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Single-molecule optical microscopy of protein dynamics and computational analysis of images to determine cell structure development in differentiating *Bacillus subtilis*

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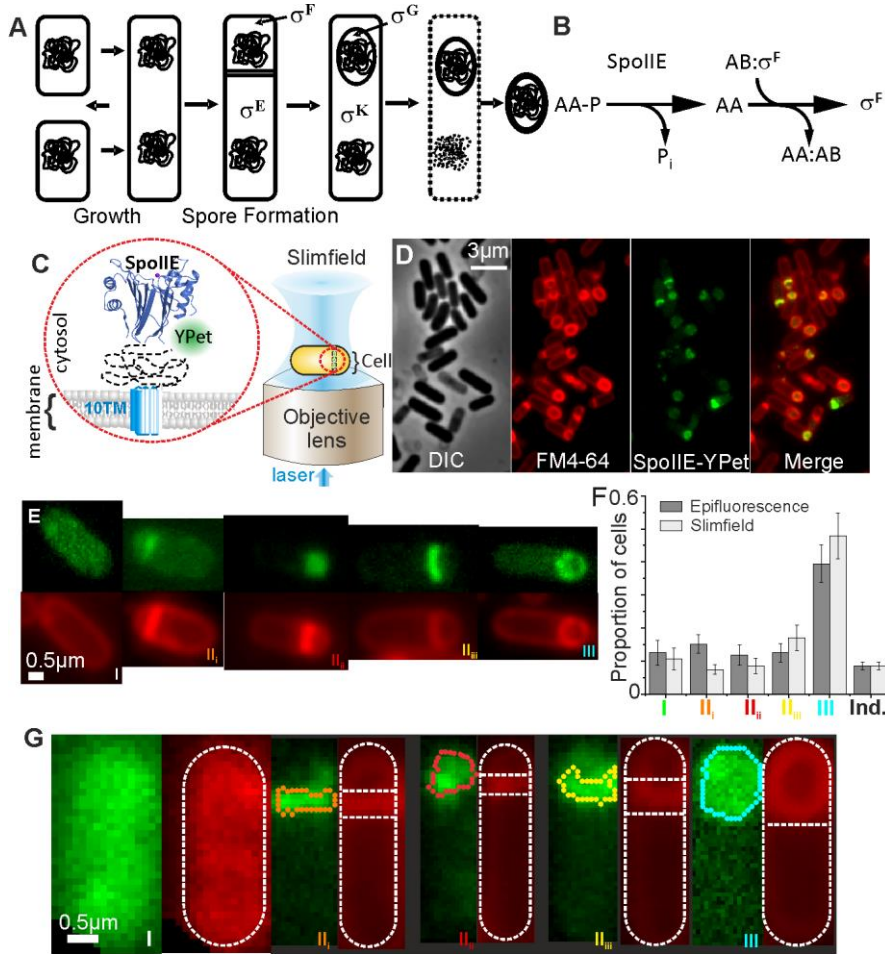
20 **Abstract**

21 Here we use single-molecule optical proteomics and computational analysis of live cell
22 bacterial images, using millisecond super-resolved tracking and quantification of
23 fluorescently labelled protein SpoIIE in single live *Bacillus subtilis* bacteria to understand its
24 crucial role in cell development. Asymmetric cell division during sporulation in *Bacillus*
25 *subtilis* presents a model system for studying cell development. SpoIIE is a key integral
26 membrane protein phosphatase that couples morphological development to differential gene
27 expression. However, the basic mechanisms behind its operation remain unclear due to
28 limitations of traditional tools and technologies. We instead used advanced single-molecule
29 imaging of fluorescently tagged SpoIIE in real time on living cells to reveal vital changes to
30 the patterns of expression, localization, mobility and stoichiometry as cells undergo
31 asymmetric cell division then engulfment of the smaller forespore by the larger mother cell.
32 We find, unexpectedly, that SpoIIE forms tetramers capable of cell- and stage-dependent
33 clustering, its copy number rising to ~700 molecules as sporulation progresses. We observed
34 that slow moving SpoIIE clusters initially located at septa are released as mobile clusters at
35 the forespore pole as phosphatase activity is manifested and compartment-specific RNA
36 polymerase sigma factor, σ^F , becomes active. Our findings reveal that information captured
37 in its quaternary organization enables one protein to perform multiple functions, extending an
38 important paradigm for regulatory proteins in cells. Our findings more generally demonstrate
39 the utility of rapid live cell single-molecule optical proteomics for enabling mechanistic
40 insight into the complex processes of cell development during the cell cycle.

41 **Keywords:** Single-molecule, sporulation, super-resolution, morphogenesis, differentiation.

42

43 **1. Introduction**



44

45

46 **Figure 1:** Fluorescence imaging and classification by sporulation stage (A) Growth and
 47 sporulation of *B. subtilis*. During spore formation, the cell divides asymmetrically producing
 48 a smaller forespore and a larger mother cell. Compartment and stage specific sigma factors
 49 are activated sequentially. The forespore is engulfed by the mother cell before maturing into a
 50 resistant spore which is released when the mother cell lyses. (B) The SpoIIIE phosphatase is
 51 the most upstream-acting of three proteins regulating the activity of the first compartment-
 52 specific sigma factor, σ^F . Dephosphorylation allows SpoIIAA (AA) to displace σ^F from its
 53 complex with an anti-sigma factor (AB) enabling forespore-specific gene expression to be
 54 established. (C) Slimfield schematic, inset showing septum-localised SpoIIIE-mYPet. (D)

55 phase/epifluorescence microscopy of SpoIIE at different stages. Membrane labelling, FM4-
56 64 (red), SpoIIE-mYPet (green). (E) **Example epifluorescence images of SpoIIE-mYPet**
57 **(green) with membrane stained with FM4-64 (red) at each stage.** (F) Detected mean
58 proportion of each stage or indeterminate (Ind.) (SEM error) of N = 4 fields of view each
59 containing approximately N = 100 cells. Proportions were statistically identical between
60 epifluorescence and slimfield data at p=0.05 (p=0.40, 0.08, 0.29, 0.72, 0.72, 0.49 for each
61 stage/indeterminate respectively) (G) Categorization of stages from Slimfield, detected
62 forespore/septa features (coloured lines), cell boundary segmentation based on fitting a
63 sausage shape to fluorescence image indicated (outer white dash) and interface between
64 forespore, septa and mother cell (horizontal white dash).

65

66 Spore formation in *B. subtilis* offers a model system for studying development,
67 differentiation, morphogenesis, gene expression and intercellular signalling in complex
68 organisms[1,2]. In nutrient rich conditions, rod-shaped cells grow and multiply by symmetric
69 mid-cell division to generate identical daughters (Fig. 1A). However, when starved, *B.*
70 *subtilis* ceases growth and is able to embark on a pathway of differentiation to form a
71 dormant cell called a spore. Spore formation begins with an asymmetric division producing a
72 smaller forespore cell next to a larger mother cell. Each compartment inherits an identical
73 chromosome, but the patterns of gene expression, orchestrated by compartment-specific RNA
74 polymerase sigma factors, differ resulting in alternative cell fates. The mother cell engulfs the
75 forespore in a phagocytosis-like process creating a cell-within-a-cell (Fig. 1A), and a
76 nurturing environment in which a robust multi-layered coat is assembled around the maturing
77 spore[3]. In the final stages, the mother cell undergoes programmed cell death releasing the
78 spore, which is resistant to multiple environmental stresses and can lie dormant until
79 favourable growth conditions are restored.

80 At sporulation onset, ring-like structures of the tubulin homologue FtsZ form at mid-
81 cell and migrate on diverging spiral trajectories towards the cell poles[4], colocalizing with the
82 membrane integrated phosphatase PP2C SpoIIE[5]. One polar ring matures into the sporulation
83 septum while the other disassembles[6]. Asymmetric division otherwise involves the same
84 proteins as vegetative cell division, though the resulting sporulation septum is thinner[7,8].
85 SpoIIE is the only sporulation-specific protein whose mutation causes ultrastructural changes
86 in the asymmetric septum; null mutants of *spoIIE* are defective in sporulation and at lower
87 frequency give rise to thicker asymmetric septa resembling the vegetative septum[7].

