

1 **Title:**

2 Imaging minimal bacteria at the nanoscale: a reliable and versatile process to perform Single
3 Molecule Localization Microscopy in mycoplasmas

4

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16 **Running Head:**

17 Super-Resolution Microscopy in Mycoplasmas

18

19 **Abstract:**

20 Mycoplasmas are the smallest free-living organisms. These bacteria are important models for both
21 fundamental and Synthetic Biology, owing to their highly reduced genomes. They are also relevant
22 in the medical and veterinary fields, as they are pathogenic of both humans and most livestock
23 species. Mycoplasma cells have minute sizes, often in the 300-800 nanometers range. As these
24 dimensions are close to the diffraction limit of visible light, fluorescence imaging in mycoplasmas
25 is often poorly informative. Recently developed “Super-Resolution Imaging” techniques can break
26 this diffraction limit, improving the imaging resolution by an order of magnitude and offering a
27 new nanoscale vision of the organization of these bacteria. These techniques have however not
28 been applied to mycoplasmas before. Here, we describe an efficient and reliable protocol to
29 perform Single-Molecule Localization Microscopy (SMLM) imaging in mycoplasmas. We provide
30 a polyvalent transposon-based system to express the photo-convertible fluorescent protein

31 mEos3.2, enabling Photo-Activated Localization Microscopy (PALM) in most *Mycoplasma*
32 species. We also describe the application of direct STochastic Optical Reconstruction Microscopy
33 (dSTORM). We showcase the potential of these techniques by studying the subcellular localization
34 of two proteins of interest. Our work highlights the benefits of state-of-the-art microscopy
35 techniques for mycoplasmology and provides an incentive to further the development SMLM
36 strategies to study these organisms in the future.

37

38 **Importance:**

39 Mycoplasmas are important models in biology, as well as highly problematic pathogens in the
40 medical and veterinary fields. The very small size of these bacteria, well below the micron, limits
41 the usefulness of traditional fluorescence imaging methods as their resolution limit is similar to the
42 dimensions of the cells. Here, to bypass this issue, we established a set of state-of-the-art “Super-
43 Resolution Microscopy” techniques in a wide range of *Mycoplasma* species. We describe two
44 strategies: PALM, based on the expression of a specific photo-convertible fluorescent protein; and
45 dSTORM, based on fluorophore-coupled antibody labeling. With these methods, we successfully
46 performed single-molecule imaging of proteins of interest at the surface of the cells and in the
47 cytoplasm, at lateral resolutions well below 50 nanometers. Our work paves the way toward a better
48 understanding of mycoplasma’s biology through imaging of subcellular structures at the nanometer
49 scale.

50

51 **Introduction:**

52 The colloquial term “mycoplasmas” refers to a set of bacteria belonging to the *Mollicutes* Class.
53 These organisms derive from a common ancestor with Firmicutes through a degenerative evolution
54 that has led to an extreme reduction in genome size (~0.6-1.35 Mbp). During this process,
55 mycoplasmas have lost a large number of genes coding for important pathways, resulting in their
56 characteristic lack of a cell wall and limited metabolic capacities (1-3). Owing to these
57 deficiencies, mycoplasmas are obligate parasites that rely on their hosts for the production of a
58 large array of essential metabolites. They have been isolated from a wide range of animals,
59 including humans, mammals, reptiles, fish and arthropods.

60 Mycoplasmas are the simplest self-replicating organisms known to date and are thought to be good
61 representatives of a “minimal” cell (4–6). They are therefore extremely interesting models in
62 fundamental biology and have been used extensively to study the basic principles governing living
63 systems and gene essentiality (7–11). These bacteria are also highly relevant in the field of
64 Synthetic Biology, as their simplicity makes them prime models for the creation of engineered
65 living systems. Mycoplasmas have been at the center of landmark studies such as the production
66 of the first cell governed by a chemically synthesized genome, and later of the first minimal
67 synthetic bacterial cell (12, 13). Mycoplasmas are also the first cells for which complete and
68 accurate predictive mathematical models have been developed (14–16).

69 In parallel to these fundamental aspects, mycoplasmas are also highly problematic organisms in
70 both the medical and veterinary fields as most of them are pathogenic for their hosts. In human,
71 two species are particularly prevalent and concerning: *Mycoplasma genitalium*, which causes
72 sexually transmissible urogenital infections (17); and *Mycoplasma pneumoniae* which causes
73 “atypical pneumonia” predominantly in children and immunocompromised patients (18). While
74 both pathogens typically cause mild diseases with low mortality, these infections are often chronic
75 and the pathogens are not completely eliminated after antibiotics treatments (19, 20). Mycoplasmas
76 also infect most livestock species and are major pathogens of cows (*Mycoplasma bovis*) (21), goats
77 and sheeps (*Mycoplasma capricolum* subsp. *capripneumoniae*) (22), pigs (*Mycoplasma*
78 *hyopneumoniae*) (23) and chickens (*Mycoplasma gallisepticum*) (24). Depending on the particular
79 bacterial species or strain, mycoplasma infections can range from chronic, low mortality
80 inflammatory diseases to peracute, highly lethal diseases. Infected animals often display heavily
81 reduced production yields, endangering farmers’ revenues and threatening food security in poorly
82 developed countries.

