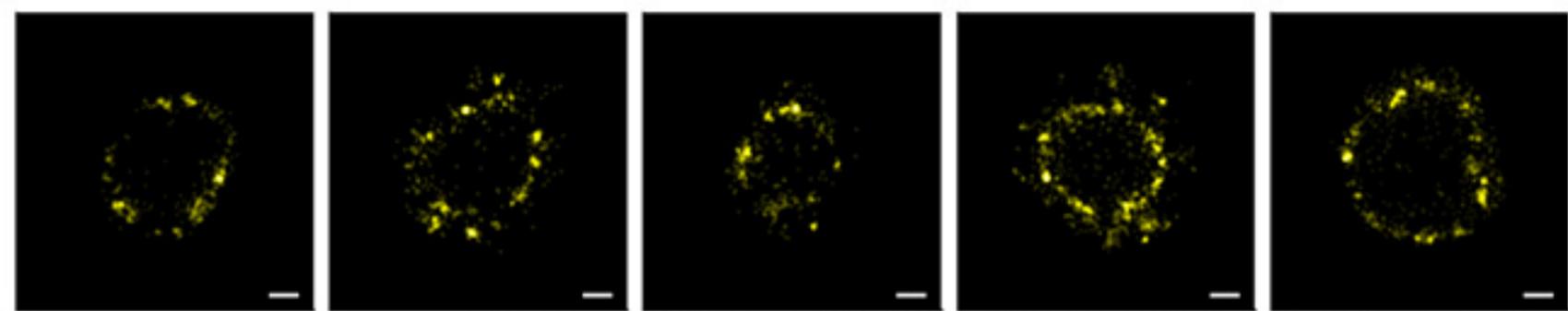
868 Appendix I -Table S3. Oligonucleotides used in this study.

Oligonucleotide name	Sequence (5' to 3')
eYFP-F	CGGCGCGCCTCAGGTTTCAGGTTTCAGGTATGGTGAGCAAGGGCGAG
eYFP-R	CGCGGCCGCTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGC
GFP-F	CGGCGCGCCTCAGGTTTCAGGTTTCAGGTATGGCTAGCAAAGGAGAAGAA CTTTTCACTGGAGTTGTCCC
GFP-R	CGCGGCCGCTTATTTGTAGAGCTCATCCATGCCATGTGTAATCCCAGCA GC
SNAP-F	GGGCGCGCCTCAGGTTTCAGGTTTCAGGTATGGACAAAGACTGCGAAATGA AGCGCAC
SNAP-R	CGAATTCTCATTAAACCAGCCCAGGCTTGCCCAGTCTG
meYFP-F	CTACCAGTCCAAGCTGAGCAAAGAC
meYFP-R	CTCAGGTAGTGGTTGTCTG
pOB-ezrA-up-F	TTTACGTACACTATCTGCAGATGCTTCTCCTCCTAATTTATCATT
pOB-ezrA-up-R	ATTCGAGCTCGGTACCCGGGTTTTAAATTAATAAAAAAACACCCACAATT
pOB-ezrA-down-F	CACTATAGAATACTCAAGCTTACTCCTTAATTTCTCATAAATGATGA
pOB-ezrA-down-R	GGATCAACTTTGGGAGAGAGAACTAGTATGTAGTTATACTTAAATAATAT GAGC
pOB-TetR-F	TAAATTAGGAGGAGAAGCATCTGCAGATAGTGTACGTAAAAAGA
pOB-TetR-R	GTATAACTACATACTAGTTTCTCTCTCCCAAAGTTGATCCC
ftsZ-eyfp-F	ACATGGCCATGTCAGGTTTCAG
ftsZ-eyfp-R	GGCGCGCCTTATTTATATAATTC
FGFtsZXhoI-F	CTCGAGATGTTAGAATTTGAACAAGG
FGFtsZEcoRI-R	TTAGAATTCACGTCTTGTCTTCTTCTTGA
FGFtsZNheI-F	GTTGCTAGCATGTTAGAATTTGAACAAGG
FGFtsZAscl-R	GTTGGCGCGCCTTATCCCAGACCCGGTTTAC

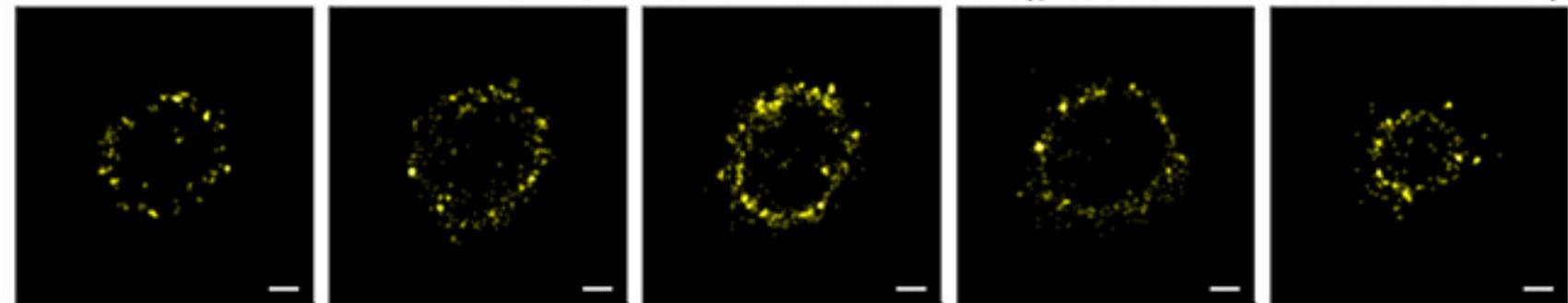
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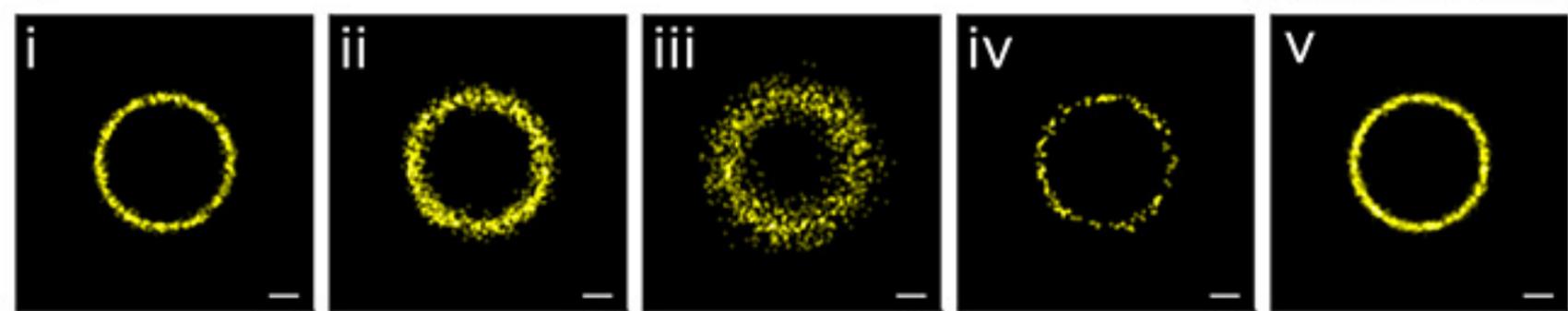
a EzrA-eYFP SH4388 (*ezrA-eyfp ΔezrA*) **f**



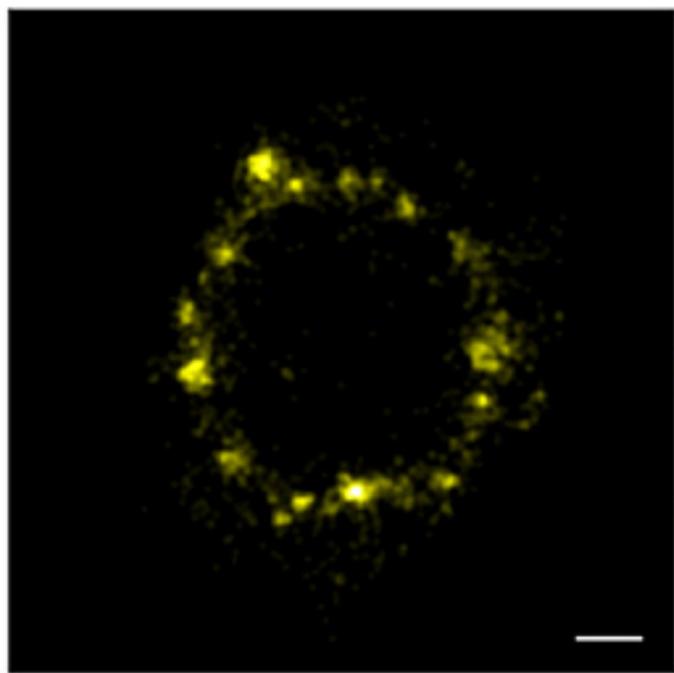
b FtsZ-eYFP SH4665 (pCQ11-FtsZ-eYFP)