88 Changes in cell morphology during sporulation are coupled to a programme of gene
89 expression, involving intercellular signalling, and the sequential activation of RNA
90 polymerase sigma factors, σ^F and σ^G in the forespore and σ^E and σ^K in the mother cell[9].
91 Forespore-specific activation of σ^F on completion of the asymmetric septum is the defining
92 step in differentiation. In pre-divisional and mother cells, σ^F resides in complex with the anti-
93 sigma factor SpoIIAB while a third protein SpoIIAA is phosphorylated. After septation,
94 SpoIIAA~P is dephosphorylated by the manganese-dependent protein phosphatase SpoIIE.
95 The resulting SpoIIAA displaces σ^F from the σ^F :SpoIIAB complex allowing RNA
96 polymerase binding and transcription of forespore-specific genes (Fig. 1B)[10,11]. This in
97 turn, triggers activation of σ^E in the mother cell and establishes alternate programmes of gene
98 expression which determine different cell fates (Fig. 1A).

99 SpoIIE has multiple roles at different sporulation stages (Fig. 1A,B). Assembly of
100 SpoIIE to form polar rings – “E-rings”, dependent on interaction with FtsZ[12], occurs during
101 stage I, defined by the formation of an axial filament spanning the cell length and comprising
102 two copies of the chromosome each tethered through its origin region to opposing cell poles.
103 Formation of the asymmetric septum is defined as stage II_i, during which SpoIIE interacts
104 with the divisome components RodZ[13] and DivIVA[14]. After closure of the sporulation

105 septum, the FtsZ ring disassembles. SpoIIE-mediated activation of σ^F correlates with release
106 of SpoIIE from the sporulation septum, marking stage II_{ii}[13]. During stage II_{iii}, SpoIIE
107 interacts with SpoIIQ[15] the forespore component of an intercellular channel[16–18], crucial
108 for later activation of σ^G . Stage III is characterized by mother cell engulfment of the
109 forespore; SpoIIE localizes around the forespore, but there are no data to suggest a specific
110 role of SpoIIE in this or later stages[15].

111 An increased concentration of SpoIIE in the forespore relative to that in the mother
112 cell has been proposed to account for the selective activation of σ^F in the emerging forespore.
113 This may occur through equipartitioning SpoIIE into the mother cell and forespore septal
114 membranes leading to a higher SpoIIE effective concentration in the forespore as a result of
115 its ~6 fold smaller volume [19]. It has also been shown that there is selective proteolysis of
116 SpoIIE in the mother cell through the action of the membrane bound ATP-dependent
117 protease, FtsH[20]. Here, it is proposed selective oligomerization at the forespore pole,
118 protects SpoIIE from proteolysis in this compartment and further increases the concentration
119 difference between the cell compartments.

120 To explore the complex function of SpoIIE further, we sought to determine its
121 dynamic molecular architecture in differentiating cells. We employ a rapid single-molecule
122 optical proteomics technique Slimfield imaging[21–23] capable of tracking single
123 fluorescently-labelled SpoIIE molecules with millisecond sampling in live *B. subtilis* cells to
124 super-resolved spatial precision. By using step-wise photobleaching of the fluorescent protein
125 tags[24] we determine the stoichiometry of each tracked SpoIIE complex and quantify the
126 precise number of SpoIIE molecules in the mother cell and forespore in each individual cell .
127 Also, by analysing the mobility of SpoIIE foci via their mean square displacement with
128 respect to time, we calculate the microscopic diffusion coefficient D , model this to determine
129 the effective diameter of SpoIIE complexes and correlate these data with measured SpoIIE

130 content. Importantly, our copy number estimates indicate that there are similar numbers of
131 SpoIIE molecules in both the mother cell and the forespore compartments when the
132 asymmetric septum forms: since the volume in the forespore is significantly smaller than that
133 of the mother cell this finding reveals an order of magnitude higher SpoIIE concentration in
134 the forespore, correlated to the increased activity of σ^F . We find that the stoichiometry and
135 diffusion of tracked SpoIIE is dependent on its interaction partners and morphological
136 changes, suggesting its roles in sporulation are influenced by oligomeric composition and
137 mobility. Interestingly, we detect higher order mobile, oligomeric SpoIIE, towards the cell
138 pole, at the stage of sporulation when σ^F becomes selectively activated in the forespore, as
139 previously proposed[20].

140

141 **2. Methods**

142 **2.1 Strains and plasmids**

143 Gene cloning in *B. subtilis*, unless specified, was performed using standard protocols[25]
144 (Table S1). To construct pSGIIE-mGFP, used in the FRAP experiments, we used previously
145 prepared pSGIIE-YPet[13]. A PCR fragment containing mGFP was prepared using
146 mGFPKpnF:

147 5' ATCATCATCGGTACCATGAGTAAAGGAGAAGAAGACTTTTCACTGGAGTTGTC 3'

148 and mGFPBamR2:

149 5' atcatcatcggatccTTATTTGTATAGTTCATCCATGCCATGTG 3'

150 primers and a plasmid derivative pSG1729 containing *mgfp* as template[26]. To yield pSG-
151 mGFP this fragment was KpnI/BamHI digested and cloned into a similarly cut pSGIIE-YPet.
152 Subsequently a 360bp KpnI fragment containing *spoIIE* C-terminus (obtained from

153 KpnI/BamHI cut pSGIIE-YPet) was cloned into pSG-mGFP digested with KpnI to yield
154 pSGIIE-mGFP.

155 *B. subtilis* liquid cultures were grown in DSM [25] supplemented with
156 chloramphenicol ($5\mu\text{g ml}^{-1}$), erythromycin ($1\mu\text{g ml}^{-1}$) and lincomycin ($25\mu\text{g ml}^{-1}$) as required.
157 Samples for microscopy on 1% agarose slides were taken 2h after sporulation onset (from our
158 measurements this would ensure that the majority of cells after the onset of sporulation would
159 have reached the start of stage II). For membrane visualization, FM 4-64 (Molecular Probes)
160 was used ($0.2\text{--}1\mu\text{g ml}^{-1}$). When necessary, cells were concentrated by centrifugation (3min,
161 $2,300 \times g$) and resuspended in a small volume of supernatant. Images and analysis were
162 obtained with an Olympus BX63 microscope (Hamamatsu Orca-R² camera) and Olympus
163 CellP or Olympus Image-Pro Plus 6.0 software. Imaging was performed at room temperature.
164 As N-terminal, cytosolic tail of SpoIIE (residues 11 to 37) is responsible for its proteolysis by
165 FtsH (20), it is not possible to determine by western blot if the protein was degraded due to
166 the fluorescent tag (Fig. S1C). It is also impossible to select only early stage sporulating cells
167 corresponding to our microscopy data in western analysis as the fusion protein construct
168 SpoIIE-mYPet localizes to the membrane and the cells sporulate at the level of the wild type
169 cells, we believe the fusion protein is expressed, is functional and is degraded as the untagged
170 version. Also, we did not detect any cytoplasmic fluorescence consistent with cleaved
171 fluorescent protein alone (background fluorescence was consistent with out of plane foci –
172 see later section). Epifluorescence images showed integration into the membrane (Fig. S1A)

173 and simulated images of membrane integrated SpoIIE were qualitatively the same as our
174 Slimfield images (Fig. S1B).

175 **2.2 Single-molecule optical proteomics**

176 A dual-color bespoke single-molecule microscope was used as described previously [23,27]
177 which utilized narrow epifluorescence excitation of 10 μm full width at half maximum in the
178 sample plane from a 514 nm 20mW laser (Obis LS, Coherent). The laser was propagated
179 through a $\sim 3\times$ Keplerian beam de-expander. Illumination was directed onto an *xyz* nanostage
180 (Mad City Labs, the Dane County, Wisconsin, USA), and emissions directed through a color
181 splitter utilizing a dichroic mirror centered on 560 nm wavelength and emission 25nm
182 bandwidth filters centered at 542/594 nm (Chroma Technology Corp., Rockingham,
183 Vermont, USA) onto an Andor iXon 128 emCCD camera, 80 nm/pixel. Brightfield imaging
184 was performed with no gain (100 ms/frame), single-molecule imaging at maximum gain (5
185 ms/frame).