83 Studying mycoplasmas is a tedious process, as these organisms are slow growing and require
84 complex and undefined culture media. In addition, the number of genetic tools available is limited,
85 except for a small set of species belonging to the *Mycopoides* cluster benefiting from techniques
86 derived from the aforementioned Synthetic Biology projects (25).

87 The physical size of mycoplasmas is also a key limiting factor, as most species have cells with
88 dimensions in the 300-800 nm range. These values are close to the resolution of diffraction-limited
89 optical microscopy, which is in the 200–300 nm range with commonly used dyes and high

90 numerical aperture oil immersion objectives. Thus, fluorescence microscopy in mycoplasmas is
91 often poorly informative, as it is extremely difficult to determine the subcellular localization of the
92 imaged component. This problem exists for most bacteria and archaea, and is exacerbated for
93 mycoplasmas.

94 Higher resolution techniques based on immunogold labelling and electron microscopy have
95 therefore been preferred to localize proteins at the cell surface or in the cytoplasm of mycoplasma
96 cells (26–30). However, these methods suffer from complex sample preparation protocols, are
97 difficult to set up for simultaneous visualization of multiple molecular species and are not
98 compatible with live-cell imaging.

99 To this date, only a handful of studies have used immuno-fluorescence to study protein localization
100 in mycoplasmas, and all of them have focused on ascertaining the polar distribution of proteins in
101 the cells of *Mycoplasma mobile*, *M. pneumoniae* and *M. genitalium* which all exhibit highly
102 polarized shapes (31–34). Similarly, a small number of studies have reported the polar localization
103 or co-localization of proteins fused to the fluorescent proteins mCherry and EYFP, again in highly
104 polarized cells (35–39). Other fluorescent proteins have successfully been expressed in several
105 *Mycoplasma* species, including GFP (40), Venus (41), mNeonGreen, and mKO2 (42), but have
106 only been used as expression reporters or transformation markers.

107 Interestingly, the last decade has seen the rapid development of multiple new fluorescence
108 microscopy techniques aimed at bypassing the diffraction limit and bridging the gap between
109 optical imaging resolution (~200 nm) and electron microscopy resolution (~1 nm). These methods,
110 broadly termed “Single Molecule Localization Microscopy” (SMLM), rely on the successive
111 imaging of individual fluorophores to mathematically determine their exact position (43–45). The
112 spatiotemporal decorrelation of fluorescence emissions can be achieved in a stochastic manner,
113 through the use of specific dyes or fluorescent proteins that can be made to randomly emit their
114 fluorescence under wide-field illumination. Techniques such as Photo-Activated Localization
115 Microscopy (PALM), Stochastic Optical Reconstruction Microscopy (STORM) or Points
116 Accumulation for Imaging in Nanoscale Topography (PAINT) can typically yield images with
117 lateral resolutions of ~10-50 nm. (46).

118 These methods have considerably expanded the possibilities of imaging in biology, allowing to
119 resolve the subcellular organization of individual molecules or molecular assemblies, such as

120 nuclear pores, chromatin complexes and cytoskeletal filaments at resolutions close to the molecular
121 scale (47). The resolution improvements offered by SMLM appear especially attractive for
122 microbiologists. These methods have been progressively adopted by the scientific communities,
123 first through their establishment in model bacteria such as *Escherichia coli* and *Bacillus subtilis*,
124 and then by gradual transfer to more specialized fields (48–50). They have however not yet been
125 applied to mycoplasmology.

126 In this report, we present the first protocols for the investigation of mycoplasmas using SMLM.
127 We demonstrate the feasibility of PALM throughout the *Mollicutes* Class by successfully
128 expressing a photo-convertible fluorescent protein in six highly relevant *Mycoplasma* species.
129 Then, working in the model *Mycoplasma mycoides* subsp. *capri* (*Mmc*), we use PALM to image
130 the subcellular localization of the cytoplasmic domain of an atypical F-type ATPase complex,
131 yielding images with a lateral resolution of ~40 nanometers. In parallel, we apply dSTORM in the
132 same model to study the localization of a surface-anchored protease involved in virulence, yielding
133 images at a lateral resolution of ~25 nanometers.

134

135 **Material and Methods:**

136 *** Bacterial strains and culture conditions:**

137 *Escherichia coli* strain NEB-5α, used for plasmid cloning and propagation, was grown at 37°C in
138 LB media supplemented with antibiotics (tetracycline 10 µg/mL; kanamycin 50 µg/mL; ampicillin
139 100 µg/mL) when selection was needed.

140 *Mycoplasma mycoides* subsp. *capri* strain GM12 (*Mmc*), *Mycoplasma capricolum* subsp.
141 *capricolum* strain 27343 (*Mcap*), *Mycoplasma bovis* strain PG45 (*Mbov*), *Mycoplasma agalactiae*
142 strain PG2 (*Maga*), *Mycoplasma genitalium* strain G37 (*Mgen*), *Mycoplasma gallisepticum* strain
143 S6 (*Mgal*) were grown at 37°C in appropriate media: SP5 (51), Hayflick modified (52) or SP4
144 modified (52, 53), supplemented with antibiotics (gentamycin 100-400 µg/mL; tetracycline 5
145 µg/mL) when selection was needed.