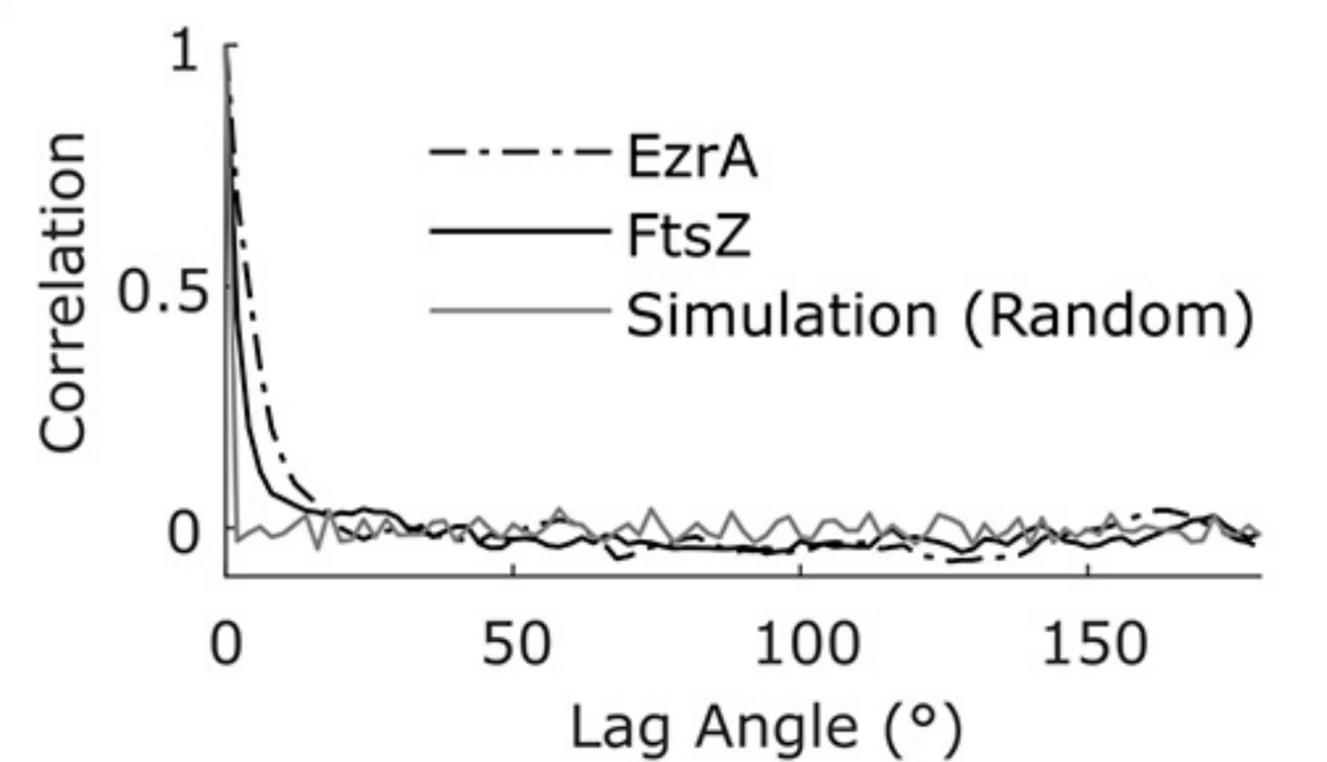
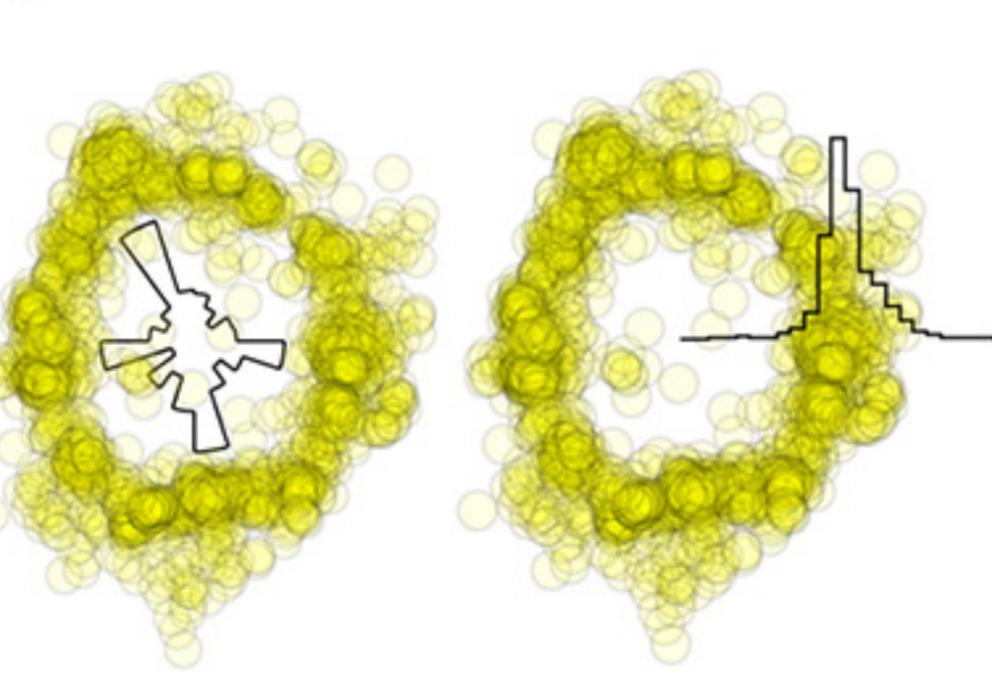
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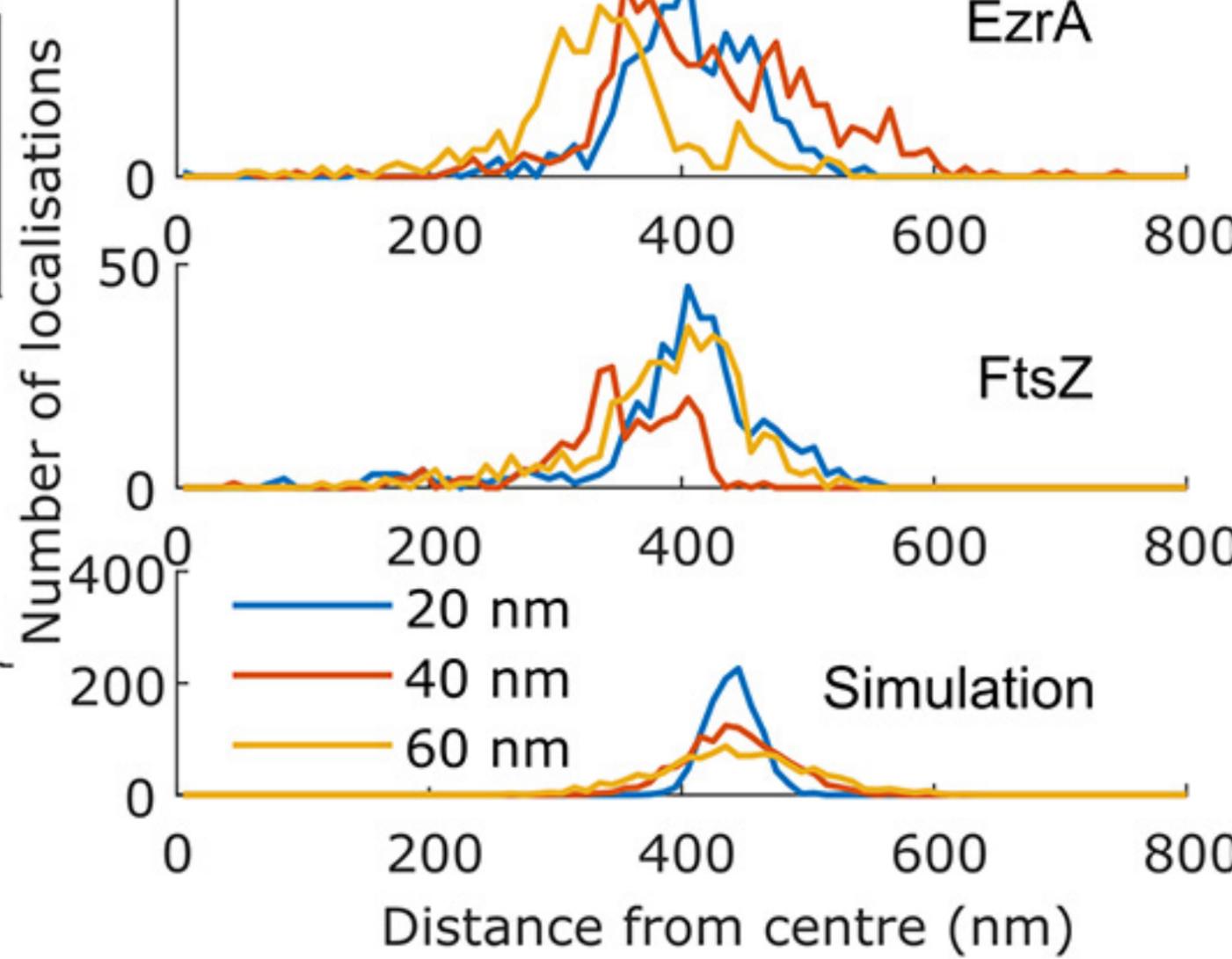
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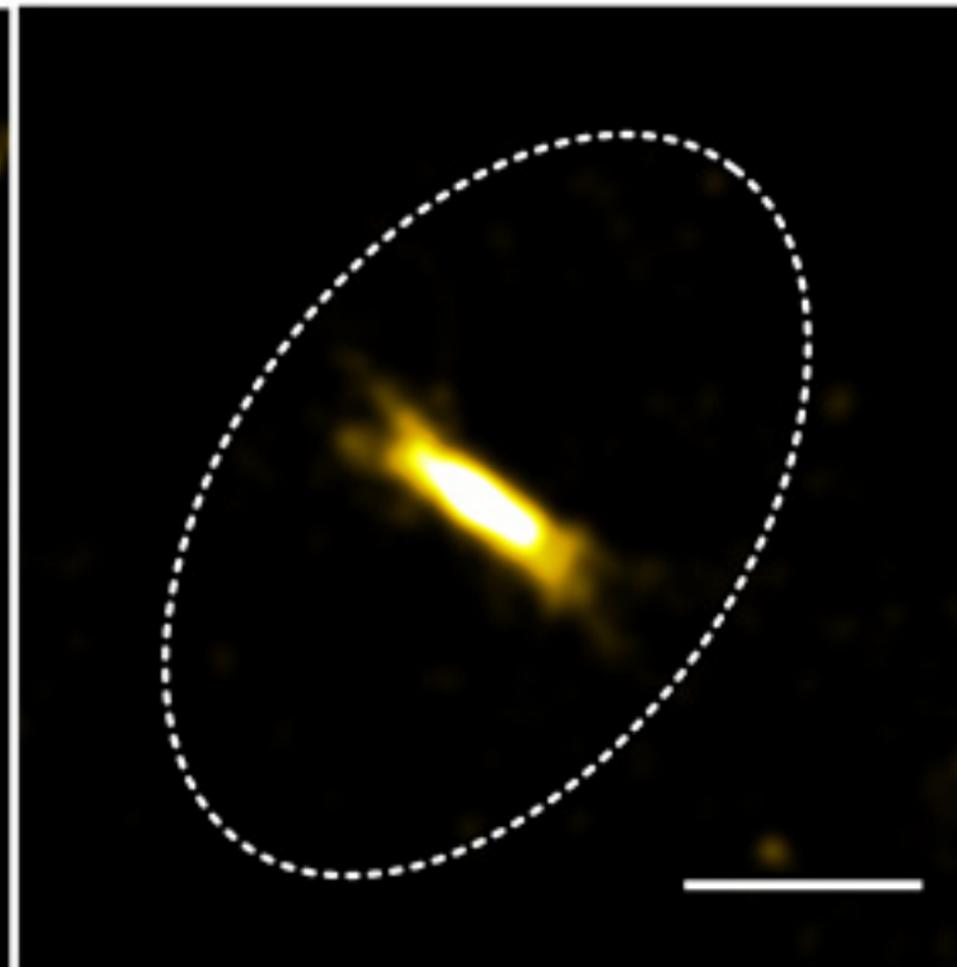
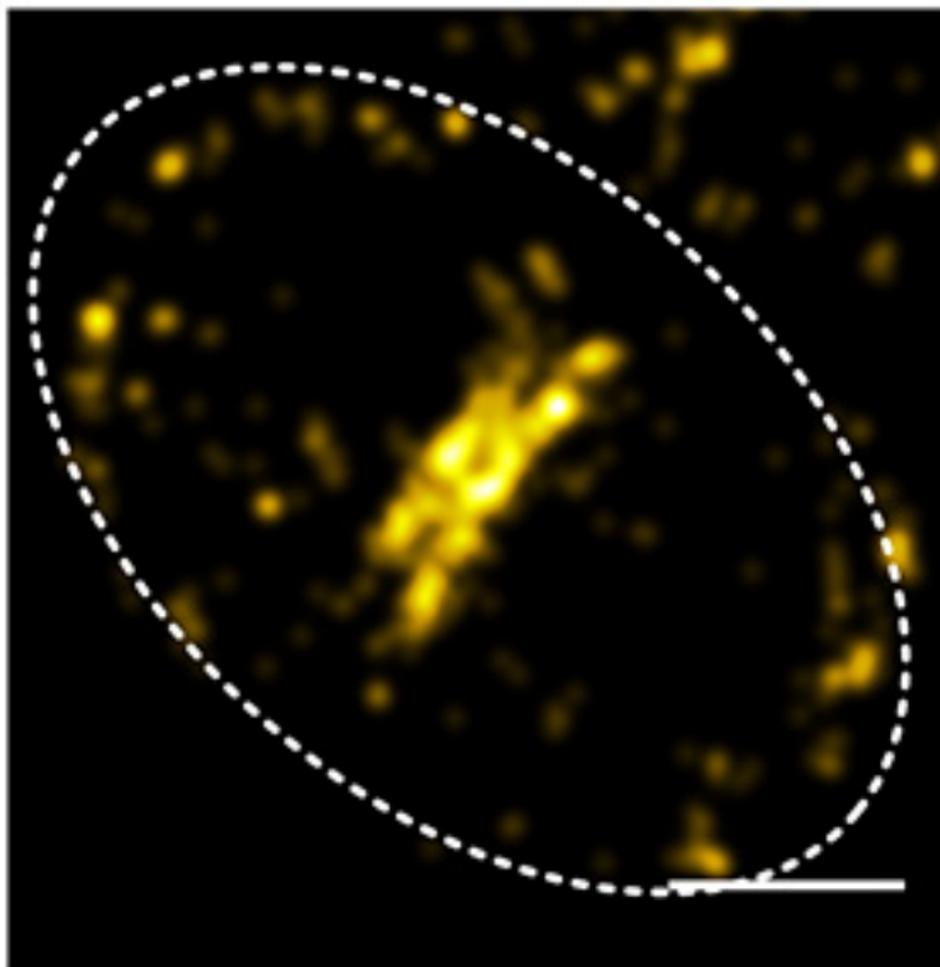
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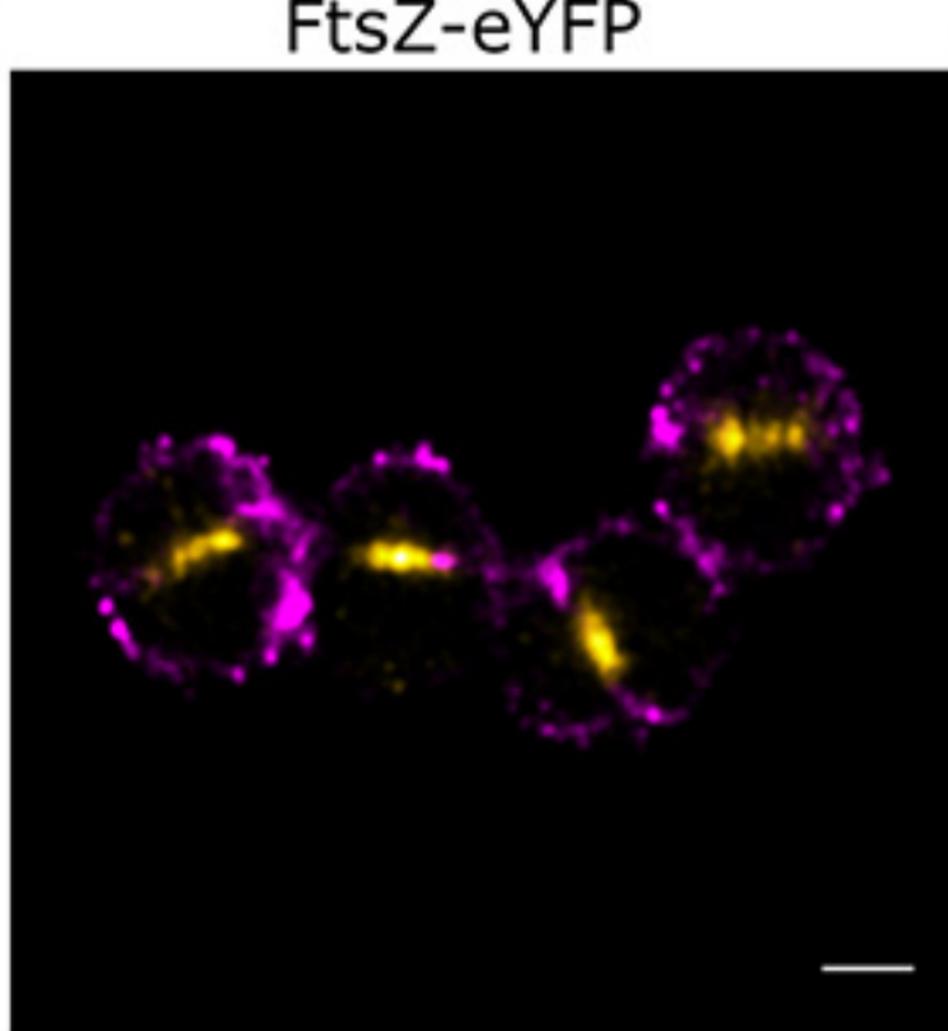
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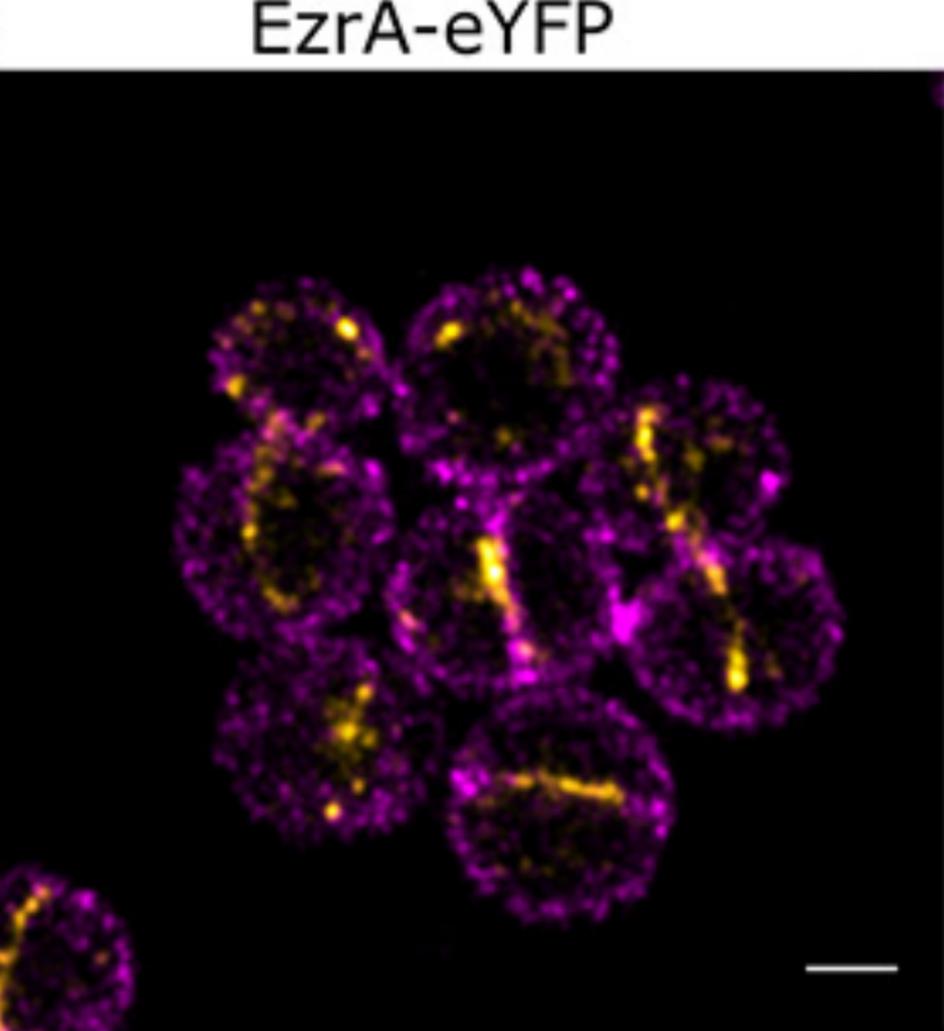
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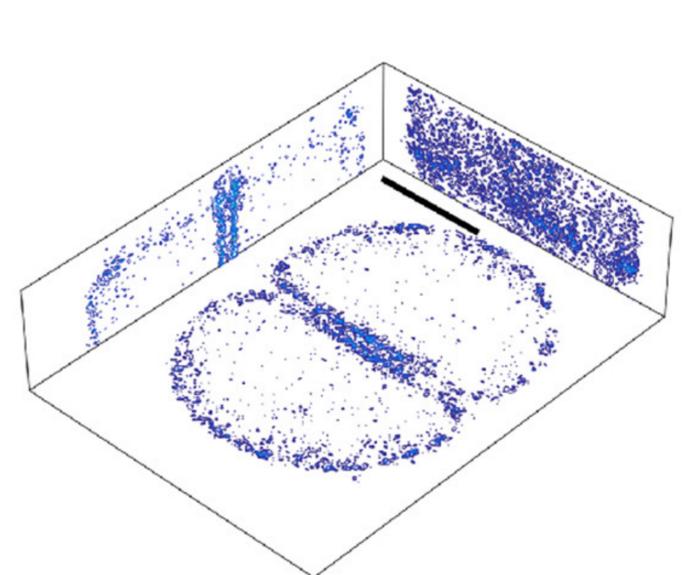
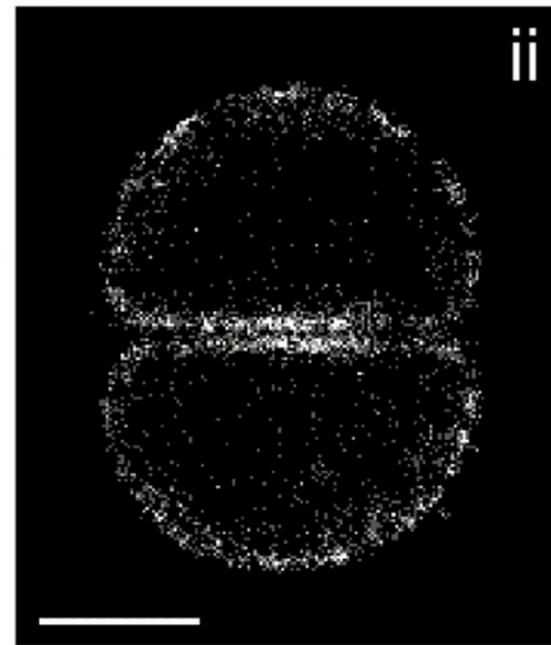
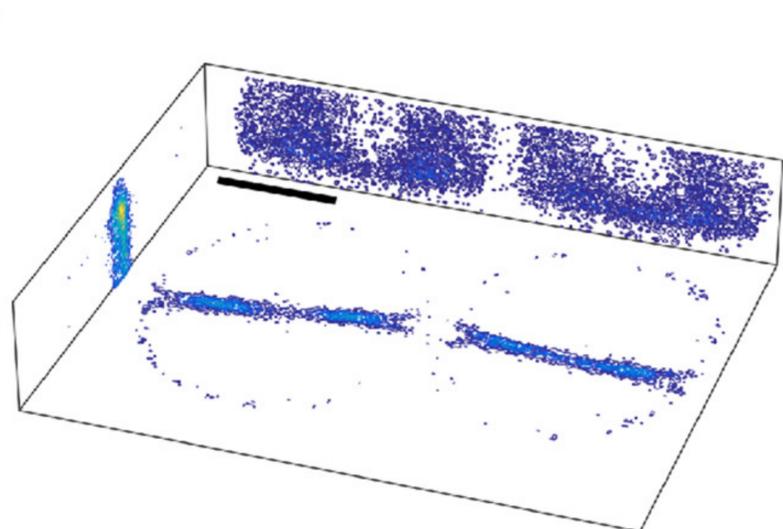
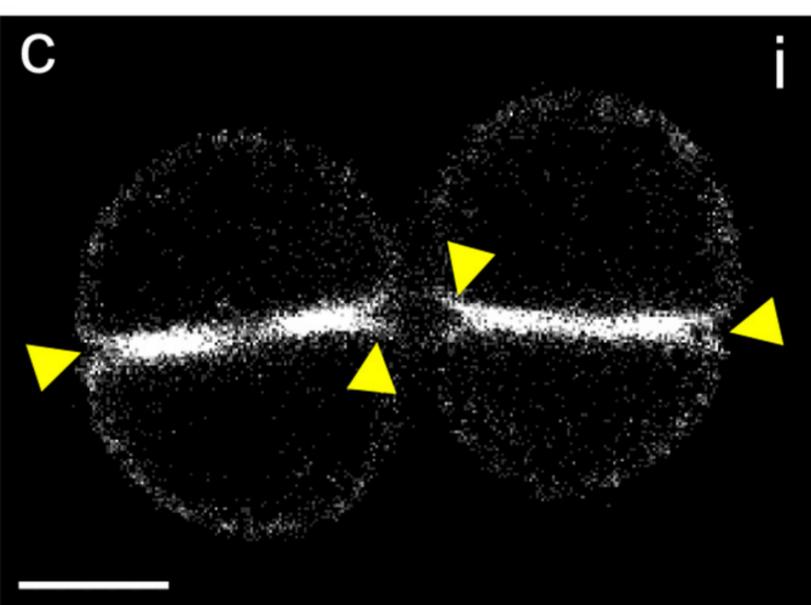
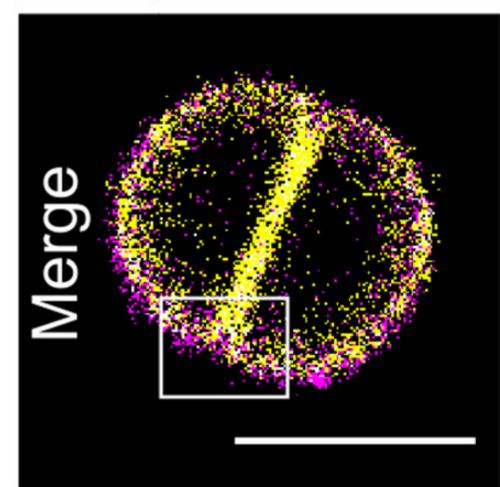
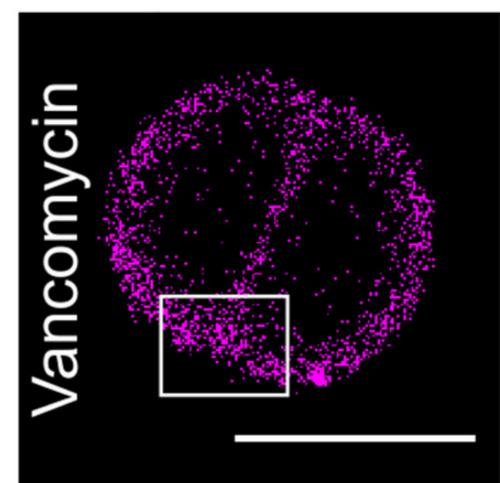
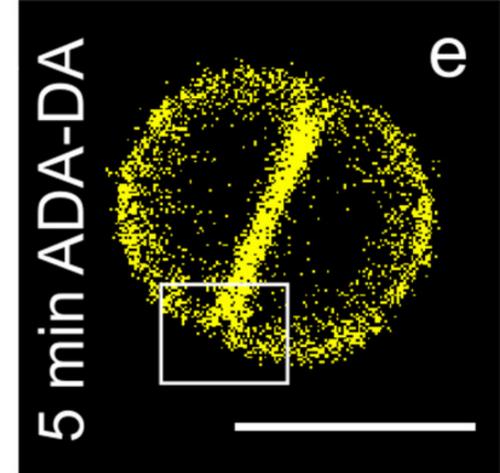
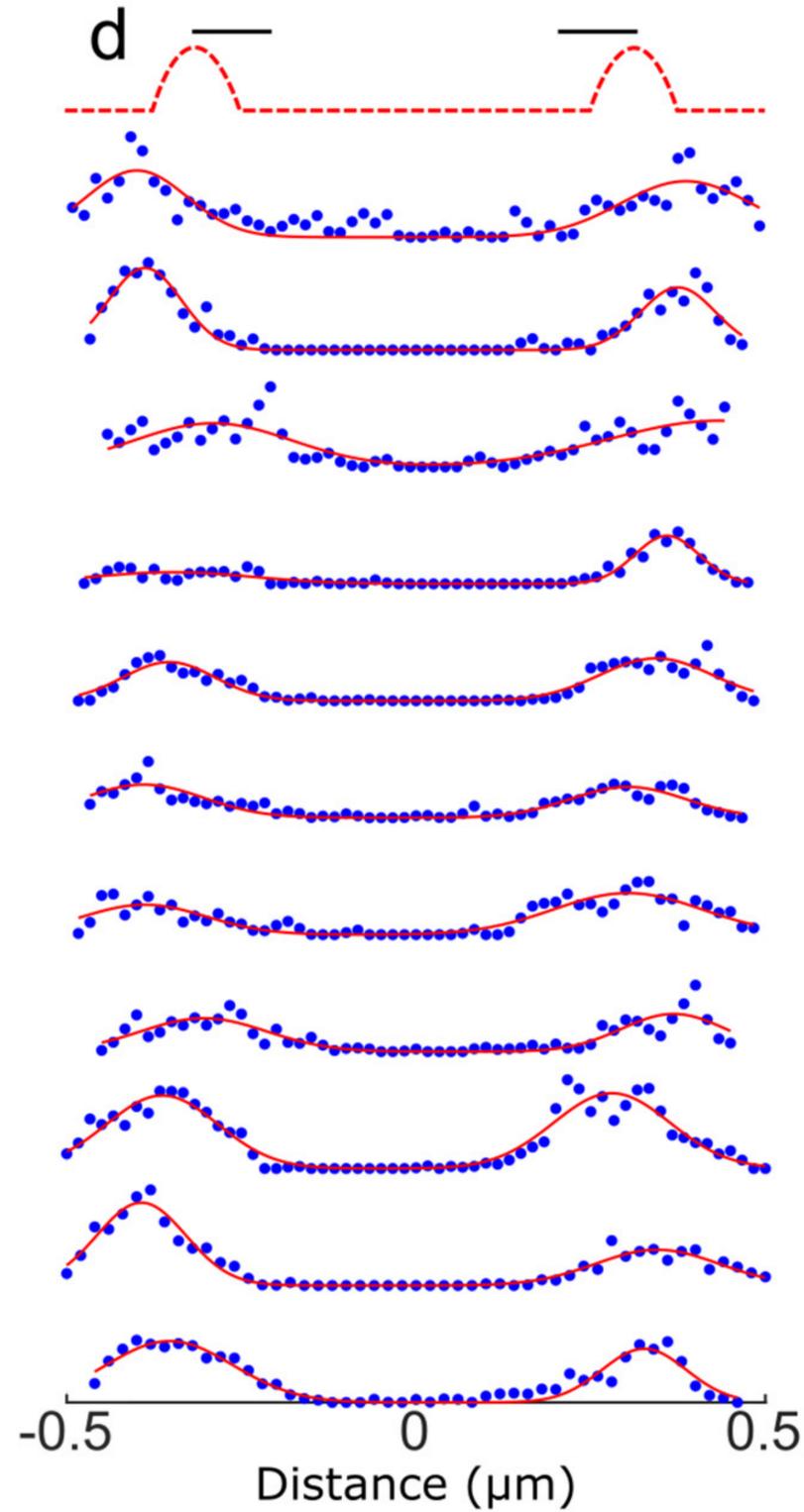
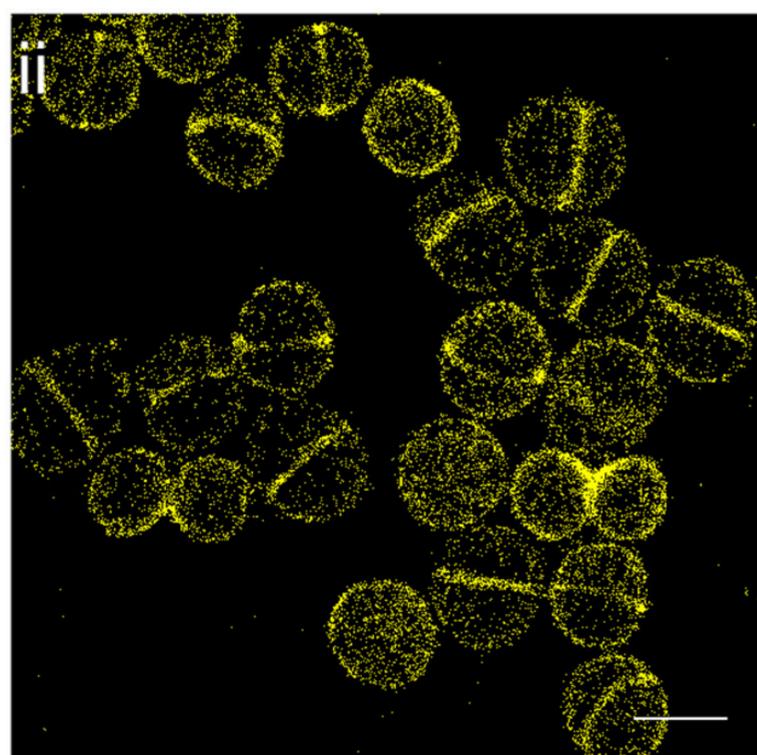
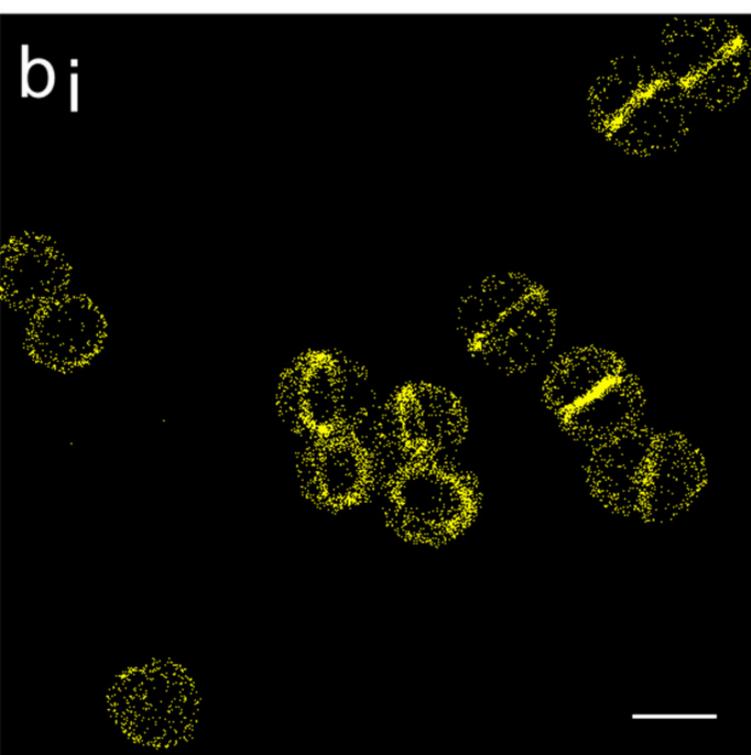
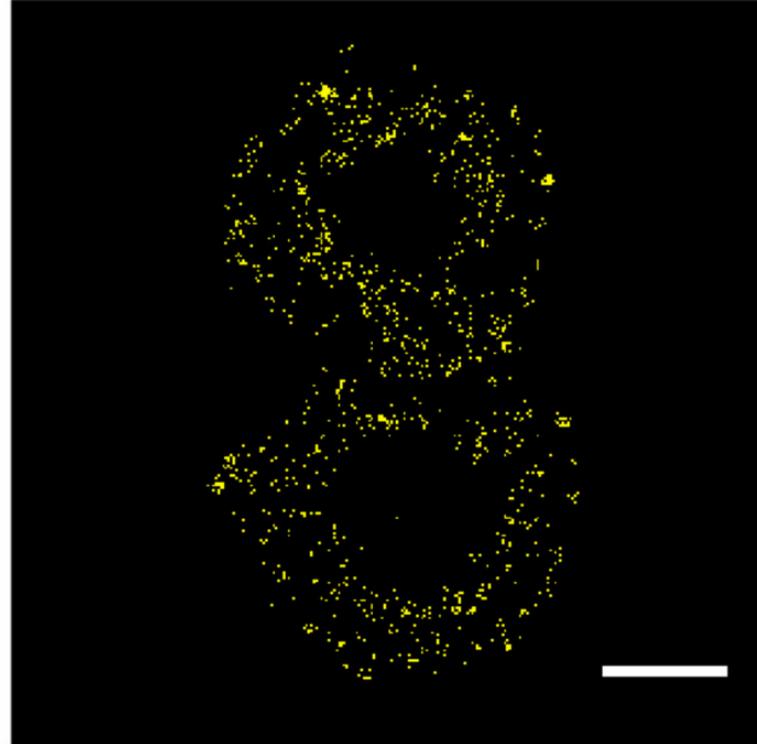
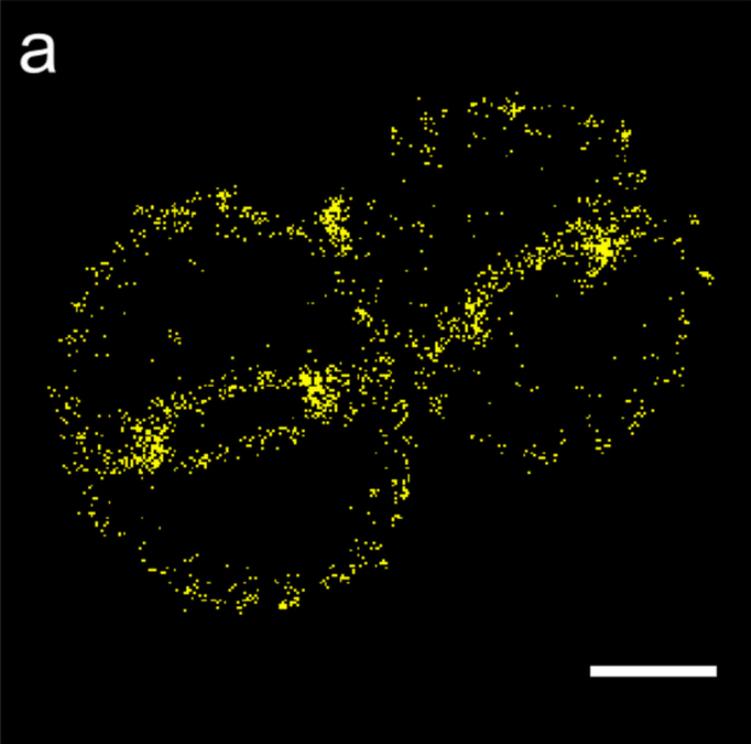