186 Foci were automatically detected using MATLAB (Mathworks) software enabling a
187 spatial localization precision of 40nm using iterative Gaussian masking, and automated *D* and
188 stoichiometry calculation. The copy number in the mother cell or forespore was determined
189 by summing pixel intensities within the compartment, correcting for low background
190 autofluorescence measured from FM4-64 labeled wild type *B. subtilis*, then dividing by the
191 characteristic SpoIIE-mYPet intensity [27]. The intensity of each foci was defined as the
192 summed intensity inside a 5 pixel radius circle corrected for the local background, defined as
193 the mean intensity in a 17 pixel square outside the circle [24]. If the signal to noise ratio of
194 the foci, defined as the mean intensity divided by the standard deviation of the local
195 background, was greater than 0.4 it was linked into an existing track if within 5 pixels,
196 approximately matching the diffraction-limited point spread function width. Only tracks with
197 4 or more points were analyzed, a commonly used criterion by us and others in the single-

198 particle tracking field[28,29]. The characteristic SpoIIE-mYPet intensity was calculated from
 199 foci intensities found towards the end of the photobleach, confirmed to be single molecule
 200 from detection of single step-wise photobleach events in individual over-tracked (i.e. tracked
 201 beyond photobleaching), Chung-Kennedy[30] filtered (an edge preserving smoothing
 202 algorithm) SpoIIE-mYPet tracks (Fig. S2). The stoichiometry of tracked foci was determined
 203 by fitting the first 4 intensity values of each track with exponential:

$$204 \quad I = I_0 \exp\left(-\frac{t}{t_b}\right)$$

205 I = foci intensity, I_0 = initial intensity, t = time since laser illuminated cell, t_b = bleach time
 206 (determined by an exponential fit to all population foci intensity to be ~100ms). I_0 was
 207 divided by the mYPet characteristic intensity to give the stoichiometry. Although sub-optimal
 208 for low stoichiometry foci, e.g. <6 molecules per focus, this exponential method is effective
 209 over a broad range of stoichiometries[31]

210 The 2D mean square displacement (MSD) was calculated from a fitted foci centroid
 211 $(x(t), y(t))$ assuming a track of N consecutive frames, and a time interval $\tau = n\Delta t$, where n is a
 212 positive integer and Δt the frame integration time [32]:

$$213 \quad MSD(\tau) = MSD(n\Delta t) = \frac{1}{N-1-n} \sum_{i=1}^{N-1-n} \left\{ [x(i\Delta t + n\Delta t) - x(i\Delta t)]^2 + [y(i\Delta t + n\Delta t) - y(i\Delta t)]^2 \right\}$$

$$= 4D\tau + 4\sigma^2$$

214 The localization precision from tracking is given by σ , which we measure as 40nm. D is
 215 estimated from a linear fit to the first three data points in the MSD vs. τ relation (i.e.
 216 $1 \leq n \leq 3$) for each accepted track, with the fit constrained to pass through a point $4\sigma^2$ on the
 217 vertical axis corresponding to $\tau = 0$, allowing σ to vary in the range 20 - 60nm in line with the
 218 experimental range.

219 **2.3 FRAP**

220 FRAP was carried out on a Zeiss LSM 510 Meta confocal system with Axiovert inverted
221 microscope, fitted with Plan Apochromat 100x /1.4 NA oil objective and temperature-
222 controlled stage. A 488nm wavelength laser excited GFP, emissions collected via a 498-
223 564nm bandpass filter. The strength of photobleaching in the region of interest was set to 10-
224 20 iterations of 100ms each to ensure maximal photobleaching of GFP inside and minimum
225 photobleaching beyond.

226 **2.4 Categorization of cell cycle stage**

227 To determine the cell cycle stage during the sporulation process, the following algorithm was
228 used:

- 229 1. Cell images were initially coarsely over-segmented by thresholding the brightfield
230 image and then using an initial ellipse shape approximation to define the cell length
231 [27]. We then manually optimised the cell width of a sausage function (rectangle
232 capped with two hemicircles) that enclosed the mYPet fluorescence intensity in each
233 cell above the level of background noise.
- 234 2. Cells were then cropped out of the original image using a bounding rectangle around
235 the segmentation and automatically rotated parallel to the horizontal axis.
- 236 3. A more precise segmentation stage then followed. This consisted of a double
237 threshold Otsu's method, applied to a 5 frame average of the mYPet fluorescence
238 image. Pixels whose intensity values were above the 2nd threshold and multiplied by
239 the segmentation contain the spore feature – either the whole forespore or septa.
- 240 4. These pixel areas were split into distinct connected components or candidate spore
241 features and their centroids and areas calculated automatically using standard
242 MATLAB functions.
- 243 5. A region was accepted as the mYPet spore feature mask if:

- 244 1. Its centroid is within 40% of either end of the cell.
- 245 2. Its centroid is within $\pm 40\%$ of the middle of the cell width.
- 246 3. The area of its centroid was >10 pixels (there was no upper threshold).
- 247 4. It had the highest summed pixel intensity of all the regions.
- 248 6. If nothing was accepted, steps 5.1-5.4 were repeated once with the previously found
- 249 regions excluded.
- 250 7. If nothing was still found then the cell is 'pre-sporulation/stage I'.
- 251 8. The FM4-64 frame average was similarly segmented but the mask multiplied by the
- 252 forespore mask to give the FM spore feature.
- 253 9. Both FM and mYPet spore feature Major/Minor Axis, Area and Orientation were
- 254 calculated by fitting the shape to an ellipse function.
- 255 10. Both were then assigned into 2 shape categories based on the aspect ratio, > 1.2 –
- 256 'septa', otherwise 'filled' structure. These correspond to fluorescence only at the
- 257 linearly extended septa or distributed about the forespore in a rounder shape.
- 258 11. If the FM segmentation was 'septa', the segmentation was morphologically 'thinned'
- 259 and its linear curvature calculated.
- 260 12. Stages were then assigned as follows:
- 261 Stage I/pre-sporulation: no mYPet spore feature detected.
- 262 Stage II_i: 'septa' FM and mYPet spore features with curvature <1 .
- 263 Stage II_{ii}: 'septa' FM and 'filled' mYPet spore features.
- 264 Stage II_{iii}: 'septa' FM and mYPet spore features with curvature >1 .
- 265 Stage III: 'filled' FM and mYPet spore features.
- 266

267 To confirm the spore categorization algorithm we tested it on a series of simulated images

268 (Fig S1C). These were generated by integrating a model point spread function (PSF) over a

269 3D model for the cell and forespore shape and subsequently noising the image with Poisson
270 noise based on real noise characteristics of our microscope [33]. The cell membrane was
271 modelled as a hollow cylinder, capped with hemisphere shells at either end with 1 pixel thick
272 walls. Stage II_i septa were modelled as cell width disks while stage II_{iii} septa were modelled
273 as hemispherical shells. Released SpoIIIE in stage II_{ii} was modelled as a hemispherical shell
274 capped by a disk while in stage III, it was modelled as a spherical shell. The relevant features
275 for 100 cells in each stage were simulated in the ‘mYPet’ and ‘FM4-64’ channels and run
276 through the categorization algorithm as if they were real data with no noise, average noise
277 and the most extreme noise observed in the data. Without noise, 100% of cells were correctly
278 identified, dropping to at worst in stage II_{ii} 79% with average noise and in the extreme case,
279 as low as 42%.

280 We attempted further confirmation using Principal Component Analysis (PCA), an
281 approach typically used to identify specific conformations or orientations in cryo-electron
282 microscopy data. Data, images in this case, can be broken down into a basis set of
283 eigenvectors or eigenimages which when summed in proportion to their eigenvalues, recreate
284 the original dataset. Its use in live cell fluorescence data is challenging due to the high
285 heterogeneity in size, shape and intensity of the images. Thus spore images were all cropped
286 to 16x16 pixels, rotated and aligned and their intensity normalised (Fig. S1H) before a basis
287 set of eigenvectors were calculated by Hotelling’s deflation [34]. The distribution of
288 eigenvalues was strongly biased towards the 1st eigenvector (Fig. S1I) however 3D scatter
289 plots of the first 3 eigenvalues did show separation of the data, further confirming our
290 categorization algorithm but not allowing us to categorise spores based on PCA alone.

291 **2.5 Determining the contribution from out-of-focus SpoIIIE-mYPet foci**

292 To quantify the contribution from out-of-focus SpoIIIE-mYPet foci (i.e. those not detected
293 during tracking) into the membrane ‘pool’ (i.e. spatially extended membranous regions of

294 fluorescence intensity not detected as distinct foci), we assumed that the number and
295 stoichiometry of detected foci from within the depth of field were the same as those without
296 and were uniformly distributed. Assuming a depth of field of ~350nm, on the basis of
297 expectations from the numerical aperture of the objective lens and peak emission wavelength,
298 a mean cell width of ~0.9 μm (61) and that the focal plane is exactly on the cell midplane we
299 estimate ~1/4 of the cell membrane lies in the depth of field of the microscope. Thus, to
300 generate indicative estimates for copy number values per cell we extrapolated the total
301 number of summed SpoIIE-mYPet in foci by a factor of 4x. For the stage II mother cell
302 (Table S2), the mean total number of molecules in foci per cell is ~32 (Mean foci
303 stoichiometry multiplied by mean number of foci per cell) which multiplied by 4 agrees with
304 the mean copy number of 82 ± 42 to within experimental error. Using the same method on
305 other stages either agrees or over or underestimates implying that there is no measurable
306 'pool' of SpoIIE i.e. all of the SpoIIE-mYPet fluorescence can be accounted for by foci.