146

147 *** Cloning the mEOS3.2 expression plasmids**

148 The mycoplasma codon-optimized version of the coding sequence for mEos3.2 was designed using
149 the online tool JCat (<http://www.jcat.de/>; parameters: input= protein sequence, reference

150 organism= *Mycoplasma mycoides* (subsp. *mycoides* SC, strain PG1), options= “Avoid prokaryotic
151 ribosome binding sites”). Further adaptation of the codon usage was performed manually based on
152 the recommendation of the Twist Bioscience synthesis tool, to enable the chemical synthesis of the
153 corresponding DNA fragment (Twist Bioscience). After PCR amplification, the coding sequence
154 was cloned by InFusion (Clontech) in the plasmid pMT85 under the control of the promoter PTufA
155 (42). Based on this pMT85-PTufA-mEos3.2 plasmid, three other variants were subsequently
156 produced by Gibson Assembly (NEB) or by site-directed mutagenesis (NEB) to replace the original
157 promoter by either the promoter PSpi (51), the promoter PSynMyco (54), or the promoter P438
158 (55)). Cloned plasmids were transformed in chemically competent *E. coli* NEB-5 α for maintenance
159 and propagation, then isolated by miniprep, verified by enzymatic restriction and finally checked
160 by Sanger sequencing (Genewiz).

161
162 * *Mycoplasmas transformation*
163 Plasmids pMT85-PSynMyco-mEos3.2, pMT85-PSpi-mEos3.2 or pMT85-P438-mEos3.2 were
164 transformed by PolyEthylene Glycol contact in *Mmc*, *Mcap* (56), *Maga* and *Mbov* (57) and *Mgal*
165 (unpublished); or by electroporation in *Mgen* (58). Transformants were subsequently plated on
166 appropriate media plates containing antibiotics for selection (see above). Transformants clones
167 were then passaged 3 times in liquid media supplemented with gentamycin (100-400 μ g/mL). The
168 presence of the mEOS3.2 coding sequence was checked by PCR.

169
170 * *Production of Mycoplasma mycoides subsp. mycoides mutants*
171 Accurate edition of the genome of *Mmc* is currently only possible through a complex process
172 involving the transfer of the bacterial chromosome in a yeast cell, its modification in the yeast, and
173 its subsequent transplantation back in a *Mcap* bacterial recipient cell (59). This process was used
174 to generate two mutant strains: *Mmc* 0582-HA in which an HA-tag coding sequence is fused to the
175 C-terminus of the coding sequence of MMCAP2_0582; and *Mmc* mEos3.2-0575 in which the
176 codon-optimized mEos3.2 coding sequence is fused to the N-terminus of the coding sequence of
177 MMCAP2_0575. To do so, we first generated two plasmids encoding guide RNAs targeting either
178 MMCAP2_0582 or MMCAP2_0575 by modification of the base plasmid p426-SNR52p-gRNA.Y-
179 SUP4t (25) using the Q5 Site-Directed Mutagenesis kit (NEB). In addition, we generated two

180 recombination cassettes that contain the modified version of the target loci flanked by 1000 bp
181 recombination arms. Both cassettes were built by first cloning the wild-type locus and 1 kb flanking
182 region in the pGEM-T plasmid (Promega) and subsequently modifying the plasmid to add the HA-
183 tag coding sequence using the Q5 Site-Directed Mutagenesis kit (NEB) or the mEos3.2 coding
184 sequence using InFusion (Clontech). Plasmids carrying the cassettes were checked by Sanger
185 sequencing (Genewiz), and the cassettes amplified by PCR. Both the guide RNA-encoding plasmid
186 and the recombination cassette were then co-transformed in the yeast carrying the *Mmc*
187 chromosome. Yeast transformants were checked for the presence of the integral bacterial
188 chromosome by multiplex PCR and for the presence of the desired mutation by PCR and amplicon
189 sequencing. The modified genome was then extracted and transplanted in the recipient cell. The
190 resulting transplants were also checked for genome integrity by multiplex PCR and for the presence
191 of the desired mutation PCR and amplicon sequencing.