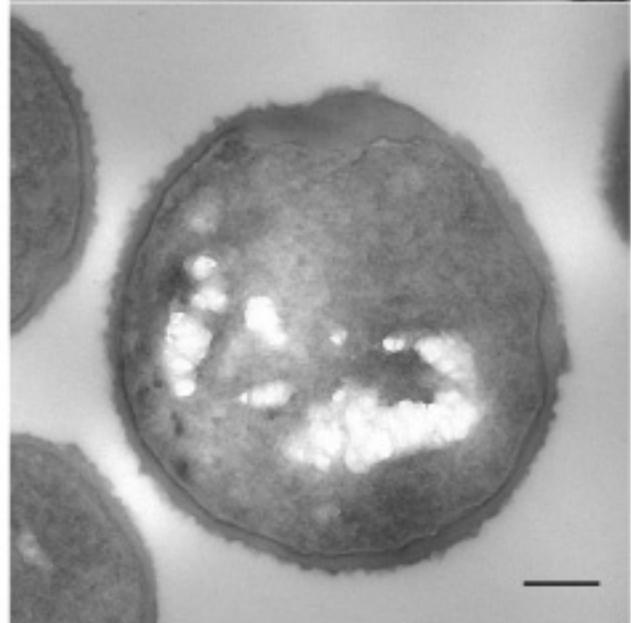
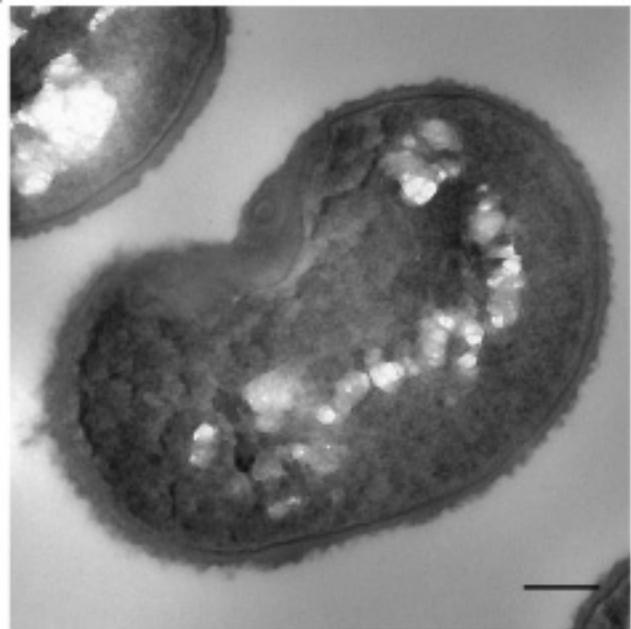
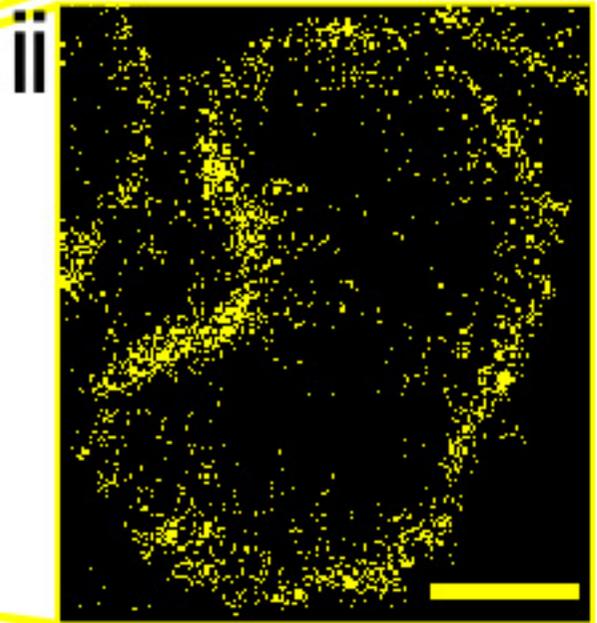
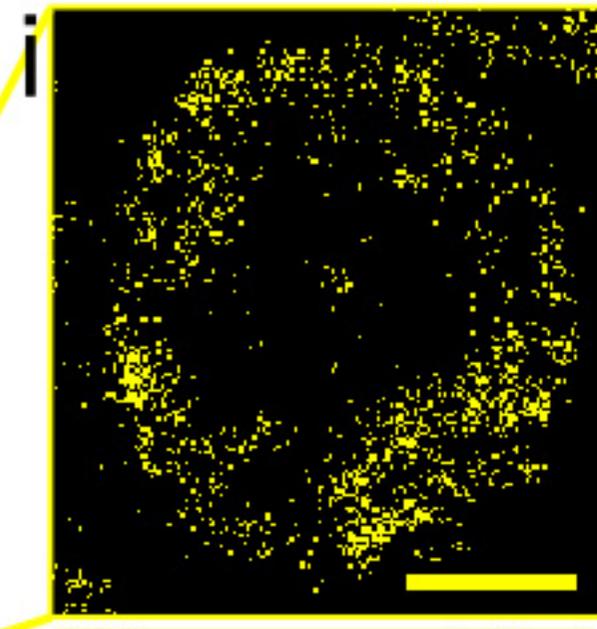
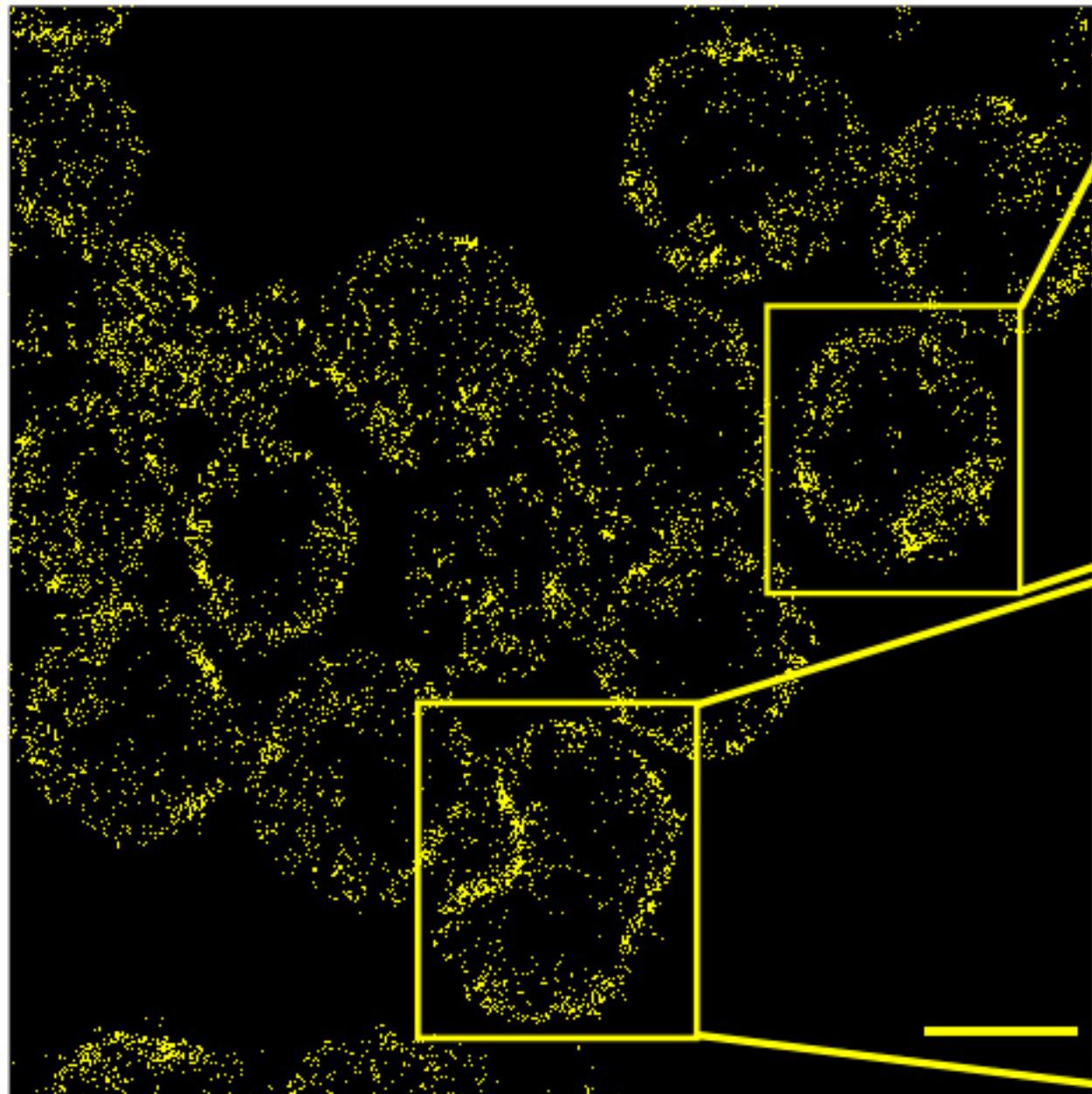
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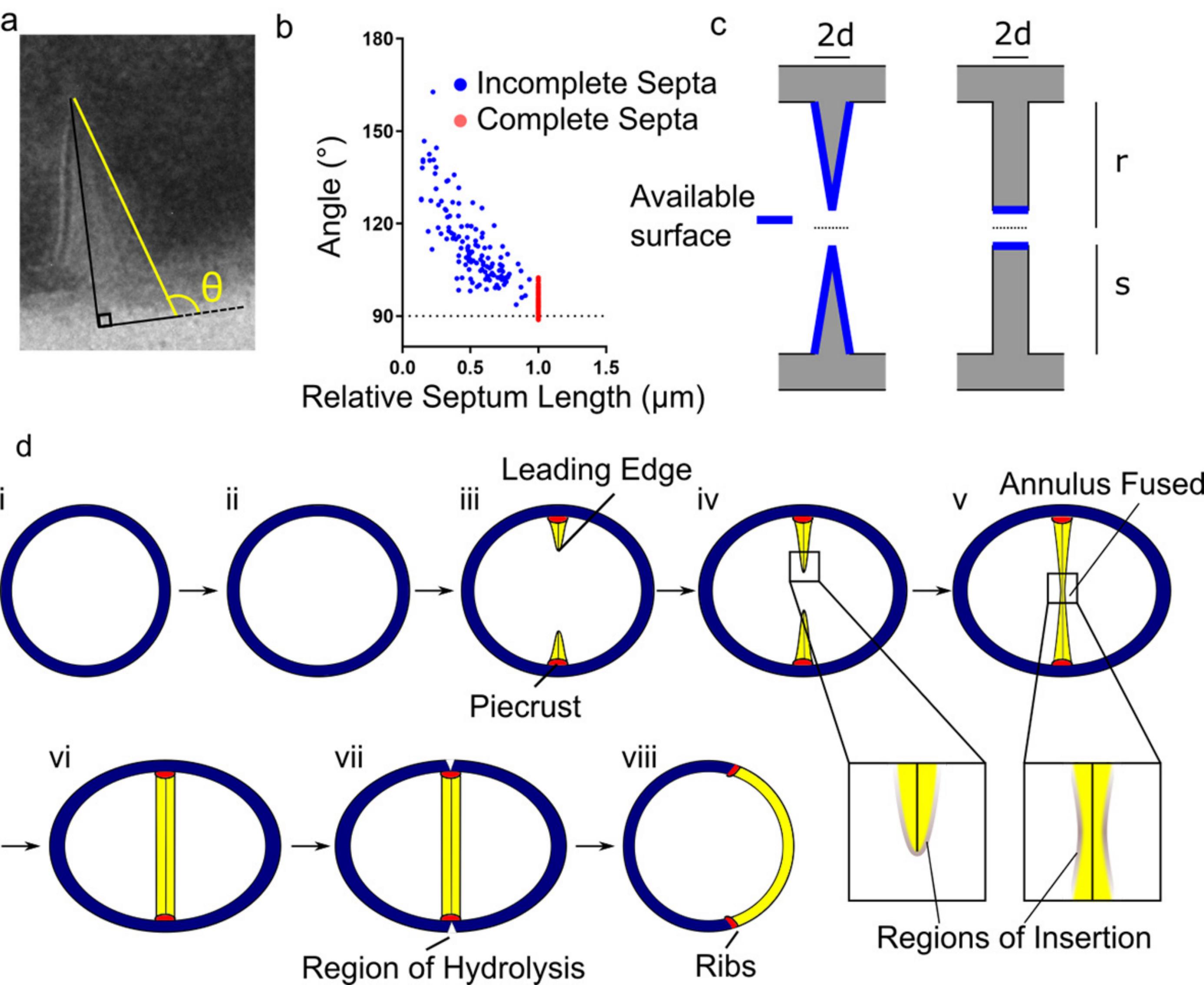