307 **2.6 Simulating the effects of different oligomeric states for SpoIIE on the predicted** 308 **stoichiometry distribution from Slimfield analysis**

309 To simulate the effects of different oligomeric states of SpoIIE-mYPet on the observed
310 stoichiometry distribution from Slimfield image data we calculated the probability of foci
311 overlap[36] in each individual cell using the number of detected foci and the area of the spore
312 feature in that particular cell. This probability was used to generate the distribution of
313 overlaps using a Poisson distribution, based on a stage specific frequency of overlap, λ . The
314 predicted apparent stoichiometry distribution was then generated by convolving the overlap
315 distribution with the intensity distribution of model stoichiometry, S (i.e. $S = 2$, dimers, $S = 4$,
316 tetramers etc.). This intensity distribution was generated from the mYPet characteristic
317 intensity distribution (Fig. S2C), re-centred on $2S$, width scaled to $S^{1/2} * \sigma$, where $\sigma = 0.675$,

318 the sigma width of Fig. S2C. Such that the probability distribution of stoichiometries $P(x)$ is
 319 given by:

$$320 \quad P(x) = \sum_{k=1}^5 \exp(-\lambda) \frac{\lambda^k}{k!} \frac{1}{\sigma\sqrt{2\pi kS}} \exp\left(-\frac{x - kS}{k\sigma^2}\right)$$

321 This model is a summation of multiple Gaussian distributions which are separated by a fixed
 322 number of molecules (for example 4 molecules in the case of the tetramer model), whose
 323 amplitude scales with a Poisson distribution, as expected from the nearest neighbour model.
 324 Here k is the number of overlapping foci – we sum up to a maximum of $k = 5$ overlapping
 325 foci since this ensured in all cases that the expectation value of foci occurrence at higher
 326 values of k was less than 1 (i.e. $P.S < 1$ focus). Finally, each of these modelled cell
 327 stoichiometry distributions was averaged over the sporulation stage population to generate
 328 the model distribution and convolved with the same 0.7 molecule width kernel as the kernel
 329 density estimates (KDEs) in the real (i.e. experimental) data. The Pearson's Chi-squared
 330 statistic χ^2 was calculated as:

$$331 \quad \chi^2 = \sum_{i=1}^{30} \frac{(O_i - C_i)^2}{C_i}$$

332 where the observed value O_i is the normalized KDE value (i.e. scaled on the probability
 333 density axis such that the total area underneath the KDE sums exactly to 1) at single molecule
 334 bin intervals up to a total of typically $n = 30$ bins, i.e. stoichiometry range tested from the full
 335 distribution is 0-30 molecules, assuming the data contained at least one recorded focus in any
 336 respective bin (if not it was discarded in the Chi-squared summation). The calculated data
 337 value C_i was taken from the normalized model fit. The degrees of freedom were equal to the
 338 number of bins used in the χ^2 calculation subtracting the 4 free model parameters (which were
 339 overlap frequency (λ), max number of overlaps (k), intensity distribution (σ) and model
 340 stoichiometry (S)). The value of the measured χ^2 was then used with the inbuilt inverse Chi-

341 squared MATLAB function `chi2cdf.m` at this equivalent number of degrees of freedom to
342 calculate the equivalent p value which corresponds to the null hypothesis that the measured
343 variation between the observed values and the model fit is random. We found that the
344 tetramer model was the only model to produce a goodness of fit corresponding to acceptable
345 p values at approximately 0.05 or less in all stages (Fig. 3 and S3).

346 **2.7 Modelling the frictional drag on SpoIIE foci**

347 We modelled the frictional drag coefficient in the cell membrane of SpoIIE foci as that due to
348 a cylinder whose height h matches the width of the phospholipid bilayer ($\sim 3\text{nm}$) with a radius
349 given by parameter a , using a generalized method established previously to characterize the
350 lateral diffusion of transmembrane proteins [37,38]. In brief, the diffusion coefficient D is
351 estimated from the Stokes-Einstein relation of $D = k_B T / \gamma$, where k_B is the Boltzmann constant
352 and T the absolute temperature, and the lateral viscous drag γ is given by:

$$353 \quad \gamma = 4\pi(\eta_1 + \eta_2)aC(\varepsilon)$$

354 where η_1 and η_2 are the dynamic viscosity values either side of the membrane, which we
355 assume here are approximately the same at η_c the cytoplasmic viscosity. C is a function of $\varepsilon =$
356 $2a\eta_c/h\eta_m$ where η_m is the dynamic viscosity in the membrane itself. Since η_m is typically 2-3
357 orders of magnitude larger than η_c [39] then ε is sufficiently small to use an approximation
358 for C of:

$$359 \quad C \approx 1/(\varepsilon \ln(2/\varepsilon))$$

360 We used these formulations to generate a look-up table between D and a for the vegetative
361 cell membrane in the mother cell, assuming $\eta_m \approx 600$ cP, and the emerging forespore cell
362 membrane, assuming $\eta_m \approx 1,000$ cP, assuming $\eta_c \approx 1$ cP throughout (Fig. 5C) [40]. We
363 estimated a consensus value for D in the mother cell from the population of unweighted mean
364 D values determined from all cell stages I-III (Table S2) of $1.05 \pm 0.06 \mu^2\text{m/s}$ ($\pm\text{SEM}$,
365 number of stages $n = 5$). We similarly estimated a consensus D value for the low mobility

366 sporulation stages II_i and II_{iii} of $0.47 \pm 0.04 \mu^2\text{m/s}$ (number of stages $n = 2$) and a consensus
367 D value for the high mobility sporulation stages II_i and III of $0.76 \pm 0.05 \mu^2\text{m/s}$ (number of
368 stages $n = 2$). We then extrapolated these consensus values and SEM error estimates using
369 the vegetative and forespore cell membrane look-up tables to determine corresponding mean
370 values and $\pm\text{SEM}$ ranges for a .

371 **2.8 Stoichiometry vs. localization**

372 To compare foci stoichiometry as a function of location in the forespore, a simplified,
373 normalised 1D coordinate was used. This was based on the generous forespore segmentation
374 which extends from the mother cell side of the septa through to the outer edge of the cell
375 pole. There was also significant variation in the size of this segmentation between cells. Thus
376 a normalised coordinate was used, 0-1 from the two most extreme points of the forespore.
377 This implied that on average both the septa and cell poles lie within the most extreme points
378 of the predicted cell outline segmentation.

379 **2.9 Structural and bioinformatics analysis**

380 CCP4mg was used to render images of structures with PDB IDs: 5MQH SpoIIE(590-827)
381 and 5UCG SpoIIE(457-827).[41]

382 **2.10 Statistics and goodness of fit**

383 Where means are presented and compared, students t-tests were run and p-values presented.
384 For data-driven models, such as the stoichiometry modelling, χ^2 and p values are presented.
385 For physical models such as FRAP and stokes fitting, the 95% confidence intervals on the fit
386 parameters are presented as goodness of fit.