192

193 * Sample preparation for SMLM experiments

194 Mycoplasmas cells, grown to late log phase at 37°C in appropriate media supplemented with
195 gentamycin (100-400 µg/mL), were harvested by centrifugation at 6800 rcf, 10°C for 10 minutes.
196 After removal of the spent media, the cells were washed twice in one volume of buffer (HEPES
197 67.7 mM - NaCl 140 mM - MgCl₂ 7 mM - pH 7.35), and subsequently resuspended in 1/3rd volume
198 of the same buffer. When necessary, cell aggregates were broken down by performing 20 passages
199 through a 26 Gauge needle. A poly-L-lysine coated 1.5H 18 mm precision coverslip (Marienfeld)
200 was placed at the bottom of a 12-well plate and equilibrated in 1 mL of wash buffer. Three
201 microliters of the cell suspension were added to the well, and the plate was subsequently
202 centrifuged in a swing-out rotor at 2500 rcf, 10°C for 10 minutes to force the cells to sediment on
203 the coverslip. The well was emptied by suction, and the coverslip was then moved to a clean well.
204 The coverslip was then washed once with 3 mL wash buffer and then incubated in 1 mL of a pre-
205 warmed 4% PFA in washing buffer, at 37°C for 30 min. Five washing steps with 3 mL of wash
206 buffer were performed to eliminate all PFA traces. Correct deposition and fixation of the cells on
207 the coverslip was checked by dark-field microscopy (Nikon Eclipse Ni microscope equipped with
208 a dark field condenser and a Teledyne Photometrics Iris 9 camera) using washing buffer as

209 mounting medium. Validated coverslips were stored at 4°C in PBS until ready to use for PALM
210 imaging (no more than 10 days).

211 For dSTORM imaging, coverslips were prepared as described above. After the final wash, each
212 coverslip was incubated 1 hour at room temperature in 1 mL of blocking buffer (PBS/BSA 1%).
213 Immuno-labeling was performed by first using a mouse anti HA-tag primary antibody (Thermo)
214 diluted at 1/500th in blocking buffer. After incubation for 1 hour at room temperature, the coverslip
215 was washed three times with 1 mL of PBS. Then, the coverslip was incubated for 1 hour at room
216 temperature with the secondary antibody (goat anti-mouse IgG conjugated to the Alexa647;
217 Jackson ImmunoResearch) diluted at 1/500th in 1 mL of blocking buffer. After three wash steps in
218 PBS, an additional fixation was performed by incubating the labeled coverslips with 2% PFA in
219 PBS for 5 minutes at 37°C. Finally, the coverslips were washed five times in wash buffer and stored
220 at 4°C in PBS until imaging (no more than 10 days).

221
222 * SMLM imaging set up and data collection
223 Imaging was performed on a LEICA DMi8 inverted microscope, mounted on an anti-vibrational
224 table (TMC, USA), using a Leica HC PL APO 100 × 1.47 NA oil immersion TIRF objective and
225 fiber-coupled laser launch (405 nm, 488 nm, 532 nm, 561 nm and 642 nm) (Roper Scientific, Evry,
226 France). Fluorescence signal was collected with a sensitive Evolve EMCCD camera (Teledyne
227 Photometrics). The coverslips bearing the fixed bacterial cells were mounted on a Ludin chamber
228 (Life Imaging Services) and 600 µL of imaging buffer was added. For PALM imaging, the buffer
229 was PBS. For dSTORM imaging, the buffer contained both oxygen scavenger (glucose oxidase)
230 and reducing agent (2-Mercaptoethylamine), and another 18 mm coverslip was added on top of the
231 chamber to minimize oxygen exchanges during the acquisition.

232 Image acquisition and control of the microscope were driven through Metamorph (Molecular
233 devices) in streaming mode using a 512 x 512 pixels region of interest with a pixel size of 160 nm.
234 Image stacks typically contained 6,000–20,000 frames, acquired at a frequency of 33 Hz for PALM
235 and 50 Hz for dSTORM. The power of the 405 nm laser was adjusted to control the density of
236 single molecules per frame, keeping the 642 nm laser intensity constant. For dual-color imaging,
237 dSTORM was performed first followed by PALM. To limit manipulation and the potential
238 resulting drift, coverslips were kept in dSTORM imaging medium during the PALM acquisition.

239
240 * SMLM data processing and analysis
241 The PALMTracer plugin for Metamorph (60–62) was used to process image stacks with a specific
242 intensity threshold for each dataset, to enable the generation of the localization tables. From these
243 tables, super-resolved images were generated with a pixel dimension of 40 nm. Pointing accuracies
244 of the PALM imaging experiments were determined by tracking individual fluorophore's motion
245 using PALMTracer. For each track with a speed below $0.01 \mu\text{m}^2.\text{s}^{-1}$, the half of the root of the
246 MSD0 value was calculated (63). PALM experiments are done with a pointing accuracy of 41 nm
247 in average). Resolution of dSTORM imaging experiments was determined by Gaussian fitting of
248 the signal of individual fluorophores attached to the coverslip. Both resolution (66 nm) and pointing
249 accuracy (25 nm) are extracted.
250 Clustering of the localizations was performed by Voronoï segmentation using SR-Tesseler (64).
251 Object detection and clustering characterization were done using a density factor of 1, and a
252 minimum cluster area of 0.05 pixel². The cell-counter plugin of Fiji was used to count the number
253 of cells present in our acquisition field. Dot plots were graphed using PlotsOfData (65).
254

255 **Results:**

256 1. Establishing a common and efficient sample preparation process for SMLM imaging in
257 mycoplasmas