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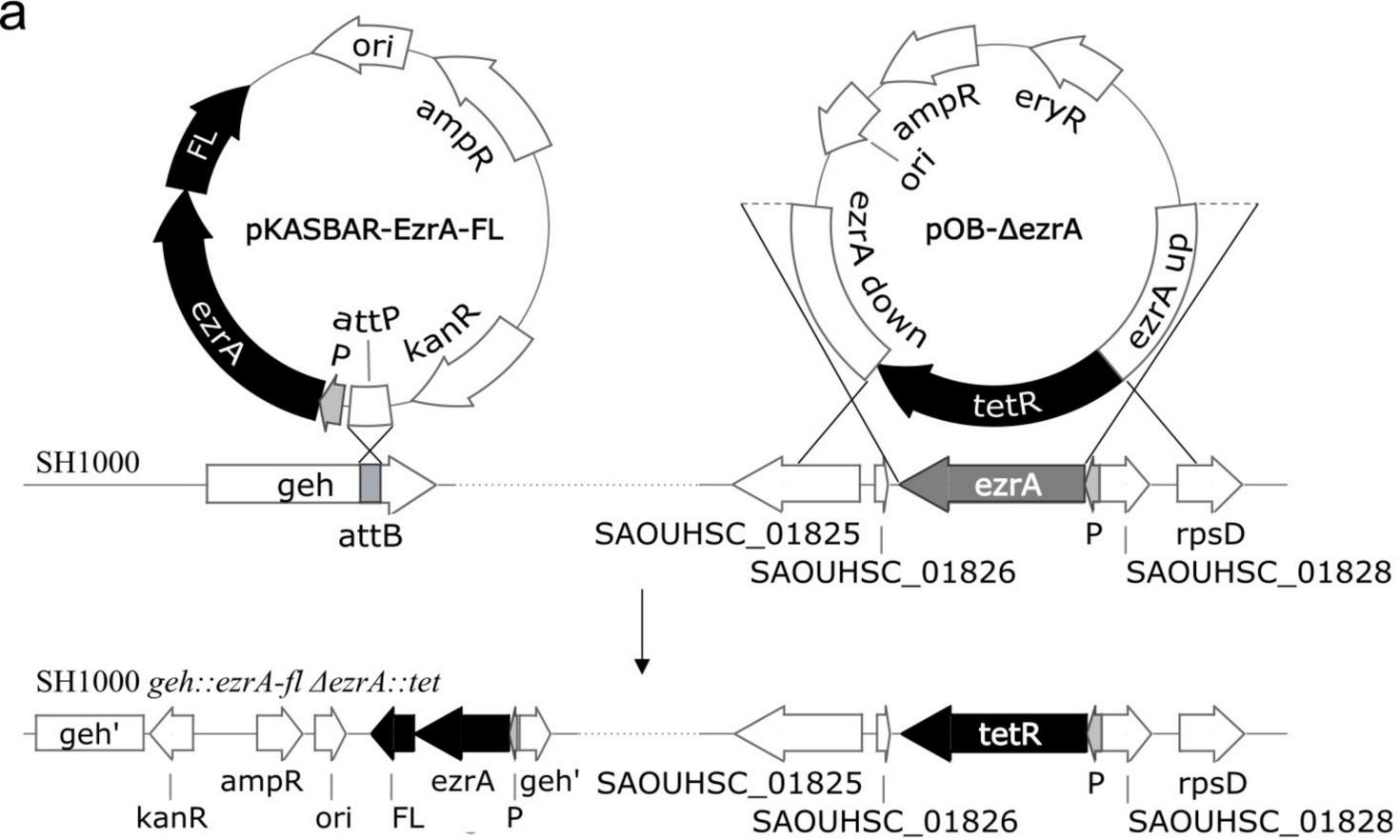




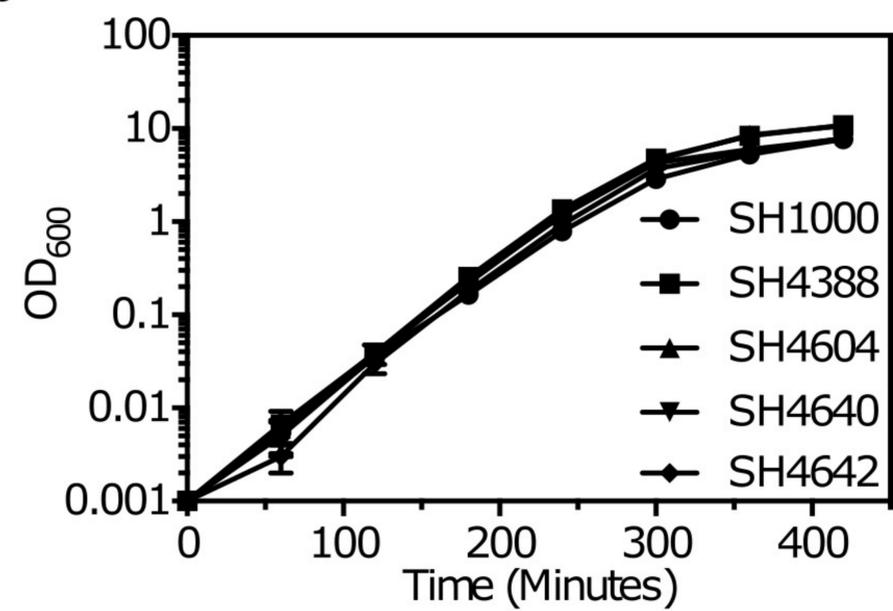
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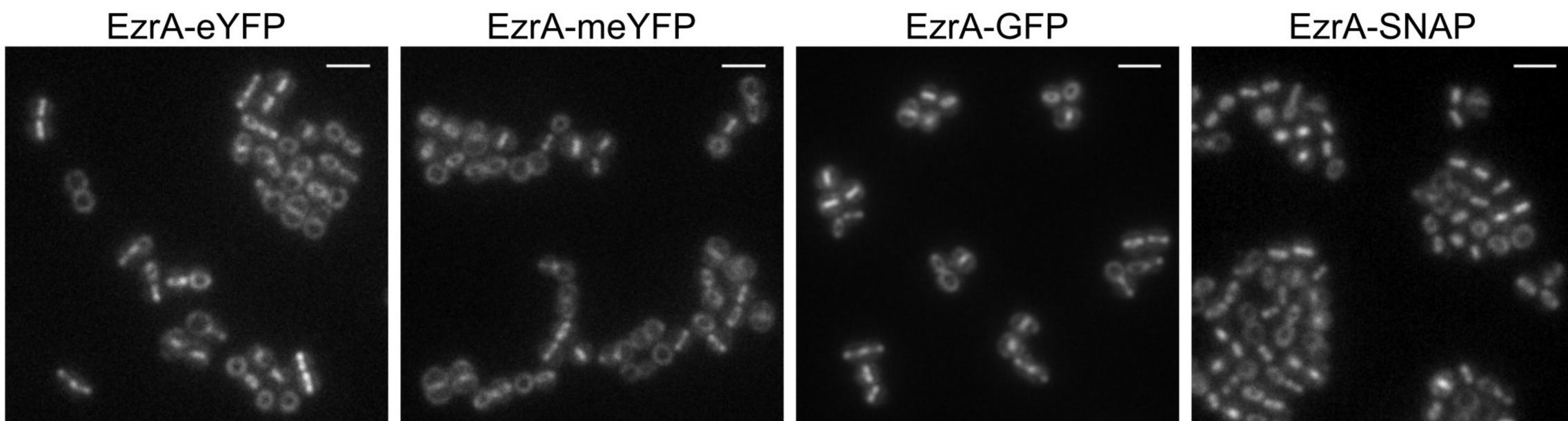
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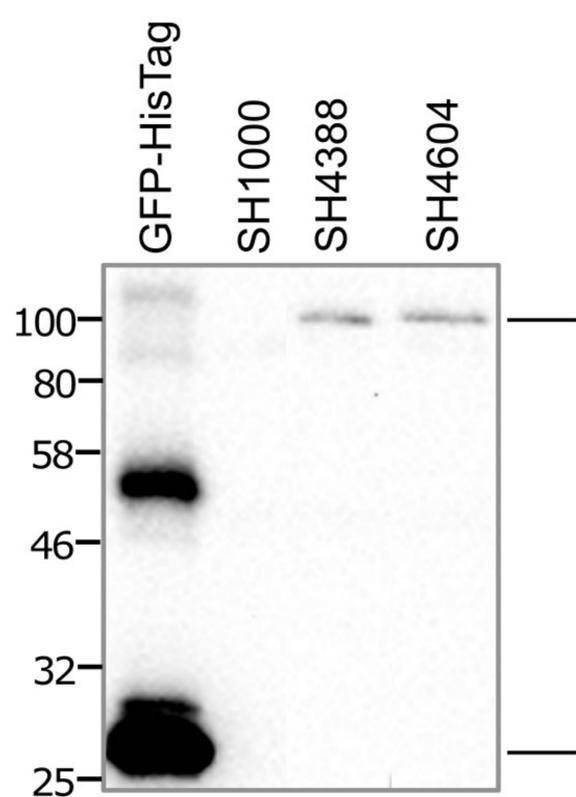
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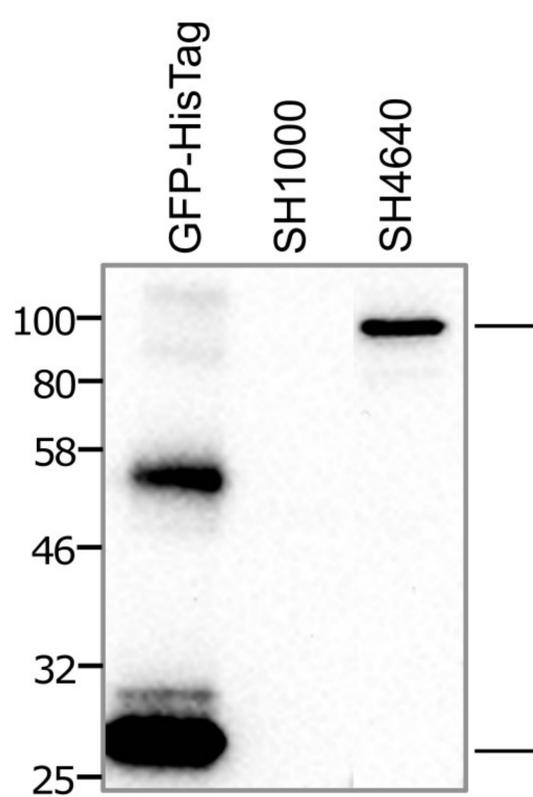
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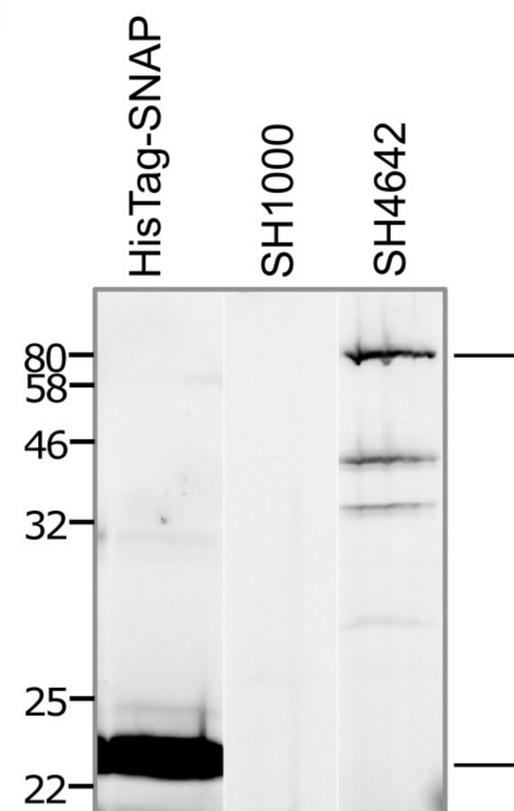
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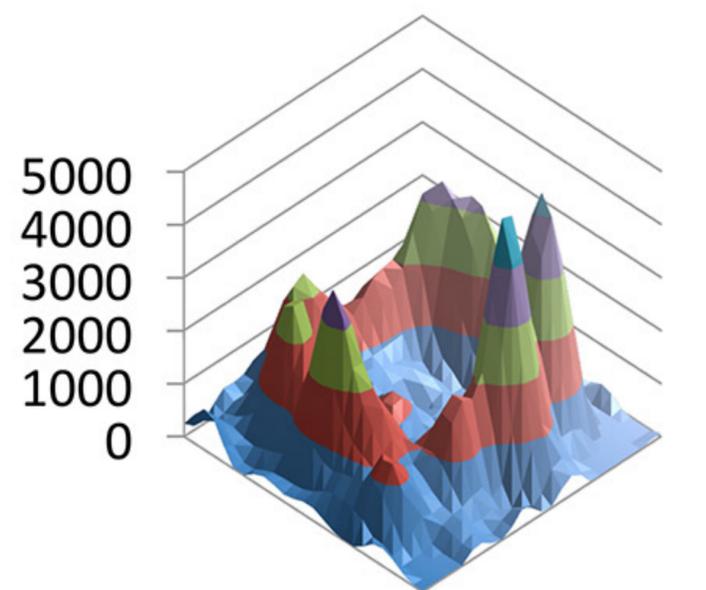
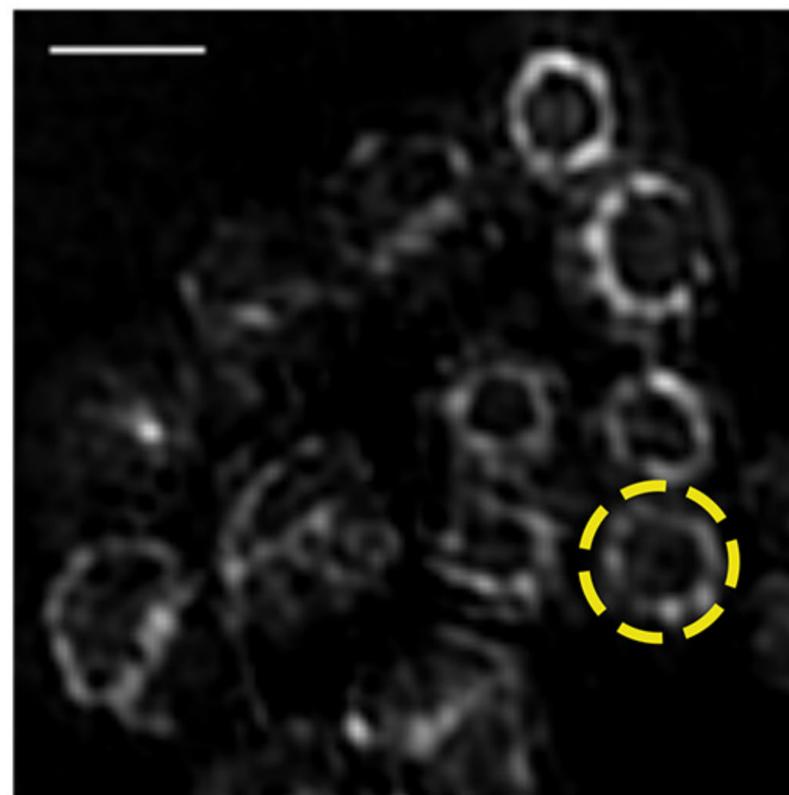