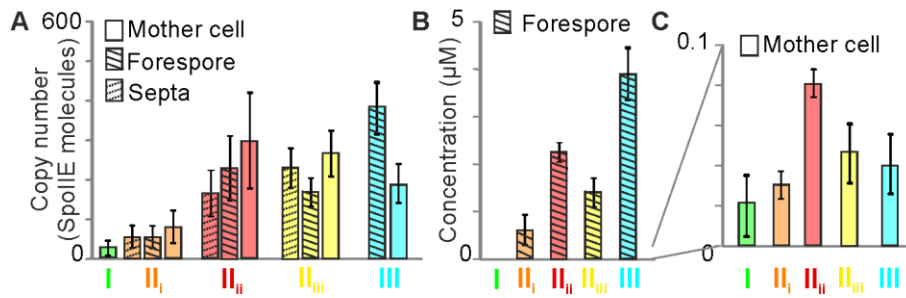
387 **3. Results**

388 **3.1 Sporulation stage can be categorized using an accurate, high-throughput automated** 389 **algorithm**

390 We generated a chromosomally encoded fusion of SpoIIE to monomeric yellow fluorescent
391 protein mYPet (a bright fluorescent protein with very short maturation time, <10 min[42]
392 compared to >2hrs sporulation time, whose long excitation wavelength results in minimal
393 contamination of cellular autofluorescence[43]) to report on SpoIIE localization (Table S1).
394 We prepared cells for sporulation using nutrient-depleted media, incubating with the red
395 lipophilic dye FM4-64 for visualizing *B. subtilis* membrane structures[44]. This allowed us to
396 observe steady-state patterns of SpoIIE-mYPet and FM4-64 localization for sporulation
397 stages I, II (with associated sub-stages) and III with single-molecule detection sensitivity via
398 Slimfield (Fig. 1C), as well as standard epifluorescence microscopy (Fig. 1D,E and S1). We
399 developed an automated high-throughput analysis framework using morphological
400 transformations[33,45] on SpoIIE-mYPet and FM4-64 data, enabling us to categorize each
401 cell into one of five different sporulation stages (I/pre-divisional, forespore formation stages
402 II_i, II_{ii} and II_{iii}, and III after engulfment), validated by simulation and principal component
403 analysis (Fig. 1F,G, Fig. S1). Our algorithm segments the SpoIIE and FM4-64 images to
404 identify septa and forespore features and categorises them into appropriate stages but does
405 not distinguish between E-ring structures in stage II[46,47] and SpoIIE localization in the
406 septa in stage II_i. The measured proportions of cells in each stage (Fig. 1G) were qualitatively
407 similar to those reported using manual, low-throughput methods[48]. Imaging a SpoIIE-
408 mYPet strain including a $\Delta spoIIIQ$ deletion (Table S1), defective in spore formation and
409 unable to progress beyond stage II_{ii}, yielded similar relative proportions of cells in stages I, II_i
410 and II_{ii} (Fig. S1). Although imperfect, resulting in some mis-characterisation (Fig. S1), our
411 software is objective and enables study of cells which are not easily categorised by eye and
412 avoids biasing our study to just previously accepted morphological features of sporulation.

413

414 **3.2 SpoIIE is concentrated in the forespore, probably through equipartitioning**



415

416

Figure 2: SpoIIE copy number (A) Mean and SEM SpoIIE copy number in mother cell,

417

forespore and septa at each stage. (B) Concentration in the forespore, excluding the septa, at

418

each stage. (C) Concentration in the mother cell, excluding the septa at each stage. Stage I

419

(green), II_i (orange), II_{ii} (red), II_{iii} (yellow) and III (cyan). Mother cell copy numbers

420

statistically higher than stage I by stage II_{ii} ($p=0.02$) and forespore copy number increases

421

significantly between stage II_i and II_{ii} ($p=0.007$).

422

423

Slimfield images revealed distinct foci, as well as a more diffuse pool of fluorescent

424

SpoIIE localized close to the cell membrane as expected (Movies S1-3). Slimfield employs a

425

high numerical aperture objective lens with a high depth of field, thus a significant amount of

426

fluorescence and even foci were detected in the middle of the cell. To check that this signal

427

was really from membrane bound SpoIIE, we simulated images of membrane bound

428

fluorophores in model *Bacillus* shaped cells and found similar patterns of localization (Fig.

429

S1B). We used bespoke single particle localization[49] on the Slimfield data to track foci

430

whose width was consistent with the measured point spread function (PSF) of our

431

microscope, ~250nm. Foci could be tracked over consecutive images up to ~0.3s using rapid

432

5ms per frame sampling to a spatial precision of 40nm[27]. Tracking of distinct foci was

433

coupled to molecular stoichiometry analysis by estimating the initial foci brightness and

434

dividing this by the brightness of a single mYPet molecule[22,24,31,50,51] (Fig. S2A-C). We

435

also observed a more diffuse pool of mYPet fluorescence, not detected as foci or caused by

436 cell autofluorescence which was negligible. Slimfield images were taken at the approximate
437 cell mid-body so foci at the top or bottom of the cell membrane are outside the depth of field,
438 generating the more diffuse fluorescence observed. By using integrated pixel intensities[27],
439 we determined the total SpoIIE copy number for each cell. Utilizing our stage categorization
440 algorithm we assigned each cell to one of the sporulation stages I-III, and also sub-divided
441 each into three sub-regions – a septum contributed by both mother cell and forespore, a
442 mother cell which excluded the septum, and a forespore which excluded the septum. We then
443 quantified the number of SpoIIE molecules specifically associated with each of these sub-
444 regions for each cell imaged (Fig. 2A, S2D).

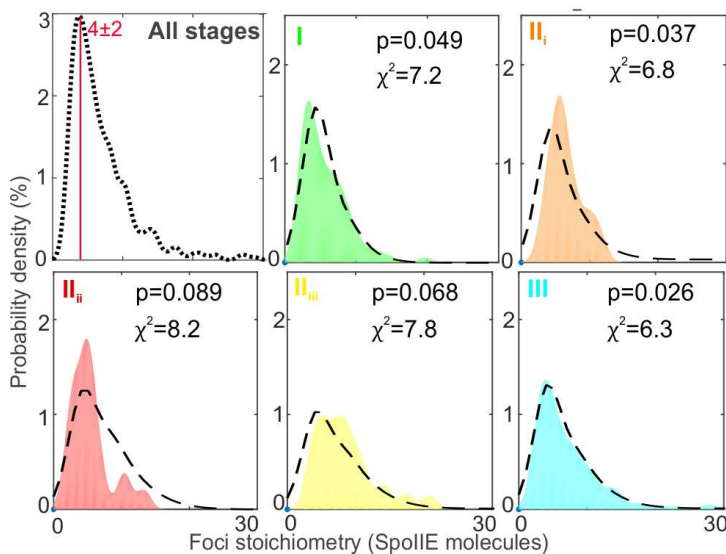
445 These analyses (Table S2) show that the total SpoIIE copy number starts at a few tens
446 of molecules per cell in stage I, increasing as sporulation progresses to ~200 SpoIIE in
447 stage II_i, then rising to 700-800 molecules per cell in stages II_{ii} and II_{iii}, before dropping down
448 to ~580 molecules per cell in stage III after spore engulfment. The mother cell sub-region
449 excluding the septum reflects this trend, increasing SpoIIE copy number from
450 20-80 molecules between stages I-II_i, peaking at ~300 molecules in stages II_{ii}-II_{iii}, then tailing
451 off to ~190 molecules in stage III. Copy number in the septum and forespore also increases
452 throughout sporulation, starting at ~60 copies of SpoIIE at stage II_i increasing to ~400 copies
453 by stage III.

454 A key question is whether the SpoIIE concentration is higher in the forespore than the
455 mother cell, providing an explanation for cell-specific σ^F activation[19]. Our results support
456 this hypothesis, although they are complicated by ambiguity in septal fluorescence, which has
457 potential contributions from both the mother cell and the forespore, since the standard optical
458 resolution limit is greater than the pixel-level precision of image segmentation algorithms.
459 Even excluding the septal region, the forespore concentration of SpoIIE is an order of
460 magnitude higher than that in the mother cell (Fig 2B and C). This increased concentration

461 would arise from equipartitioning of SpoIIE between the mother cell and forespore combined
 462 with the ~6 times smaller volume of the forespore. We use volume as the simplest model here
 463 but similar results are obtained using the ~3 times smaller surface area also to account for
 464 SpoIIE being membrane bound. Intriguingly, arbitrarily attributing the septal fluorescence
 465 equally to the mother cell and forespore, the simplest model considering the ambiguity in
 466 which side it is on, results in approximately equal copy numbers in the two. It has been
 467 shown with an *in vitro* reconstituted system that a ~10-fold increase in the phosphatase
 468 activity of SpoIIE towards SpoIIAA~P is sufficient to release 90 % of σ^F from its inhibitory
 469 complex[52]. However, this imbalance in SpoIIE concentration cannot be immediately
 470 decisive *in vivo* as σ^F activation is delayed until stage II_{ii}. This suggests that following
 471 septation either SpoIIE is not immediately active as a phosphatase, or that following its
 472 dephosphorylation by SpoIIE, SpoIIAA is delayed in its capacity to displace σ^F from its
 473 inhibitory complex with the anti-sigma factor.