258 The production of high-quality samples is generally regarded as a critical step for the acquisition
259 of high-quality SMLM data, and multiple reviews provide important guidelines to follow (66, 67).
260 Here, the first step of the process was to ensure the reliable immobilization of the mycoplasma cells
261 to high-precision glass coverslips. We initially attempted to grow the mycoplasmas directly onto
262 poly-L-lysine coated coverslips by immersing the coverslips in inoculated media. However, this
263 approach failed as cells remained planktonic and did not adhere to the glass. We thus developed an
264 efficient and reliable centrifugation-based process to force the cells to sediment and attach to the
265 coverslip (Figure S1). Briefly, mycoplasma cells are harvested and washed to form a homogenous
266 medium-density suspension (approximately 10^5 - 10^7 cfu.mL⁻¹) in which the coverslip is immersed,
267 in a 12 well plate. Centrifugation at low speed in a swing-out rotor forces the cells to sediment and
268 adhere to the glass. After washing to remove unbound cells, fixation is performed using a solution

269 of 4% paraformaldehyde. The coverslip quality is checked by observation with a dark-field
270 microscope. A good sample is characterized by bacterial cells deposited in a monolayer, regularly
271 spaced and separated from each other by a few microns. These samples can either be imaged
272 directly using PALM or further processed by immuno-labeling in order to later perform dSTORM.
273

274 2. Establishing a polyvalent mEos3.2 expression system for PALM in multiple mycoplasmas

275 PALM imaging is based on the expression of specific fluorescent proteins that are either
276 photoactivatable (irreversible off-to-on) or photo-convertible (wavelength A to wavelength B)
277 (68). This process is driven by light, and can be tuned to occur at a slow rate, thus enabling imaging
278 of individual fluorophores. Here, we elected to use the fluorescent protein mEos3.2 (69), which
279 can be converted by illumination with a near-UV wavelength (405 nm) from a green state (Ex=
280 507 nm, Em= 516 nm) to a red state (Ex= 572 nm, Em= 580 nm).

281 To assess the functionality of mEos3.2 in a wide range of *Mycoplasma* species, we first designed
282 a mEos3.2 codon-optimized coding sequence using the codon usage table of *Mycoplasma mycoides*
283 subsp. *mycoides* strain PG1 as reference. This coding sequence was subsequently cloned in the
284 plasmid pMT85 (42, 58, 70–74). The plasmid backbone carries a transposon derived from Tn4001
285 (75), the insertion of which can be selected through the gentamicin resistance gene *aacA-aphD*
286 placed under the control of its natural promoter. In order to drive the expression of the mEos3.2,
287 we used the recently developed synthetic regulatory region PSynMyco (54). These three elements
288 (transposon, selection marker and SynMyco regulatory region) have all been shown to be
289 functional in a wide range of *Mollicutes* species, and should yield a “mycoplasma-universal”
290 mEos3.2 expression vector.

291 The resulting plasmid pMT85-PSynMyco-mEos3.2 (Figure 1A) was transformed in six
292 *Mycoplasma* species, relevant to either the veterinary or medical fields, and covering the three main
293 *Mollicutes* phylogenetic sub-groups (Figure 1B): *i*) *Mycoplasma mycoides* subsp. *capri* strain
294 GM12 (*Mmc*) and *Mycoplasma capricolum* subsp. *capricolum* strain 27343 (*Mcap*), Spiroplasma
295 group; *ii*) *Mycoplasma bovis* strain PG45 (*Mbov*) and *Mycoplasma agalactiae* strain PG2 (*Maga*),
296 Hominis group; and *iii*) *Mycoplasma genitalium* strain G37 (*Mgen*) and *Mycoplasma gallisepticum*
297 strain S (*Mgal*), Pneumoniae group. Transformants carrying the transposon were obtained for all
298 six species, and sample coverslips were prepared for each.

299 We then assessed the ability of the mEos3.2 to be converted to its red state by performing PALM
300 imaging. Cells were observed in the red wavelength, with low power illumination at 405 nm to
301 sparsely and stochastically photo-convert the mEos3.2. Based on the acquired image stacks, the
302 localization of each individual fluorescence emitter was determined through the PALMtracer
303 plugin under Metamorph, then, super-resolved images are reconstructed and analyzed by automatic
304 Voronoï-based segmentation of these localizations (Figure 1C, Figure S2). The principle of
305 tessellation analysis is to extract objects with a similar density of localization. Here, with the first
306 level of segmentation we isolated the property of individual mycoplasma cells, with the second
307 level yielding data on clustering of the protein of interest.