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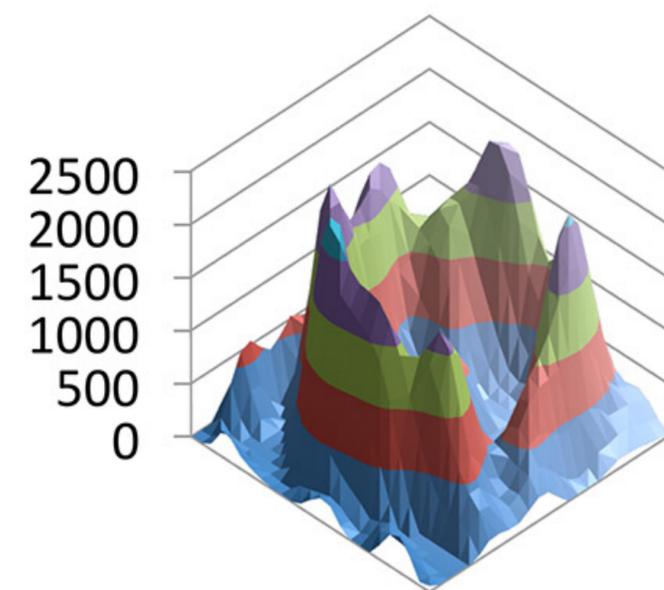
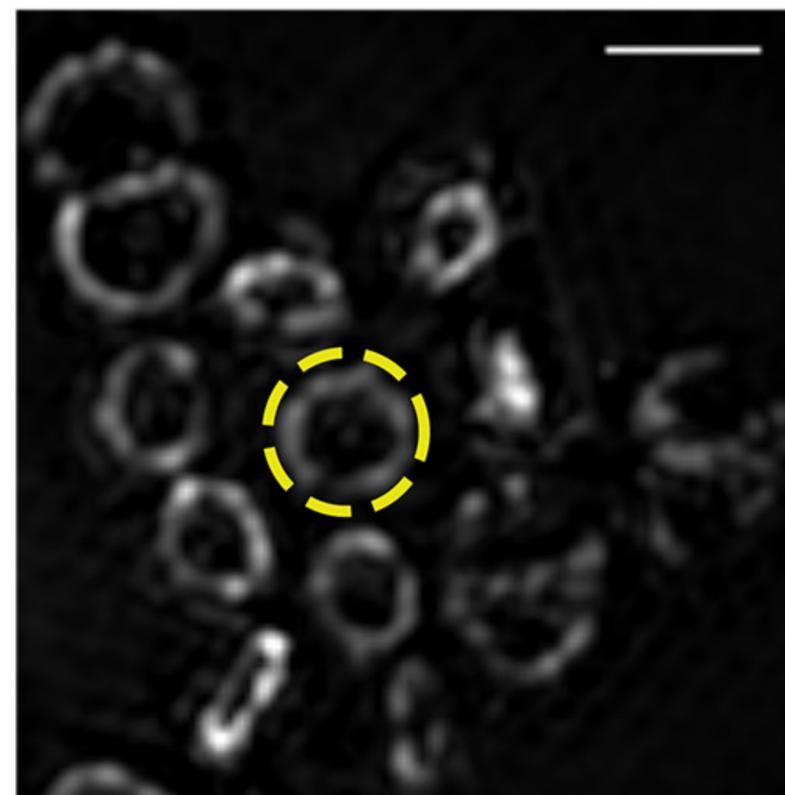


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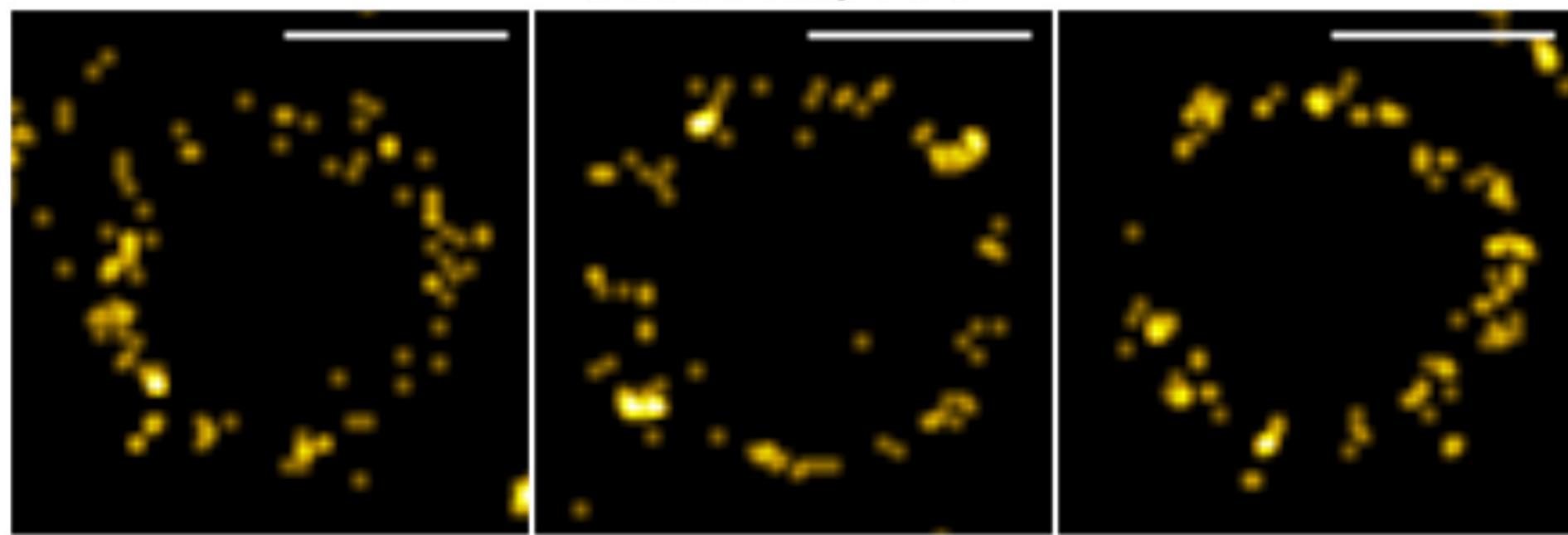