474

475 **3.3 SpoIIE is a tetramer whose quaternary organization depends on spatial and**
 476 **temporal localization**

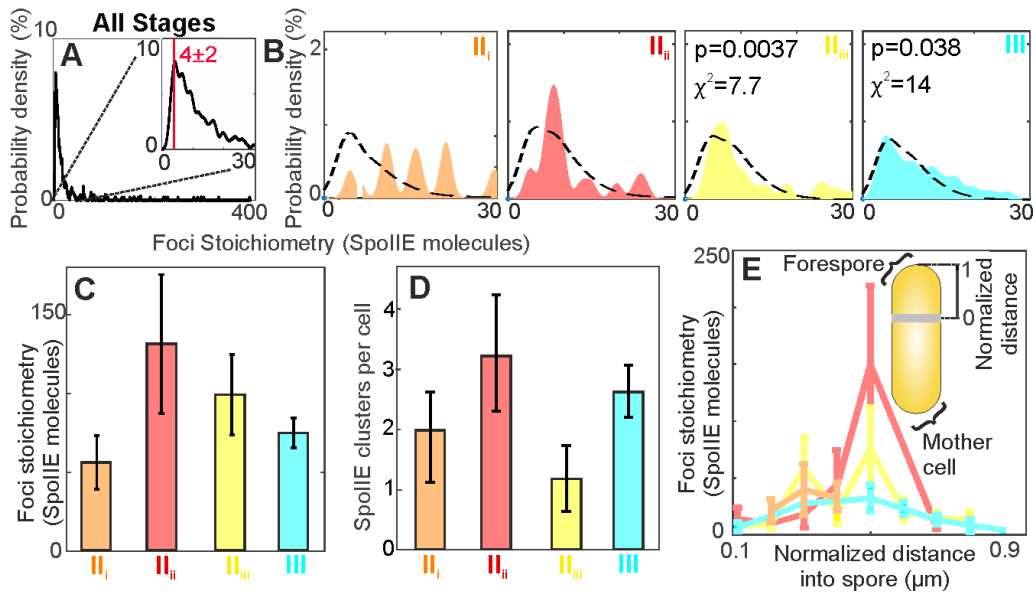


477

478 **Figure 3:** Stoichiometry of SpoIIE in the mother cell. Kernel density estimation (KDE) of the
479 stoichiometry (i.e. number of SpoIIE molecules per detected fluorescent focus) in mother cell
480 with predicted overlap tetramer model (black dotted line). Chi-squared χ^2 and probability of
481 confidence p values indicated. Stage I (green), II_i (orange), II_{ii} (red), II_{iii} (yellow) and III
482 (cyan).

483

484 Next, we sought to characterize the molecular architecture of functional SpoIIE by measuring
485 the stoichiometry of fluorescent foci. In the mother cell, the apparent stoichiometry of tracked
486 foci ranged from as few as two up to several tens of molecules, but with a clear peak at
487 4 ± 2 SpoIIE molecules, conserved throughout stages I-III (Fig. 3). Using a randomized
488 Poisson model for nearest-neighbour foci distances, whose key parameters comprise SpoIIE
489 copy number and foci density, we calculated the probability of foci being separated by less
490 than the optical resolution limit (thus detected as single foci of higher apparent stoichiometry)
491 to be 20-40% in the mother cell. Overlap models which used the raw SpoIIE-mYPet intensity
492 distribution (Fig S2C) in monomers, dimers, hexamers or octamers do not account for the
493 observed stoichiometry distribution (Fig. S3). By contrast, we find that a tetramer overlap
494 model generates reasonable agreement within experimental error for stages I-III in the mother
495 cell (Fig. 3, dashed lines) for all stages, with a corresponding mean probability of confidence
496 value of $p = 0.05$. Thus, we believe the most likely model among those trialled is that SpoIIE
497 in the mother cell comprises predominantly tetramers.



498

499 **Figure 4:** Stoichiometry of SpoIIE in the forespore. (A) Kernel density estimate (KDE) of
 500 forespore stoichiometry pooled for all data, and zoom-in (inset), (B) in separate stages with
 501 overlap tetramer model (black dotted line). (C) Bar chart of mean foci stoichiometry for foci
 502 which have >20 molecules. Stage II_{iii} statistically higher than II_i (p=0.007) (D) Mean number
 503 of foci detected per forespore. (E) Mean foci stoichiometry vs. normalised distance into spore
 504 for each stage, (inset) schematic of forespore distance normalization. Stage I (green), II_i
 505 (orange), II_{ii} (red), II_{iii} (yellow) and III (cyan).

506 For SpoIIE foci in the forespore or the septum, we find the same tetramer peak in the
 507 measured stoichiometry distribution but with a longer tail of higher stoichiometry clusters
 508 extending up to hundreds of SpoIIE molecules per focus (Fig. 4A, B). We adapted the
 509 overlap model to account for different sizes and shapes of sporulation features at each stage
 510 resulting in differences in the density of SpoIIE foci (Fig. 4B). The overlapping tetramer
 511 model accounts only for low apparent stoichiometries near the tetramer peak and only in
 512 stages II_{iii} and III. More generally, accounting for the apparent stoichiometry in the forespore
 513 requires populations of higher order oligomeric SpoIIE clusters in the model fit, in addition to
 514 tetramers. Excluding free tetrameric foci, we observe 1-3 clusters per cell (Fig. 4D) with the

515 mean cluster stoichiometry peaking in stage II_{ii} at >100 molecules per focus (Fig 4C) before
516 decreasing as the proportion of free tetramers increases again in stage III (Fig, S5A, B). We
517 find for foci present in the forespore the measured stoichiometry in all stages was periodic,
518 with a characteristic interval spacing of ~4 molecules (Fig. S4), suggesting that higher order
519 clusters are composed of associating SpoIIE tetramers.

520 Aspects of these *in vivo* observations are consistent with previous *in vitro* experiments.

521 Analytical ultracentrifugation experiments using a soluble fragment of SpoIIE, in which the
522 N-terminal 319 residues, which includes the 10 putative transmembrane segments, were
523 truncated, suggested that SpoIIE(319-872) formed hexamers and larger assemblies composed
524 of multiples of hexamers. [20] A more recent study of a similarly truncated protein
525 SpoIIE(325-872) fragment fused to maltose binding protein demonstrated reversible
526 manganese-dependent oligomerisation as evidenced by changes in sedimentation behaviour
527 and the observation of extended structures (50 nm x 10 nm) using electron microscopy[53] ,
528 although these authors did not speculate on the oligomeric state of the species involved.
529 Fragments of SpoIIE are challenging to express and purify (see also Lucet et al., 2000[12])
530 and their behaviour is sensitive to the size of the truncation. It is therefore not surprising that
531 the full length protein present in the membranes of living cells assembles in a different
532 manner. Whether SpoIIE forms oligomers *in vivo* in the absence of manganese would be an
533 interesting topic of further study.

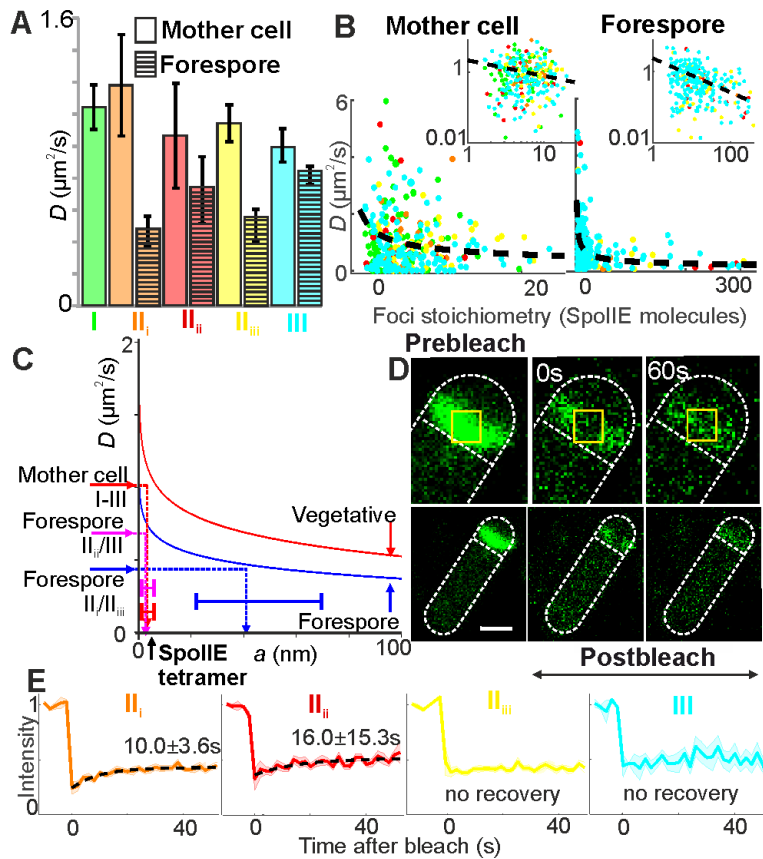
534 We also observed that the stoichiometry of foci in the forespore was influenced by
535 their distance from the septum. We normalized the distance parallel to the long axis of each
536 cell from the mother cell side of the asymmetric septum through to the distal outer edge of
537 the cell containing the smaller forespore cell for all tracked foci and plotted this distance
538 against foci stoichiometry (Fig. 4E and S5C)., For stage II_i, foci are localized to the septum,
539 within ~300 nm , however, other stages contain foci which are delocalized over the full