308 For all the six *Mycoplasma* species studied, wild type and mutant, a comparable number of cells
309 per field of view is observed in transmission light (Figure S3A). For each first level segmented
310 object, the number of underlying detections was compared between the wild-type and mEos3.2
311 expressing mycoplasmas. Similar results were obtained for each of the six *Mycoplasma* species
312 studied (Figure 1D). For the wild-type cells, only a small number of individual objects were
313 identified (*Mmc*: 240, *Mcap*: 291, *Mbov*: 79, *Maga*: 99, *Mgen*: 110, *Mgal*: 50), with each being
314 supported by a small number of detections (median detections/object: *Mmc*: 42, *Mcap*: 31, *Mbov*:
315 43, *Maga*: 63, *Mgen*: 61, *Mgal*: 79). Amongst them, a large fraction of the objects were found
316 outside of the cells, suggesting that they are imaging artifacts rather than signals emitted by the
317 mEos3.2 (Figure S3B). Comparatively, the mEos3.2 expressing cells yielded one to two orders of
318 magnitude more objects (*Mmc*: 1487, *Mcap*: 1183, *Mbov*: 814, *Maga*: 3761, *Mgen*: 919, *Mgal*:
319 326), each supported by one order of magnitude more detections (median detection/object *Mmc*:
320 245, *Mcap*: 132, *Mbov*: 152, *Maga*: 237, *Mgen*: 493, *Mgal*: 1826; Figure 1D). In the mEos samples,
321 most objects are found inside the boundaries of the cells (Figure S3B). Taken together, the data
322 collected indicate that the mEos3.2 is functional in a wide range of *Mycoplasma* species, thus
323 enabling PALM imaging of proteins of interest in these organisms.

324

325 3. PALM imaging of an atypical F-ATPase in *Mmc*

326 To showcase the interest of performing PALM to study biological processes in mycoplasmas, we
327 worked in our model organism *Mmc*. We were particularly interested in localizing an atypical F-
328 type ATPase called “F₁-like-X₀” (76), putatively involved in a mechanism of immune evasion (59).

329 Leveraging the genome engineering tools available for this species, we generated a mutant strain
330 *Mmc* mEos3.2-0575 in which the fluorescent protein is fused to the N-terminus of the β -subunit of
331 the F₁-like domain. Diffraction-limited images taken by epifluorescence in the green wavelength
332 (before photo-conversion) did not yield any meaningful information, as they only show dimly lit
333 cells with no apparent distribution of the signal (Figure 2A). In stark contrast, the super-resolved
334 images reconstructed from the PALM datasets reveal that most of the signal detected for each cell
335 is localized in a small number of clusters that are found predominantly at the periphery of the
336 cytoplasm (Figure 2A). This localization is in accordance with pre-existing information gathered
337 on the association of the F-domain of this ATPase with the internal face of the plasma membrane
338 (76). The first level of tessellation successfully delineated the cells, and automatic segmentation of
339 the localizations indicated that each cell typically presents 2 to 4 clusters of detections (mean= 3.3,
340 median= 3) (Figure 2B). Meanwhile, cells expressing mEos3.2 as a monomer in the cytoplasm
341 presented mainly a single cluster (Figure S4). The median area of the clusters formed by mEos-
342 tagged ATPase is 5609 nm² (Figure 2C) which corresponds to a circle with a diameter of 84 nm.
343 This cluster area corresponds on average to ~2% of the median cell area, estimated to 324518 nm²
344 (equivalent to a circle of 640 nm of diameter).

345 In order to estimate the accuracy of our detections, we used PALM-tracer to track individual
346 emitters and calculate the Mean squared displacement (MSD) of the trajectories (Figure 2D, inset).
347 The fit of the first points of the MSD gives access to both the median speed of the molecule and its
348 pointing accuracy (77). As our cells are fixed, we directly obtained the pointing accuracy of the
349 fluorophore (63). The majority of pointing accuracies were between 30 and 60 nm, with a median
350 of 41 nm (Figure 2D). The imaging process was highly reproducible, with similar results obtained
351 for data collected from two regions of the same coverslip or from two independent coverslips
352 (Figure S5: Median clusters per object: 3; Median object area: 329971-464727 nm² – equivalent to
353 a circle with a diameter of 648-770 nm; Median cluster area: 6188-8795 nm² – equivalent to a
354 circle with a diameter of 88-106 nm; Median pointing accuracy: 39-51 nm).

355

356 4. dSTORM imaging of a surface protease in *Mmc*

357 In parallel to PALM, we also performed dSTORM imaging in *Mmc*. In this method, a fluorophore-
358 conjugated probe, such as an antibody, is used to label the protein of interest to image. The