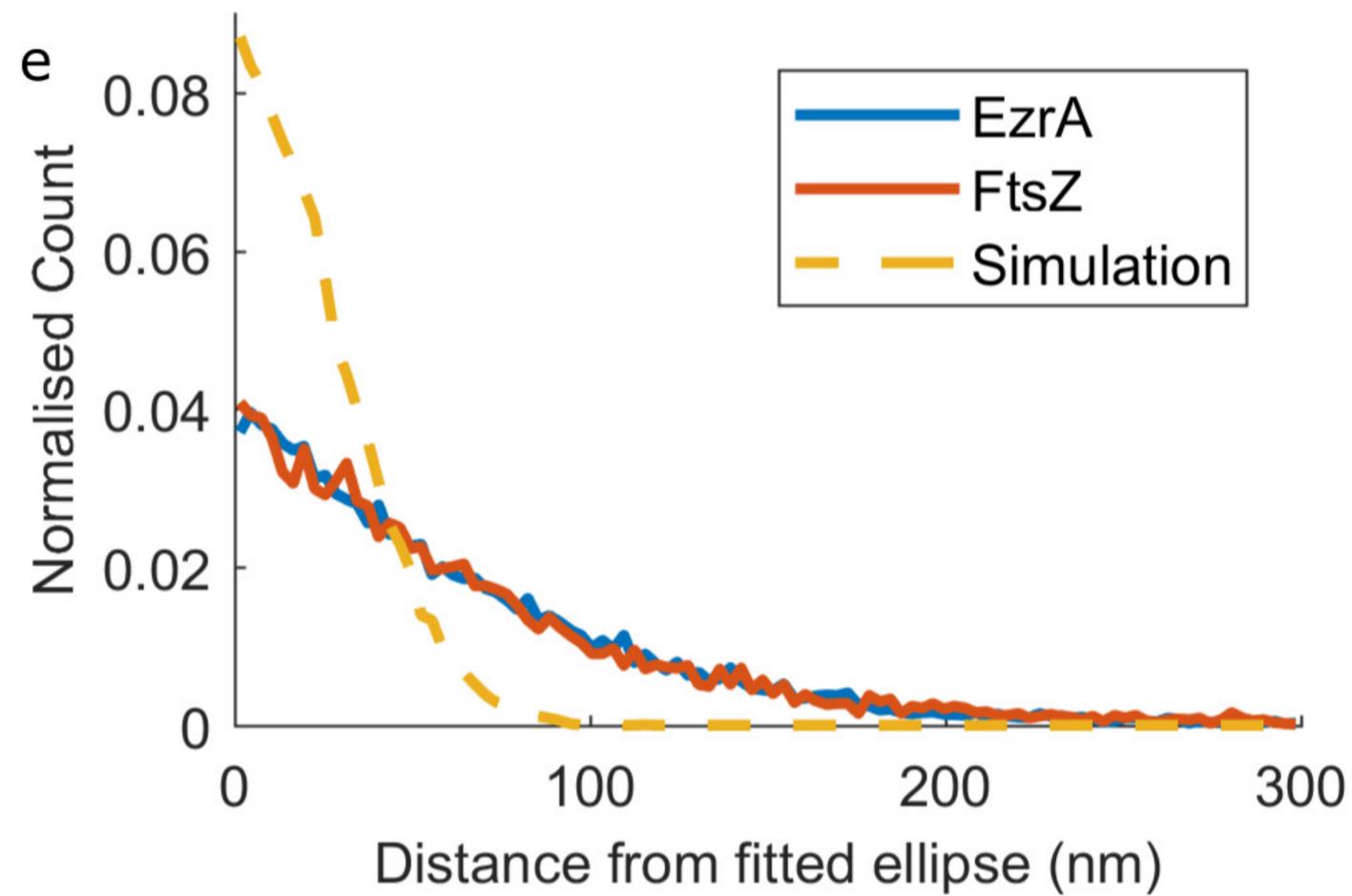
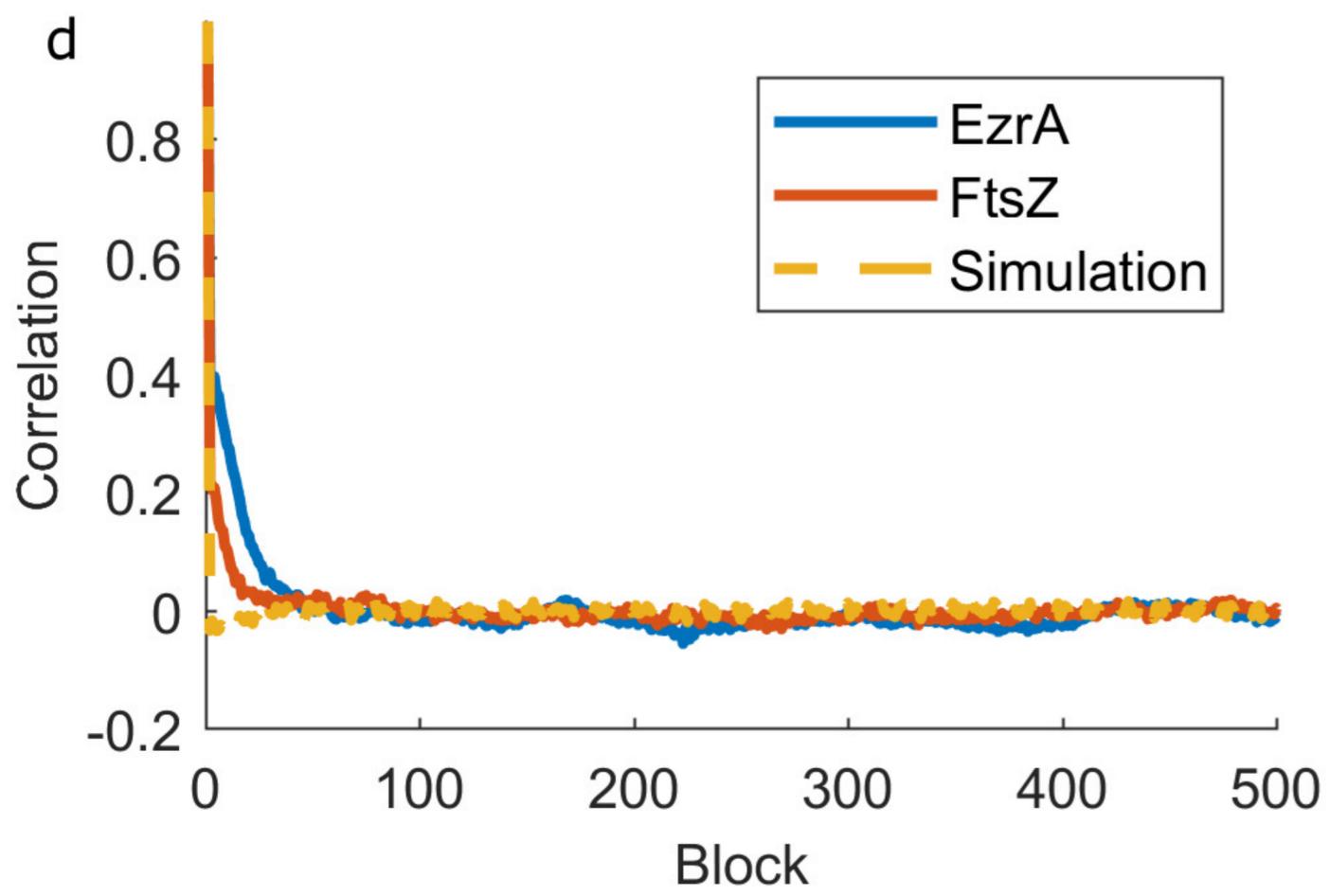
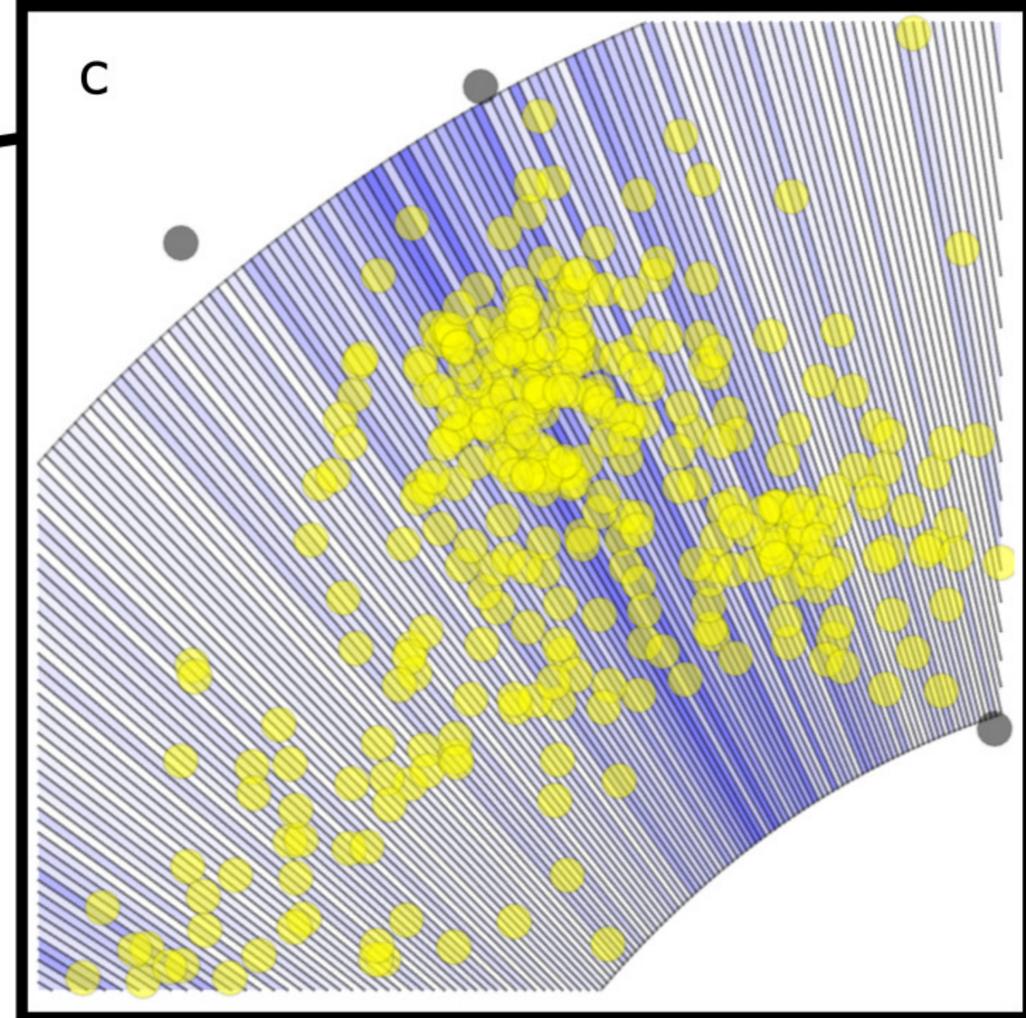
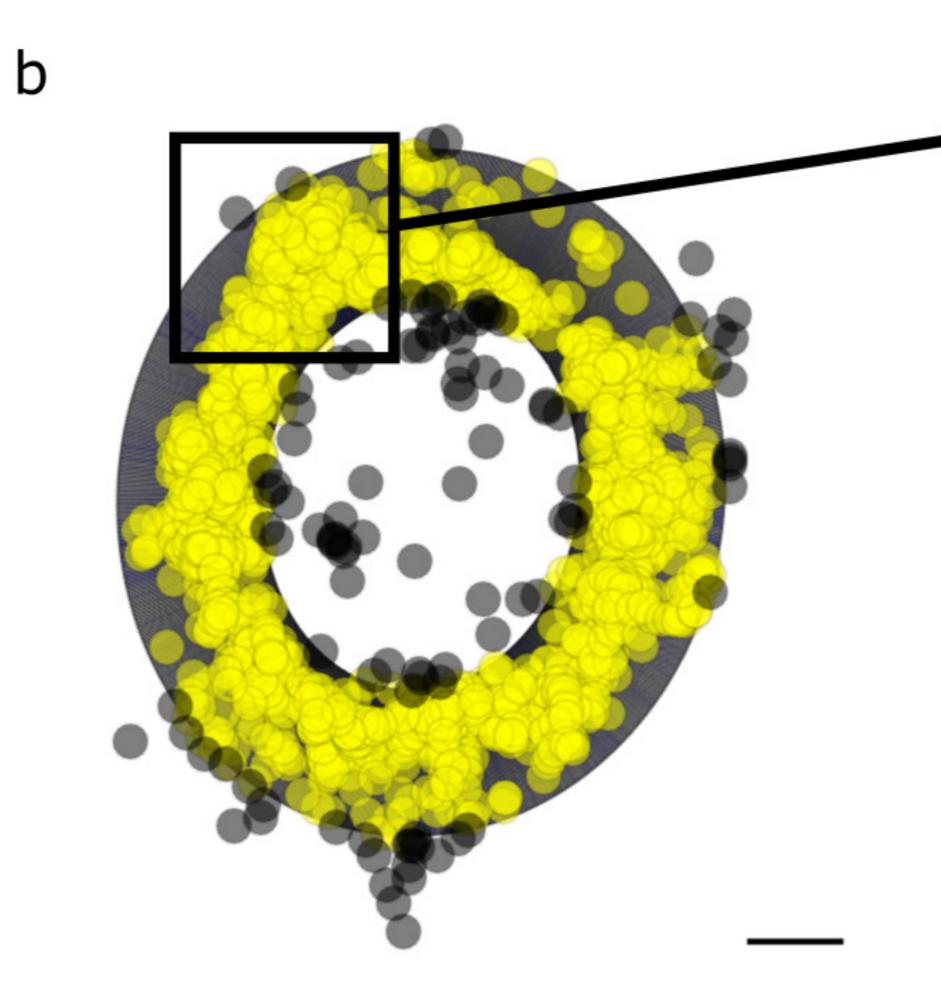
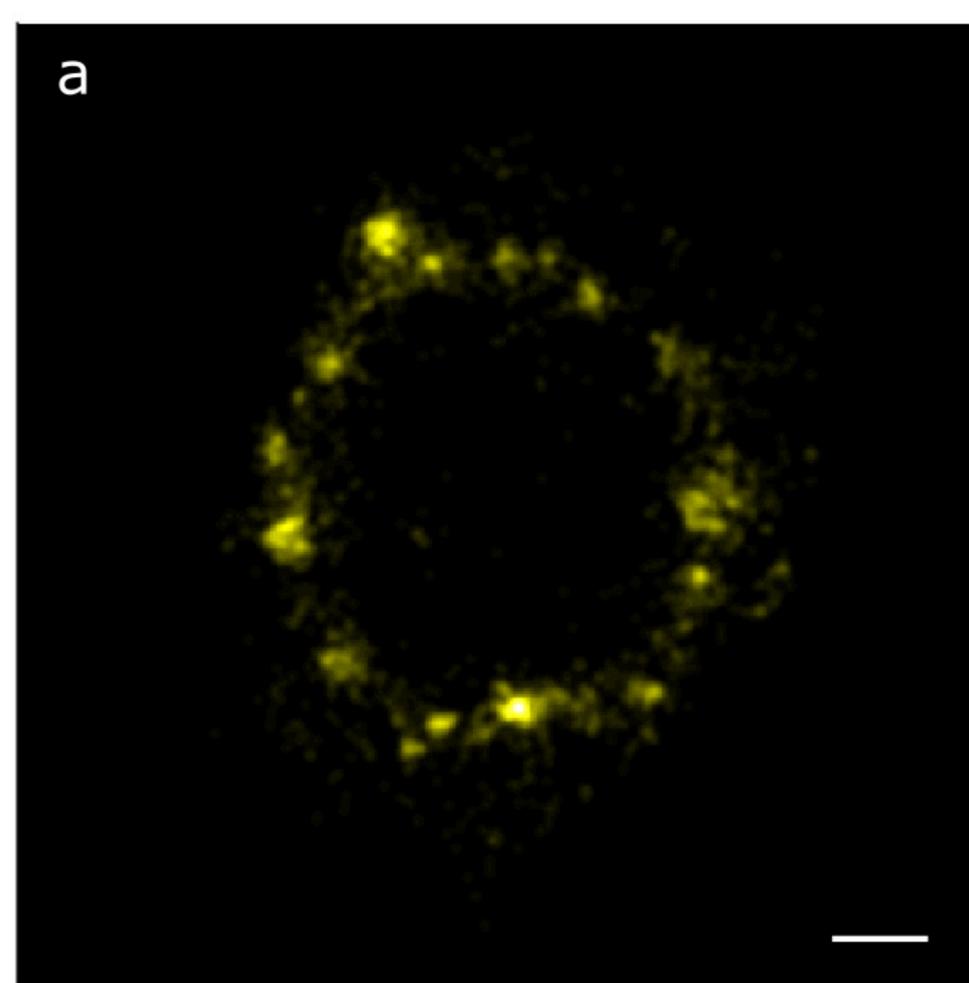
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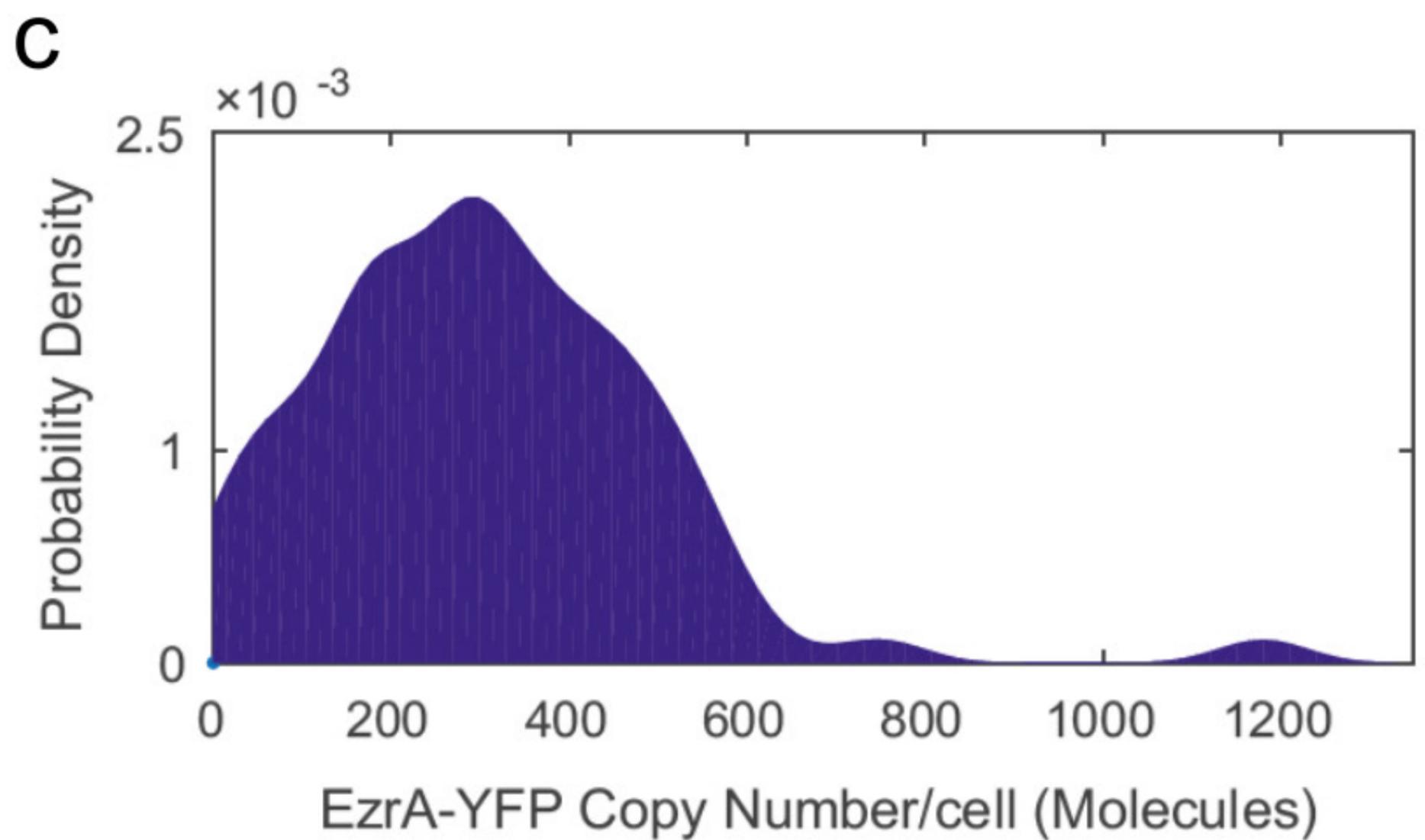
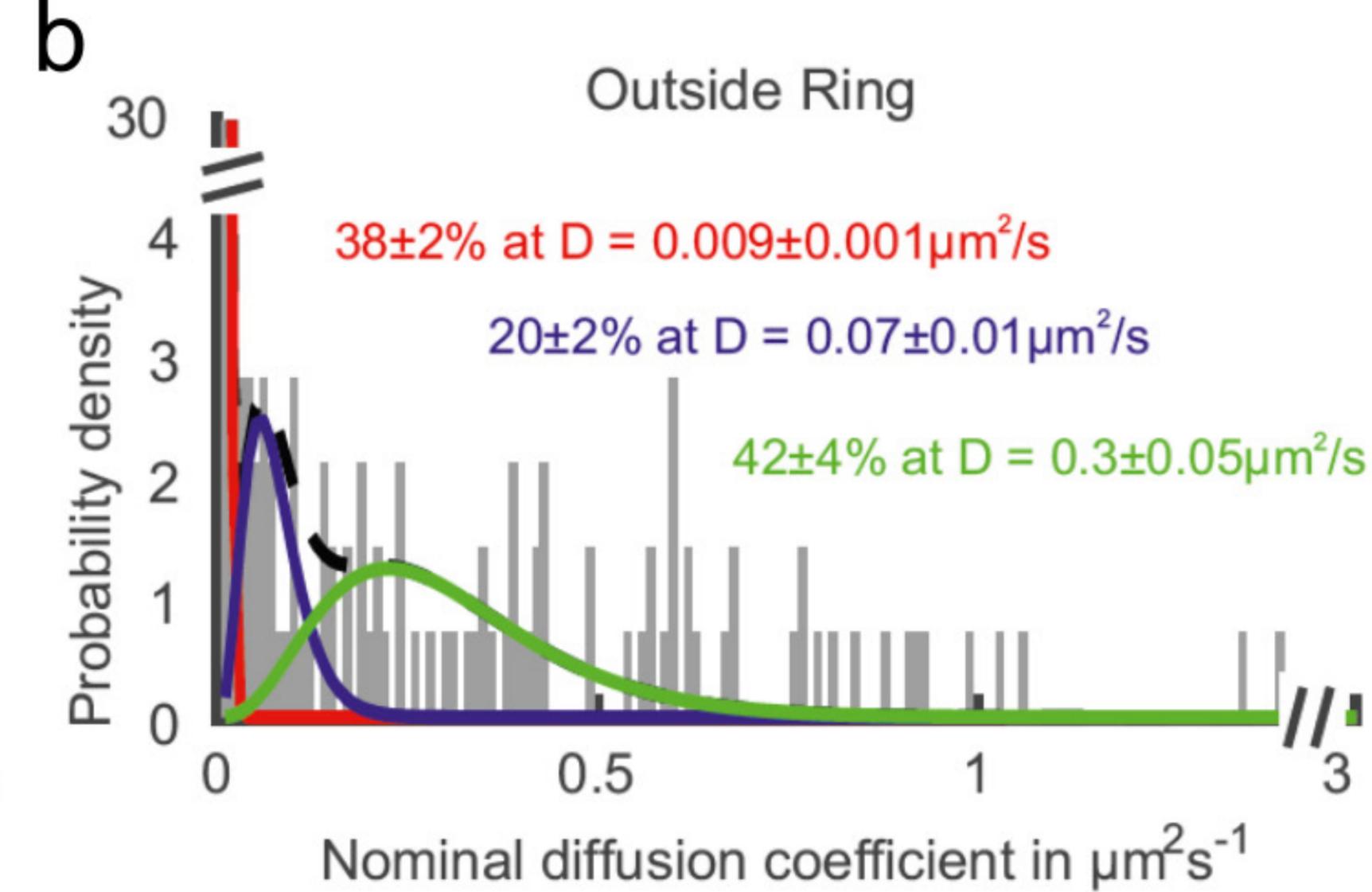
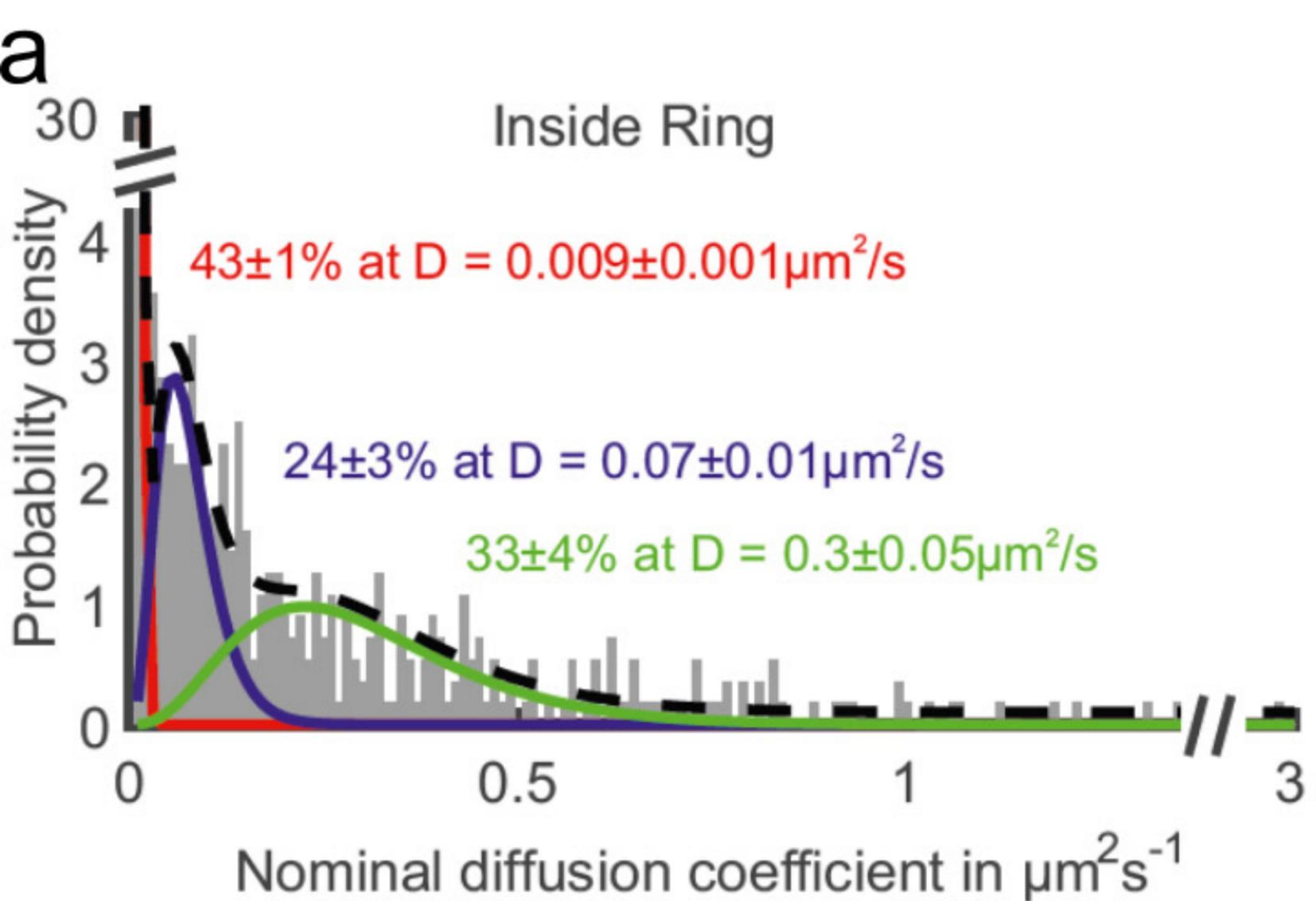