540 extent of the emerging forespore (Fig. 4E); we find that the mean SpoIIE stoichiometry for
541 these foci increases from ~12 to 150 molecules per focus (a factor of ~12) for stage II_{ii}. This
542 observation supports the recently proposed mechanism for σ^F activation regulation[20]
543 through clustering of SpoIIE in the direction of the pole at stage II_{ii}

544

545

546 **3.4 SpoIIIE foci mobility suggests that large multi-protein assemblies are present in**
 547 **stages II_i and II_{iii}**



548
 549 **Figure 5:** SpoIIIE mobility (A) Bar chart of mean D for each compartment and stage, SEM
 550 errors. Forespore statistically different to mother cell in stages II_i and II_{iii} ($p=0.018, 0.284,$
 551 $0.004, 0.160,$ respectively for each stage. No significant change between each stage in the
 552 forespore, except III, $p=0.003$). (B) Scatter plots of stoichiometry vs. D for all stages in
 553 mother cell and forespore, log-log axes inset (power law model black line). (C) Variation of
 554 D with effective cluster cylinder radius a from frictional drag model, vegetative (i.e. as
 555 opposed to sporulating, shown in red) and forespore (blue) indicated with interpolations from
 556 D to a made from mother cell (all stages), and forespore stages (II_i/II_{iii}) and (II_{ii}/III), SEM
 557 errors. (D) FRAP from representative cell in stage II_i, immediately pre/post-bleach, and ~60s
 558 post-bleach, bleached region indicated (yellow squares). (E) Mean normalised fluorescence
 559 recovery for each stage, SEM bounds shown (shading). Dotted lines show best exponential fit

560 where recovery was detected, t_r and 95% confidence intervals indicated. Stage I (green), II_i
561 (orange), II_{ii} (red), II_{iii} (yellow) and III (cyan). N=10-20 cells per stage.

562

563 We sought to determine the composition and function of clusters by analyzing their mobility
564 in live cells. We find that SpoIIE fluorescent foci mobility in general was consistent with
565 Brownian (i.e. normal) diffusion over short timescales irrespective of cell compartment or
566 stage (Fig. S6, S7). In the mother cell, the mean value of the microscopic diffusion
567 coefficient D was 0.9-1.2 $\mu\text{m}^2/\text{s}$ while that in the forespore was lower by a factor of ~ 2 (Fig.
568 5A, Fig. S7 and Table S2). At the onset of sporulation in stage II_i foci mobility in the
569 forespore is at its lowest with a mean D of $0.43 \pm 0.08 \mu\text{m}^2/\text{s}$, which increases during stage II_{ii}
570 to $0.67 \pm 0.19 \mu\text{m}^2/\text{s}$, then decreases in stage II_{iii} to $0.50 \pm 0.09 \mu\text{m}^2/\text{s}$ before increasing again in
571 stage III to $0.76 \pm 0.05 \mu\text{m}^2/\text{s}$, although only statistically significant in stage III. For stages I-
572 III, D shows a dependence on stoichiometry S , indicating a trend for decreasing D with
573 increasing SpoIIE content (Fig. 5B). Modelling this dependence as $D \sim S^\alpha$ indicates a power-
574 law exponent α of 0.48 ± 0.18 , with no measurable difference within error for each stage
575 (Fig. S7).

576 Calculations of frictional drag on SpoIIE foci, using a consensus value for D from
577 stages I-III for the mother cell, indicate an average Stokes radius (the radius of equivalent
578 cylinder in the membrane) in the range 3-8nm (Fig. 5C, red dashed line). The N-terminal 330
579 residues of SpoIIE are predicted to form a membrane binding domain with 10 transmembrane
580 α -helices[54]. A close packed circular arrangement of these helices, each with a diameter of
581 1.2 nm, would produce a SpoIIE tetramer comprising 40 transmembrane helices with a ~ 4 nm
582 radius, consistent with our experimentally-derived estimate. By contrast, a 'mean' ~ 50 -mer
583 SpoIIE cluster has a Stokes radius of ~ 13 nm. Thus the Stokes radius provides an estimate for

584 the real size of the diffusing SpoIIE complex, including any other protein partners diffusing
585 along with it.

586 For the forespore, the mean value D for higher SpoIIE mobility stages II_{ii} and III
587 indicates a range for Stokes radius consistent with clusters composed solely of SpoIIE
588 tetramers (Fig. 5C, magenta dashed line). However, the low SpoIIE mobility stages II_i and
589 II_{iii} indicate a Stokes radius approximately an order of magnitude higher at ~40 nm (Fig. 5C,
590 blue dashed line), far more than expected for a cluster of only 100 SpoIIE molecules. This
591 observation supports a model in which SpoIIE interacts with other proteins or complexes,
592 with these other unlabeled proteins here forming ~5x the SpoIIE foci surface area in the
593 membrane, increasing the apparent Stoke's radius. In stage II_i interactions would be with
594 components of the divisome[12–14] while in stage II_{iii} they would be with SpoIIQ, the
595 forespore component of an intercellular channel formed with proteins encoded on the *spoIIIA*
596 operon expressed in the mother cell[15]. In stage II_{ii} we find clusters of SpoIIE are likely not
597 associated with a protein partner as the Stokes radius is consistent only with the SpoIIE
598 present. This finding is also consistent with σ^F activation regulation[20].

599

600 **3.5 Forespore SpoIIE turnover depends on sporulation stage**

601 Using confocal microscopy of a similar cell strain but using monomeric GFP labelled
602 SpoIIE (i.e. SpoIIE-mGFP) we performed fluorescence recovery after photobleaching
603 (FRAP) experiments to photobleach the asymmetric septum at different stages and monitor
604 any subsequent fluorescence recovery (Fig. 5D). During stages II_i and II_{ii} there is a relatively
605 slow recovery with mean exponential recovery time t_r of 10 ± 3.6 s and 16.0 ± 15.3 s respectively
606 (Fig. 5E, S7). Our finding that t_r is not directly correlated to D in each stage suggests that
607 turnover here is reaction- as opposed to diffusion-limited; it may be limited by an effective

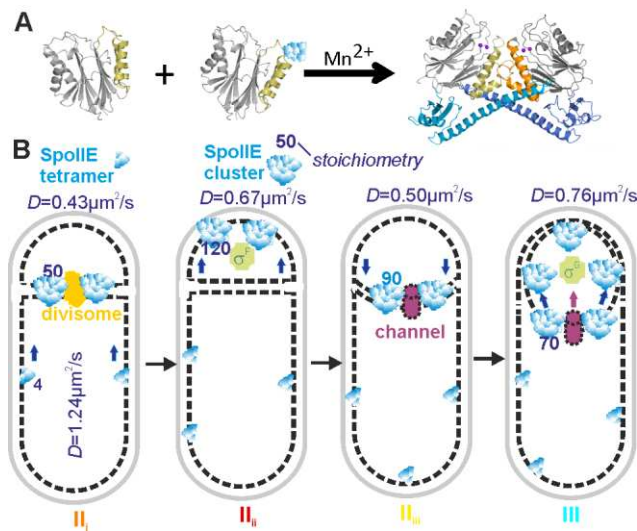
608 off-rate as observed in other complex bacterial structures such as components of the flagellar
609 motor or replisome[55,56]. In subsequent stages II_{iii} and III, no recovery is detectable within
610 error, though lower levels of fluorescence and numbers of cells in stage III result in higher
611 measurement noise which limits the sensitivity for detecting low levels of putative recovery.

612 Divisome components such as FtsZ have been shown to turnover in similar FRAP
613 studies[57], consistent with our stage II_i findings when SpoIIE associates with the divisome.
614 Turnover is also expected at stage II_{ii} when SpoIIE is released. At stage II_{iii} SpoIIE interacts
615 with the SpoIIQ-SpoIIIAH channel which may account for the lack of turnover. A similar
616 absence is unexpected at stage III when SpoIIE is released and has no known function. This
617 suggests that at stage III, SpoIIE is released quickly then anchored into the spore, or that the
618 viscosity in spore itself has changed as has been shown to occur during sporulation[40].