359 fluorophores used in dSTORM have the ability to spontaneously transition in and out of a dark
360 state, under strong excitation illumination and in a reducing and oxygen-depleted buffer. To test
361 this method in mycoplasma, we first generated a mutant strain of *Mmc* in which the HA epitope
362 tag was fused to the C-terminus of the protease MIP₀₅₈₂ (59, 78). This immunoglobulin-specific
363 protease is anchored to the cell surface and belongs to the same immune evasion system as the F-
364 ATPase studied above. Coverslips samples of *Mmc* 0582-HA were immuno-labeled with a mouse
365 anti-HA primary antibody and a goat anti-mouse Alexa647-conjugated secondary antibody.
366 Acquisition of diffraction-limited data was performed by illuminating the sample with the
367 excitation laser at a low power which is not sufficient to cause blinking of the fluorophores. Again,
368 no meaningful information could be extracted as the cells appeared dimly fluorescent, with a
369 slightly more intense signal at the periphery in some cases (Figure 3A). Conversely, super-resolved
370 images reconstructed from the dSTORM datasets reveal that the fluorescence signal is localized in
371 clusters that are predominantly at the periphery of the cell (Figure 3A). This is in accordance with
372 the known localization of the protease at the cell surface.
373 Automatic segmentation of these localizations indicates that cells typically present 5-10 clusters
374 (median= 6), and a significant proportion of cells exhibit more than 10 clusters (10%) (Figure 3B).
375 The median cluster area is 3254 nm² (Figure 3C) which corresponds to a circle with a diameter of
376 64 nm. Interestingly, these clusters are smaller than those measured by PALM for the ATPase, with
377 a median area inferior by ~40% and a corresponding circle diameter inferior by ~25%, probably
378 due to the higher pointing accuracy obtained with dSTORM technique (25 nm) compared to PALM
379 (41 nm). However, the first level of tessellation objects, which can be assimilated to the cells, are
380 very similar to those observed by PALM, with a median area of 371130 nm² (equivalent to a circle
381 with a diameter of 687 nm). Again, the imaging process showed high reproducibility, with similar
382 results from data collected from two regions of the same coverslip or from two independent
383 coverslips (Figure S6: Median clusters per object: 5-7; Median object area: 353506-422145 nm² –
384 equivalent to a circle with a diameter of 670-733 nm; Median cluster area: 3582-3944 nm² –
385 equivalent to a circle with a diameter of 67-70 nm).
386
387 5. Dual-color and 3D SMLM in *Mmc*

388 One of the key advantages of fluorescence imaging is the ability to perform multi-color
389 experiments to locate multiple proteins of interest by combining fluorophores with different
390 excitation/emission spectra. Here, we leveraged the spectral compatibility between the mEos3.2
391 red state and the Alexa647 emissions/excitations wavelengths to perform dSTORM/PALM dual
392 color imaging in *Mmc*. To do so, the strain *Mmc* 0582-HA was transformed with the plasmid
393 pMT85-PSynMyco-mEos3.2. The transformants were then imaged sequentially, first by dSTORM,
394 then by PALM. Datasets were processed as above. Super-resolved images were successfully
395 reconstructed from both datasets, yielding results similar to those observed during single-color
396 imaging: the mEos filling the cytoplasm in PALM, and 10-20 small clusters of MIP0582-HA at the
397 cell periphery in dSTORM (Figure 4A). This experiments clearly demonstrate the ability of super-
398 resolution technique to discriminate two types of organization in the minute mycoplasma cells,
399 while classical microscopy failed.

400

401 6. Using mEos3.2 as a reporter and PALM imaging to measure physical parameters

402 In addition to the acquisition of localization data for proteins of interest, we also demonstrated that
403 PALM imaging process could be used to gather information on protein expression levels. Indeed,
404 similarly to what can be done with classical fluorescent proteins, the amount of mEos3.2 in a given
405 cell is proportional to the number of detections. We compared the relative strength of various
406 promoters by building modified versions of the plasmid pMT85-PSynMyco-mEos3.2 in which the
407 PSynMyco promoter was replaced by the P438 or PSpi promoter, respectively. The three plasmids
408 were transformed in *Mmc*, yielding transformants that were subsequently imaged by PALM.
409 Analysis of the data revealed that each promoter drives the expression of mEos3.2 at different
410 levels (Figure 5A), with P438 producing a signal slightly above the background noise (P438: 46
411 detections/object, WT: 23 detections/object), PSynMyco one order of magnitude higher (104
412 detections/object) and PSpi giving the highest signal (858 detections/object).

413 Interestingly, the overexpression of mEos3.2 alone can also be used to estimate the absolute
414 physical size of individual cells. Indeed, for each of the hundreds to thousands of cells found in the
415 field of view, the cytoplasm can be reliably discriminated from the background based on the
416 localization of the fluorescence signal. The surface occupied by the cytoplasm can be then
417 accurately measured (length, width, area) (Figure S7). As an example, we analyzed a population

418 of 358 cells of *Mmc* pMT85-PSpi-mEos3.2 (Figure 5B), showing a normal distribution of sizes
419 ranging from 107 to 1490 nm (mean: 768 nm, median: 754 nm) on the major axis and 87 to 1179
420 nm (mean: 540 nm, median: 540 nm) on the minor axis.

421

422 **Discussion:**

423 Super-resolution microscopy techniques have revolutionized biology by enabling fluorescence
424 imaging well beyond the diffraction limit. Establishing these methods in mycoplasmas represents
425 a significant milestone for the investigation of these minute bacteria with atypical properties and
426 broad biological relevance.