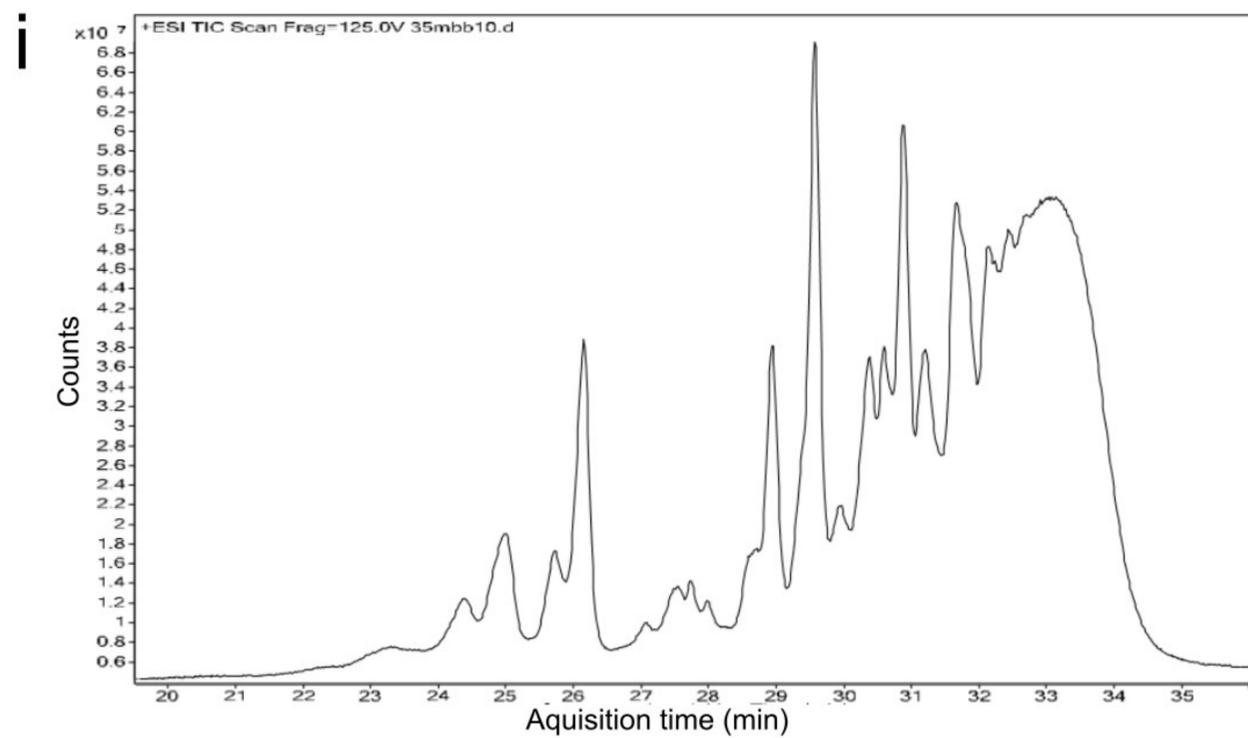
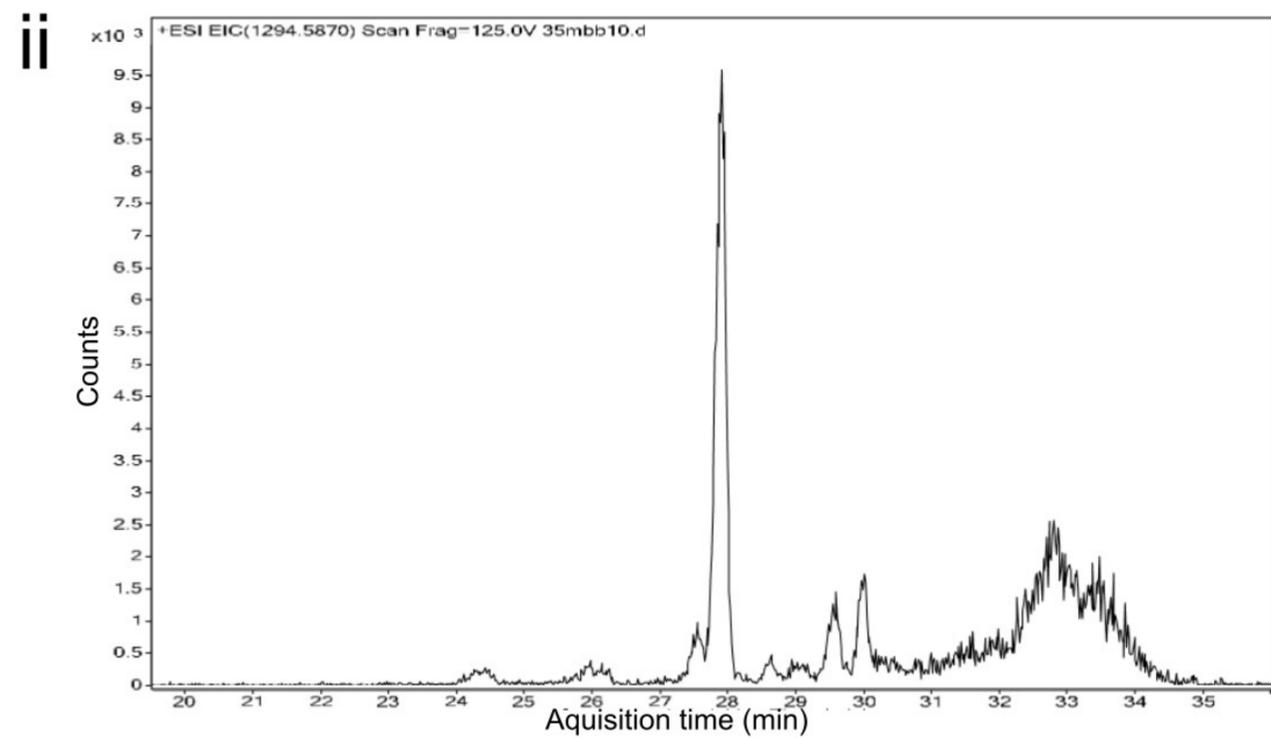
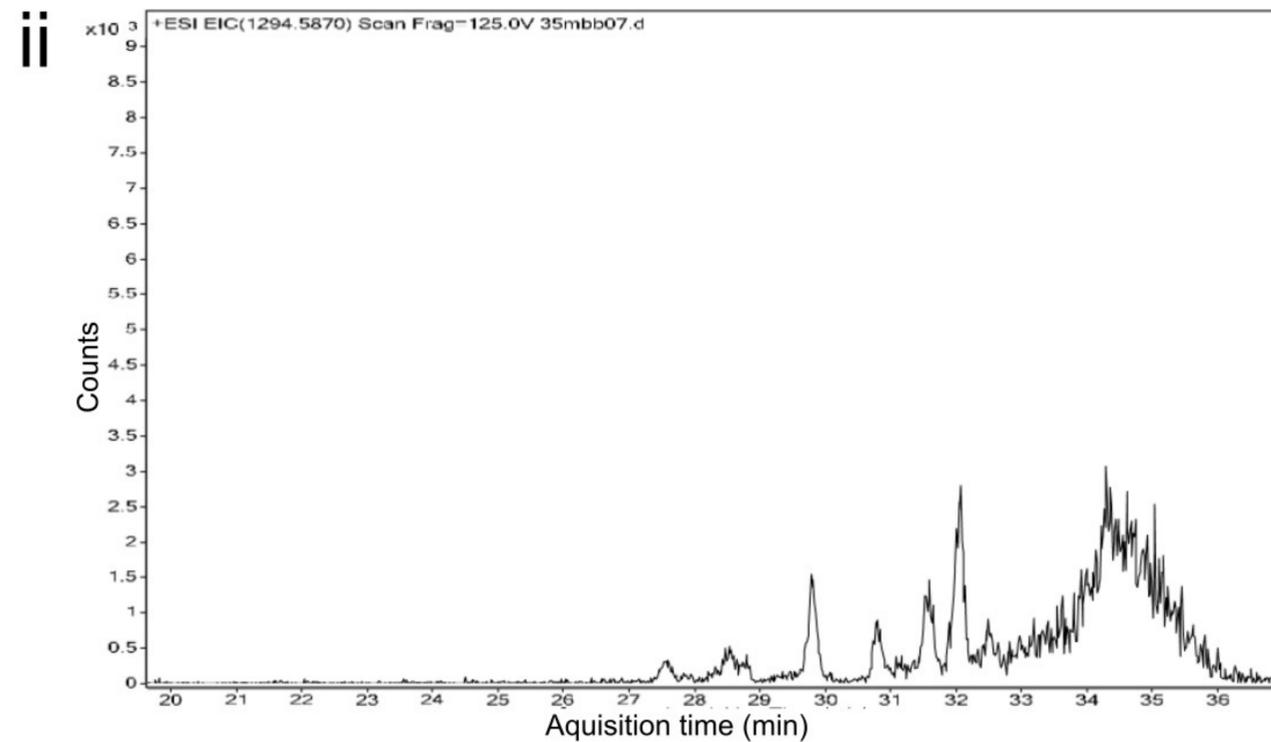
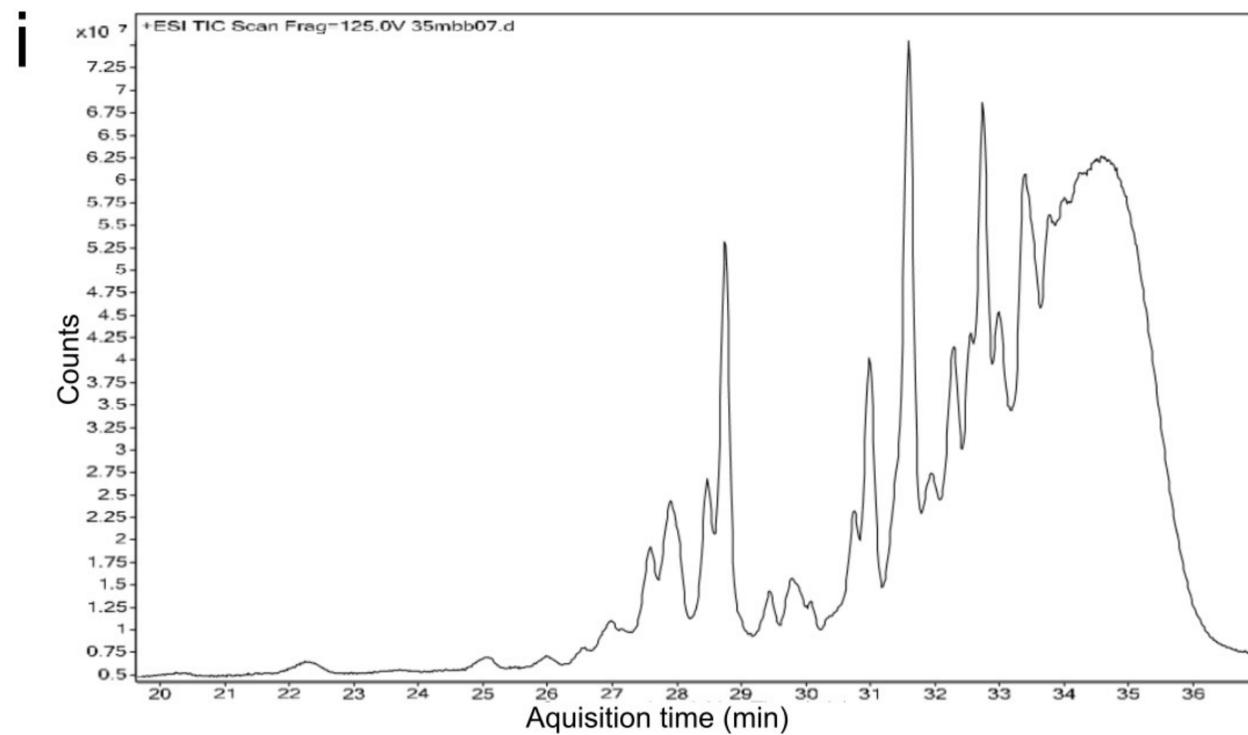
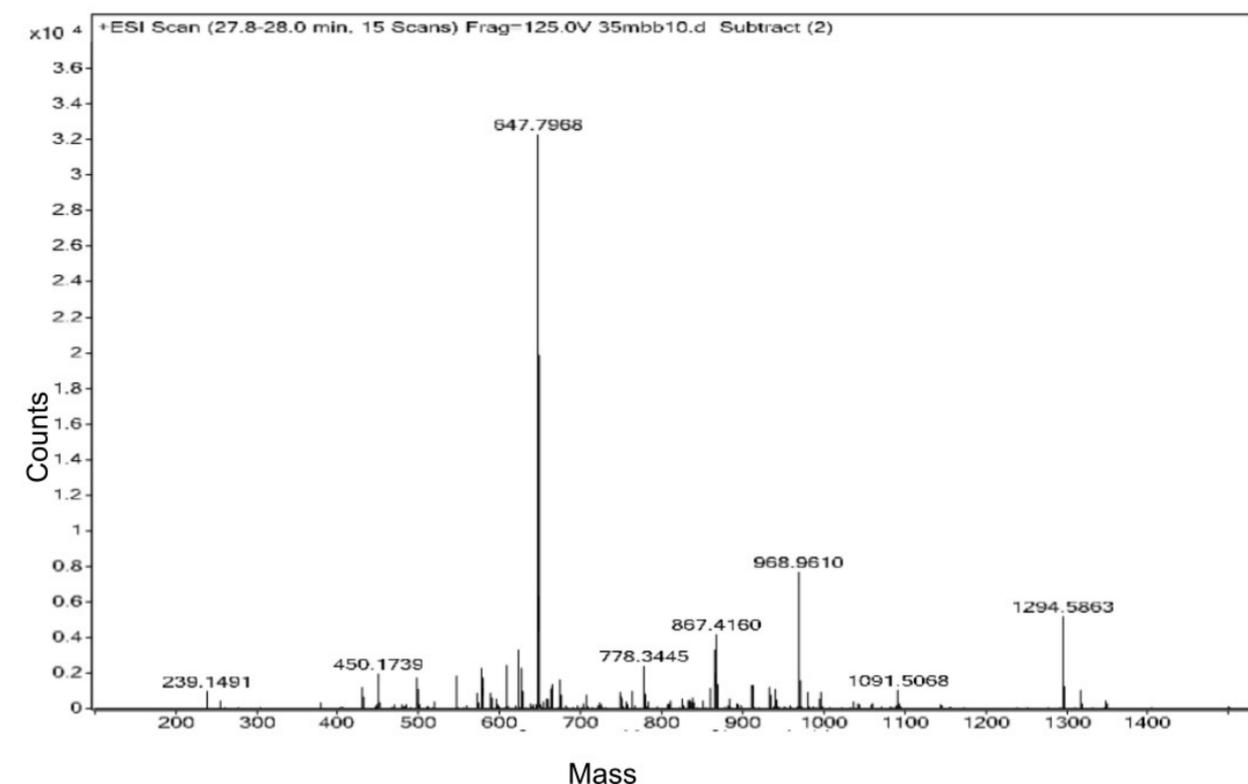
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EzrA-meYFP