619

620

621 **4. Discussion**



622

623 **Figure 6:** Model of SpoIIE dynamics during sporulation. (A) Activation of phosphatase
 624 involves dimerization, and recruitment of Mn²⁺ ions. Isolated phosphatase, SpoIIE(590-827)
 625 (PDB: 5MQH), is monomeric while a longer fragment, SpoIIE(457-827) (PDB: 5UCG),
 626 forms dimers about an interface dominated by a long helix spanning residues 473-518.
 627 Subunits in dimer distinguished by shading: PP2C domains (gray), switch helices (orange),
 628 regulatory domains (blue), Mn²⁺ ions (purple spheres, modeled onto structure following
 629 superposition of PstP structure from *M. tuberculosis* PDB: 1TXO). (B) Schematic of SpoIIE
 630 architecture and *D* at each stage: SpoIIE tetramers (blue), divisome (yellow), activated sigma
 631 factors (green), and SpoIIQ channel (purple) shown. Arrows indicate SpoIIE's release or re-
 632 capture to the septa.

633 SpoIIE performs multiple important functions. For example, it is essential to form a
 634 proper sporulation septum as well as to activate SigF. Without SpoIIE no spore can be
 635 formed and also there are many point mutations characterized in *spoIIE* which cause
 636 complete arrest of cell differentiation. However, how it switches roles at different stages has
 637 been unclear. It is not known how SpoIIE localizes to the polar septum, how it causes FtsZ to
 638 relocalize from mid-cell to one of the cell poles, what role it plays in septal thinning, or how

639 its SpoIIAA-P phosphatase activity is controlled so that σ^F activation is delayed until the
640 asymmetric septum is completed[7,11]. How SpoIIE brings about forespore-specific
641 activation of σ^F is a subject of particular interest[58]. Plausible suggested mechanisms
642 include preferential SpoIIE localization on the forespore face of the septum[59], transient
643 gene asymmetry leading to accumulation of a SpoIIE inhibitor in the mother cell[58], and the
644 volume difference between compartments leading to higher specific activity of
645 equipartitioned SpoIIE[60,61]. Most recently, it was shown that mother cell restricted
646 intracellular proteolysis of SpoIIE by the membrane bound protease FtsH is important for
647 compartment-specific activation of σ^F [20]. Our findings indicate that SpoIIE operates as an
648 oligomer whose stoichiometry and mobility switch in the forespore according to specific
649 sporulation stage, driving morphological changes, as opposed to changes being primarily
650 dependent on the differential effective concentration of SpoIIE in either mother cell or
651 forespore. In particular, complexes comprising four SpoIIE molecules predominate in the
652 mother cell and at multiple stages in the forespore. Crucially, we observe reversible assembly
653 of these tetrameric SpoIIE entities into higher order multimers during stage II_{ii} when the
654 protein localizes towards the pole and its latent protein phosphatase activity is manifested.

655 Unlike previous microscopy of YFP-labelled SpoIIE which suggested a pattern of
656 localization almost exclusively in the forespore following asymmetric septation[20], our
657 higher sensitivity shows SpoIIE content is at most 10-30% greater in the early forespore and
658 septum compared to the mother cell if all of the SpoIIE in the septum is assigned to the
659 forespore. An equipartition of septal SpoIIE results in approximately equal copy number in
660 the mother cell and forespore. However, the more than 6 times smaller forespore volume[19]
661 results in a higher SpoIIE concentration by a factor of ~6-8, depending on partitioning of
662 septal SpoIIE. It was shown previously that a 10-fold difference in SpoIIE phosphatase
663 activity towards its substrate SpoIIAA~P could account for all-or-nothing compartmental

664 regulation of σ^F activity[62]. The bias towards higher copy number values in the forespore
665 aligns with the recent suggestion that SpoIIE captured at the forespore pole is protected
666 against proteolysis[20]. In this model, SpoIIE sequestered in the polar divisome, is handed-
667 off to the adjacent forespore pole following cytokinesis. This forespore polar SpoIIE is
668 protected from FtsH-mediated proteolysis by oligomerisation, which is clearly described by
669 our observations. Compartment specificity results from the proximity of the forespore pole to
670 the site of asymmetric division.

671 Crystallographic and biophysical studies reveal that SpoIIE(590-827), comprising the
672 phosphatase domain, is a monomer while SpoIIE(457-827), comprising the phosphatase plus
673 part of the upstream regulatory domain, is dimeric (Fig. 6A)[63,64]. Comparison of these
674 structures and mapping of mutational data onto them led to the proposal that PP2C domains
675 in SpoIIE(590-827) and SpoIIE(457-827) crystals represent inactive and active states
676 respectively. Activation is accompanied by a 45° rigid-body rotation of two ‘switch’
677 helices[64]. This switch is set by a long α -helix in the regulatory domain which mediates
678 dimerization (Fig. 6A). Movement of the switch helices upon dimerization translates a
679 conserved glycine (Gly629 in SpoIIE) into the active site where it can participate in
680 cooperative binding to two catalytic manganese ions. These ions are conserved in PP2C
681 phosphatases and here would be expected to activate a water molecule for nucleophilic attack
682 at the phosphorus of the phosphorylated serine 57 residue in SpoIIAA-P. The increase in
683 SpoIIE stoichiometry observed upon activation *in vivo* is consistent with these structural
684 findings although clearly larger assemblies are implied. We can speculate on the basis of the
685 data presented here these larger assemblies arise from further homomeric quaternary
686 interactions mediated by the substantial membrane binding domain and/or the component of
687 the regulatory domain which has yet to be fully characterized. The results are consistent with
688 the hand-off model[20] in which release from the divisome allows SpoIIE tetramers to

689 diffuse away from the septum and self-associate to form high stoichiometry clusters in a
690 spontaneous process with similarities to that observed for the plasmalemmal protein
691 syntaxin[65]. We speculate that the free energy of reassembly is used to flip the helical
692 switch, allowing manganese acquisition and activation of phosphatase activity.

693 Changes in oligomeric state and quaternary organization of proteins are widespread
694 mechanisms for regulating biological activity. These can be induced variously by binding of
695 allosteric ligands, covalent modification, proteolytic processing and reversible interactions
696 with protein agonists or antagonists. SpoIIE, which transitions between complexes which are
697 unusually large, is transiently active as a phosphatase after its release from an inhibitory
698 complex with the divisome. Regulation of phosphatase activity through sequestration is also
699 seen in adaptation to drought in plants; the phosphatase HABI dephosphorylates the kinase
700 SnRK2, inhibiting transcription of drought tolerance genes until the complex of the hormone,
701 abscisic acid and its receptor, PYR, binds to and inhibits the phosphatase HABI[66].

702 Our findings show more generally that we can combine robust cell categorization with
703 single-molecule microscopy and quantitative copy number and stoichiometry analysis to
704 follow complex morphologies during differentiation (Fig. 6B). Importantly, these tools
705 provide new insight into the role of SpoIIE by monitoring its molecular composition and
706 spatiotemporal dynamics, linking together different stages of cell development. Our findings
707 show that the function of a key regulatory protein can be altered depending upon its state of
708 multimerization and mobility, enabling different roles at different cell stages. Future
709 applications of these methods may involve multicolor observations of SpoIIE with other
710 interaction partners at different sporulation stages. Optimising these advanced imaging tools
711 in the model Gram-positive *B. subtilis* may ultimately enable real time observations of more
712 complex cellular development, paving the way for future studies of tissue morphogenesis in
713 more challenging multicellular organisms. More generally our findings demonstrate that the

714 application of super-resolved single-molecule optical proteomics biotechnology can enable
715 new mechanistic insight into complex cell stage dependent processes in single living cells
716 which are technically too challenging to achieve using traditional methods. Such findings are
717 made possible by a range of innovative computational tools to categorise cell cycle stage and
718 to quantify single-particle tracks, and enable not only new understanding of the dynamic
719 patterns of spatial localization of a key protein used in triggering cell development, but also
720 in posing questions about its structural properties at different cell cycle stages.

721

722

723 **Data availability.** Data included in full in main text and supplementary files. Raw data
724 available from authors.

725

726 **Software access.** Code written in MATLAB available from Sporulationanalyser at
727 <https://sourceforge.net/projects/york-biophysics/>

728

729 **Declaration of Interests** The authors declare no conflict of interest.

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