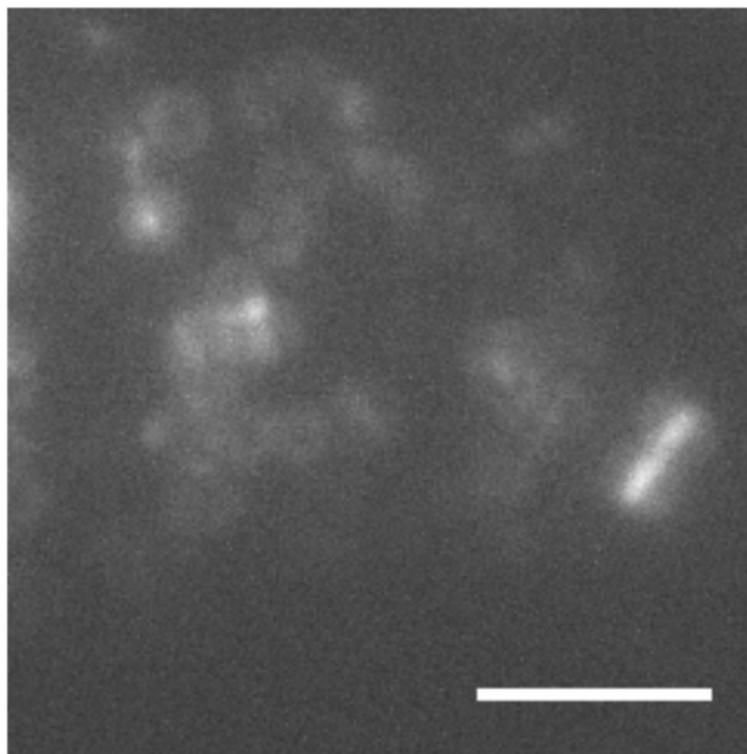




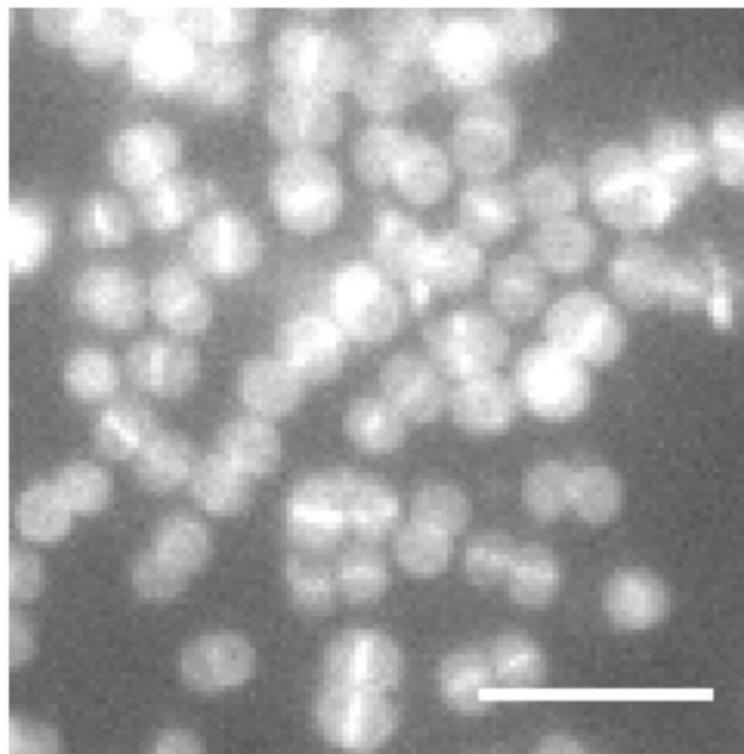


a**Total Ion Count****Extracted Ion Count****b****c**

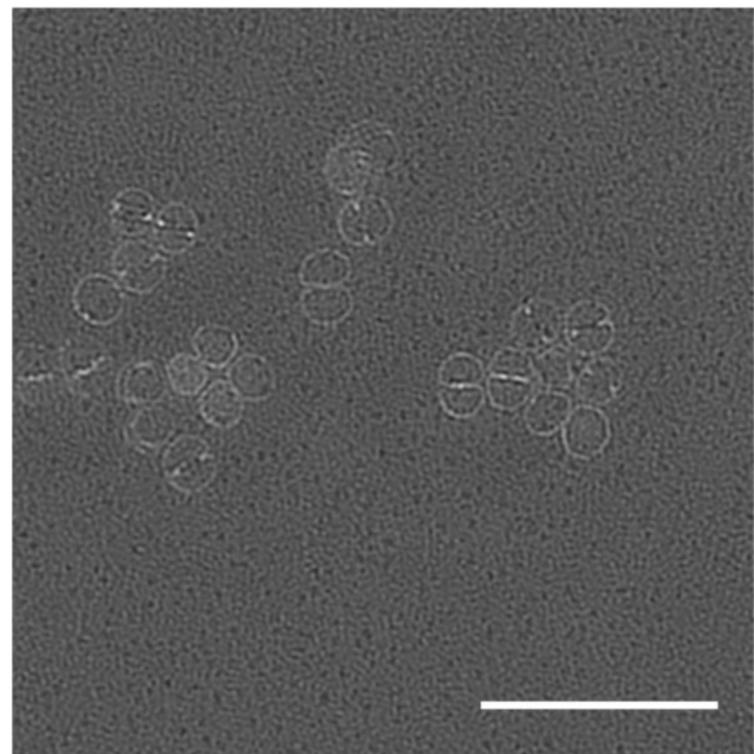
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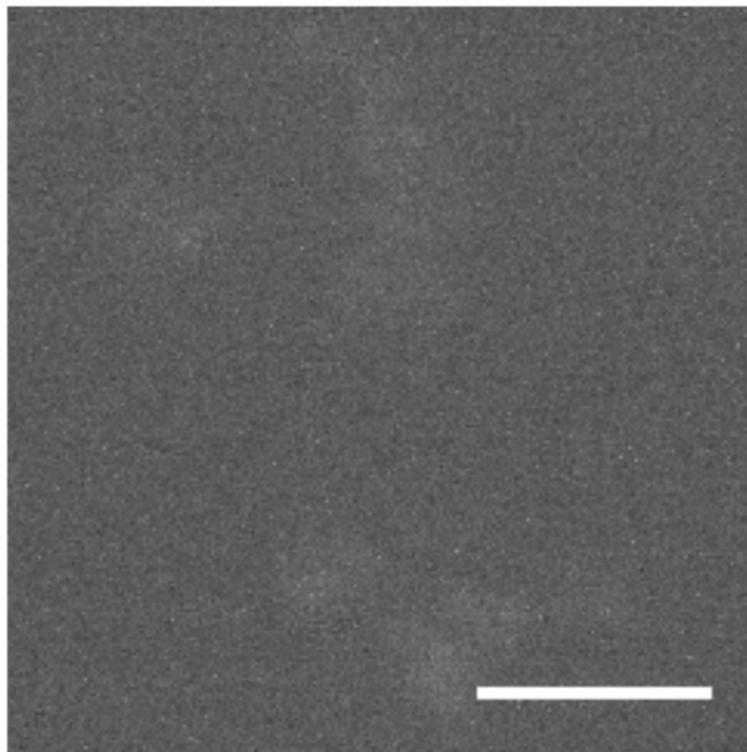
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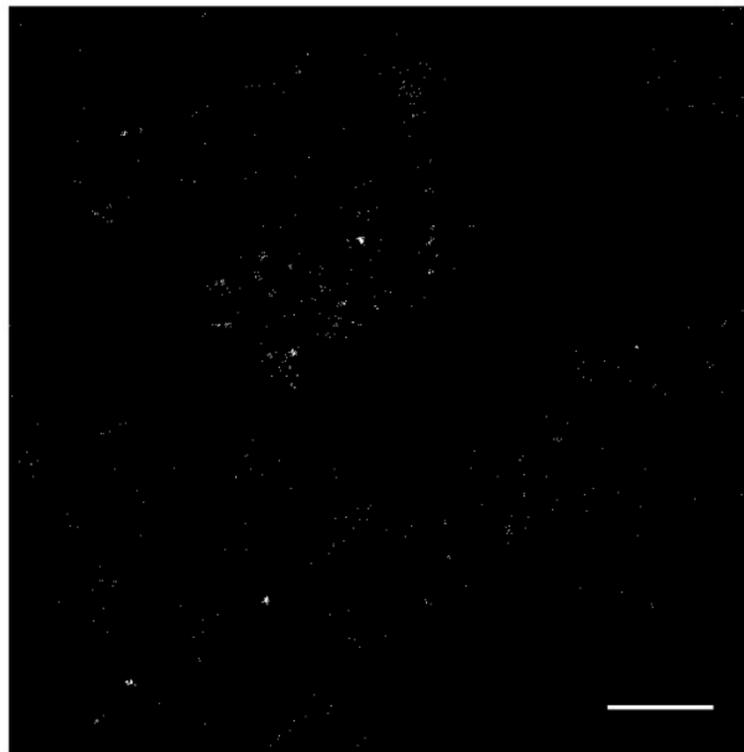
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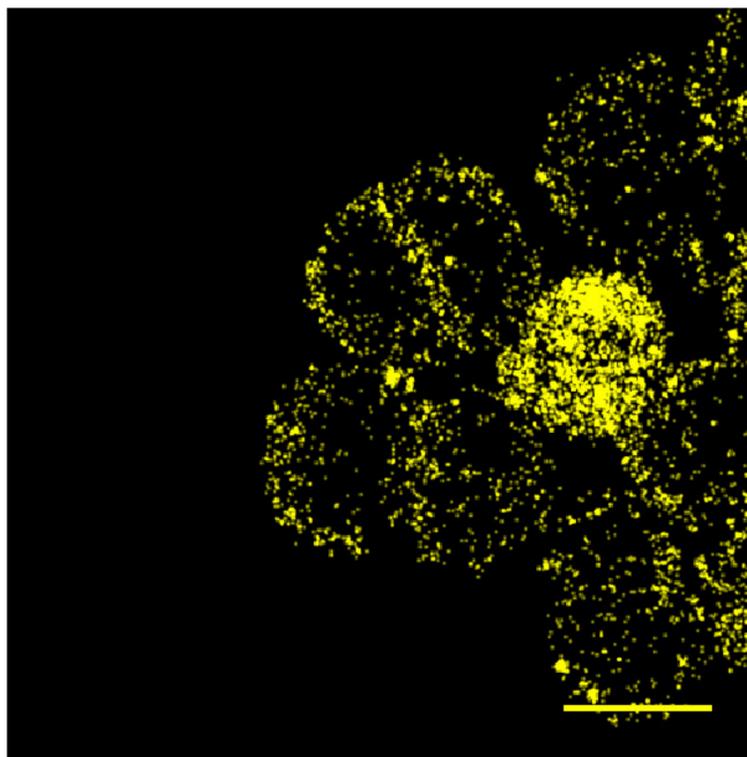
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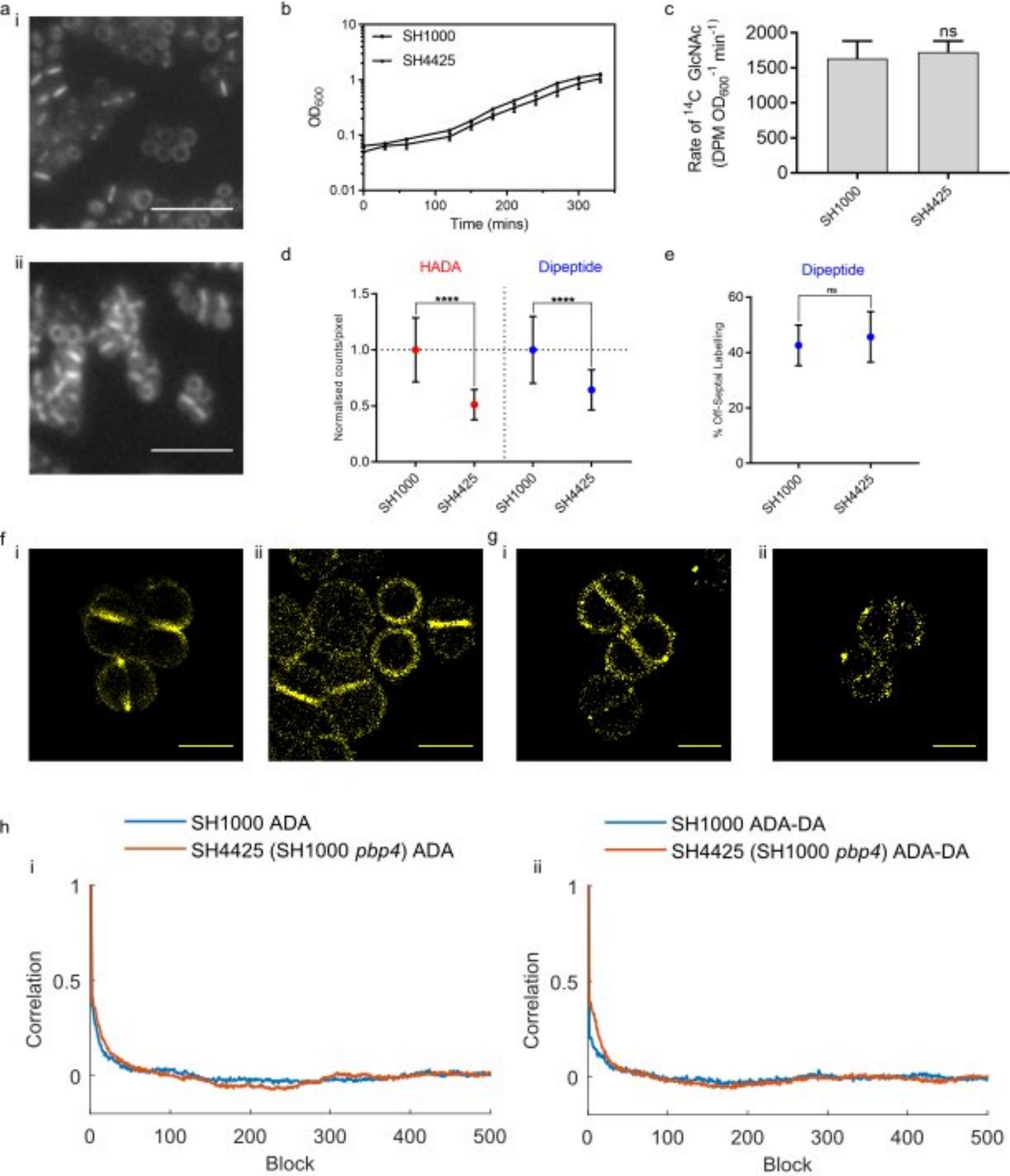


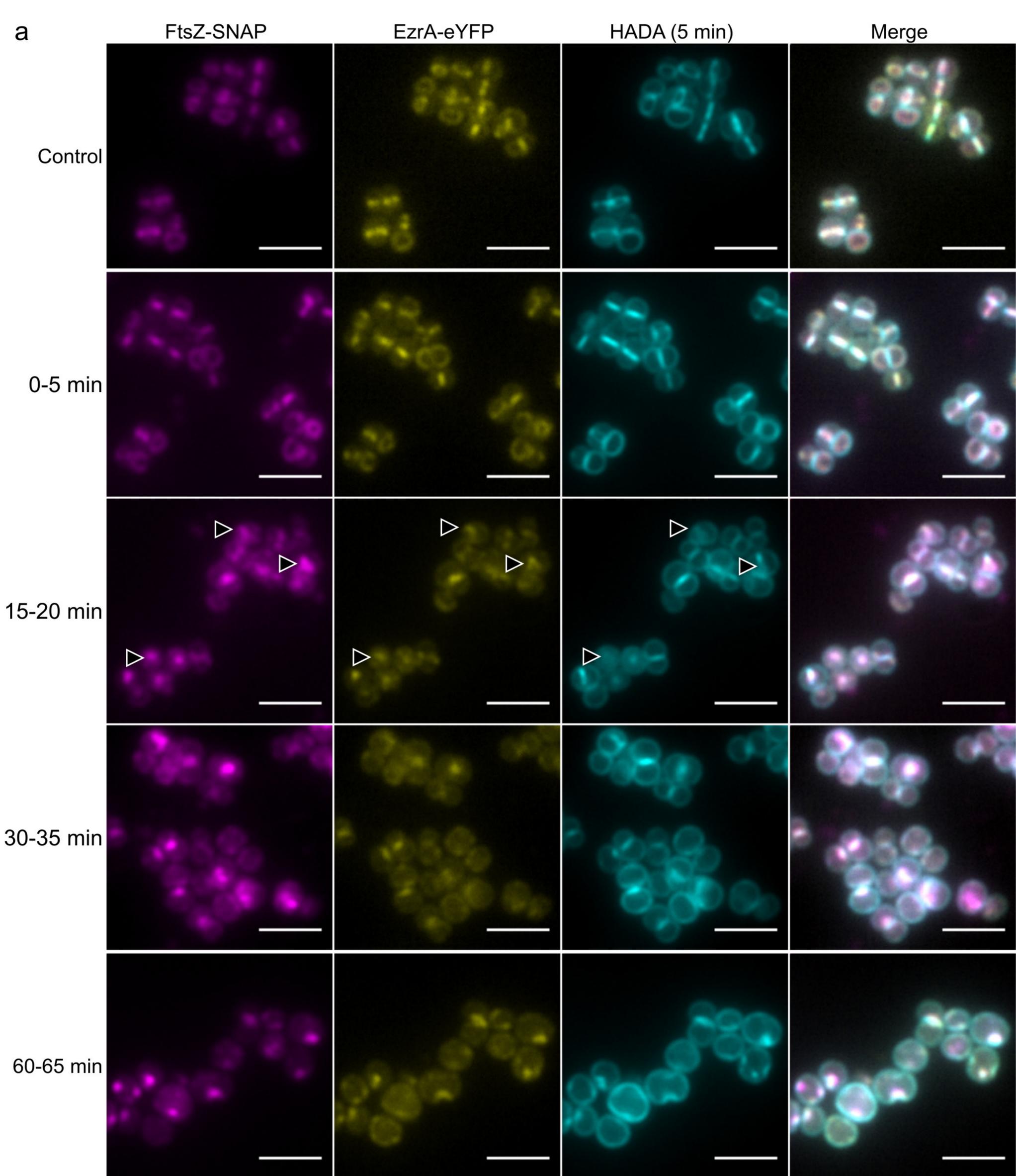
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d







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