427 Here we report the first application of PALM and dSTORM, two mature SMLM techniques, in
428 mycoplasmas. We describe a common, simple and reliable method to generate high-quality,
429 SMLM-ready, fixed samples and showcase the interest of super-resolution imaging in our model
430 species *Mycoplasma mycoides* subsp. *capri*. This bacterium, in addition to being the etiologic agent
431 of Contagious Caprine PleuroPneumonia, has emerged as a major model in both synthetic and
432 fundamental biology. Indeed, it is the original organism from which the synthetic cells JCVI Syn1.0
433 and Syn3.0 have been derived through genome engineering and reduction (12, 13). As a result, the
434 SMLM strategies developed here in *Mmc* are likely to be applicable in its man-made descendants.
435 We imaged two proteins of interest in *Mmc*, one cytoplasmic and one at the cell surface, with a
436 pointing accuracy below 50 nm (more than 6 times the diffraction limit). The images reconstructed
437 from our PALM and dSTORM datasets provide qualitative and quantitative data that were
438 previously only accessible through immuno-gold electron microscopy. Our imaging process
439 proved fast, as sample preparation takes ~3 hours for PALM and ~6 hours for dSTORM, followed
440 by ~10-15 minutes of data acquisition for each field of view. Given that each field of view contains
441 several hundred to several thousand cells, it is possible to collect data at a medium-throughput and
442 to get an accurate description of a biological process across a large population of cells. In addition,
443 we note that our process is highly reproducible, with similar results obtained across multiple fields
444 of view acquired on the same coverslip, or across independent coverslips, prepared from
445 independent biological replicates and imaged at different dates.

446 In this study, we focus our analysis on the localization of virulence factors associated with the cell
447 membrane. However, SMLM can be applied to study any protein of interest, including those

448 involved in fundamental biological processes such as transcription, translation or cell division, and
449 has the potential to become a key tool for the mycoplasmologist community. In order to foster a
450 quick adoption of SMLM, we have validated our sample preparation process in a total of six
451 different *Mycoplasma* species, relevant to both the medical and veterinary fields and covering the
452 three main *Mollicutes* phylogenetic subgroups.

453 We also provide the plasmid pMT85-PSynMyco-mEos3.2 which allows the expression of the
454 photo-convertible fluorescent protein mEos3.2 under the control of the SynMyco regulatory
455 element, enabling PALM imaging in all the tested *Mycoplasma* species. We then produced two
456 variants of the plasmid in which the mEos3.2 expression is driven by the alternative promoters
457 PSpi (51) or P438 (55), which are well known and used throughout the *Mollicutes* field. These
458 three plasmids are available through the repository Addgene (references 173894, 173895, 173896)
459 for rapid distribution, and evaluation in other species. It should be noted that the mEos3.2 is
460 generally regarded as one of the best fluorophores for PALM imaging, as it exhibits multiple traits
461 that are desirable for SMLM: it is monomeric, has a fast maturation and has a relatively high
462 photostability. Its green and red excitation and emission wavelengths also make it compatible for
463 multicolor imaging with widely used DNA dyes such as Hoechst 33342 (361/497 nm), membrane
464 dyes such as Nile Red (552/636 nm) and far-red organic dyes such as Alexa 647 (650/670 nm).
465 Alternative fluorescent proteins could be expressed from our pMT-85 backbone, and multiple
466 options are available in the literature, including the well-characterized mMaple3 (79), PAmCherry
467 (80) or Dendra2 (81). These alternatives should nonetheless be properly benchmarked, as it was
468 shown the performances of different fluorescent proteins can vary greatly in a given microbial
469 species (82).

470 Meanwhile, in order to perform dSTORM we relied on the expression of an epitope-tagged variant
471 of our protein of interest which was subsequently immuno-labeled. This choice was guided by the
472 possibility to use commercial, well-characterized, high affinity monoclonal anti-HA antibodies
473 enabling us to have a good labeling of the protein of interest and a low background noise. However,
474 genetic edition strategies allowing the precise modification of a genomic locus for epitope tagging
475 are only available in a limited number of *Mycoplasma* species. This issue can however be bypassed
476 by using custom, protein-specific antibodies. It is noteworthy that our protocol used a primary
477 antibody and fluorescent-labeled secondary antibody. This method, while practical and widely

478 used, introduces a significant displacement between the targeted protein and the reporting
479 fluorophore due to the physical size of the antibodies (83). This linkage error is approximately of
480 15-20 nm and can be mitigated through the utilization of either a fluorophore-conjugated primary
481 antibody or alternative small probes such as nanobodies or aptamers which offer a ~5 nm linkage
482 error (84-86).

483 Finally, it should be noted that super-resolution microscopy is still a developing field, which sees
484 continuous improvements in resolution through new techniques. For instance, strategies based
485 around Expansion Microscopy (a process in which the sample is physically expanded in an
486 isotropic fashion), could help reach the nanometer scale by decrowding biomolecules in dense
487 samples (87). Meanwhile, the recently developed MINFLUX promises imaging at 1-3 nm lateral
488 resolution, in multicolor and in 3D (88). These improvements, coupled with the commercialization
489 of “turnkey” microscopy systems will further drive the adoption of SMLM and in the long term
490 will benefit our understanding of mycoplasma’s biology.

491

492 **Author contribution:**

493 Funding acquisition: E.H. and Y.A.; Project administration: Y.A.; Supervision: E.H. and Y.A;
494 Conceptualization: F.R., E.H. and Y.A; Resources: E.V. and E.H.; Investigation: F.R., A.V., P.B.,
495 E.H., and Y.A.; Formal analysis: F.R., E.H.; Visualization: F.R., E.H. and Y.A.; Writing: F.R.,
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497

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Figures:

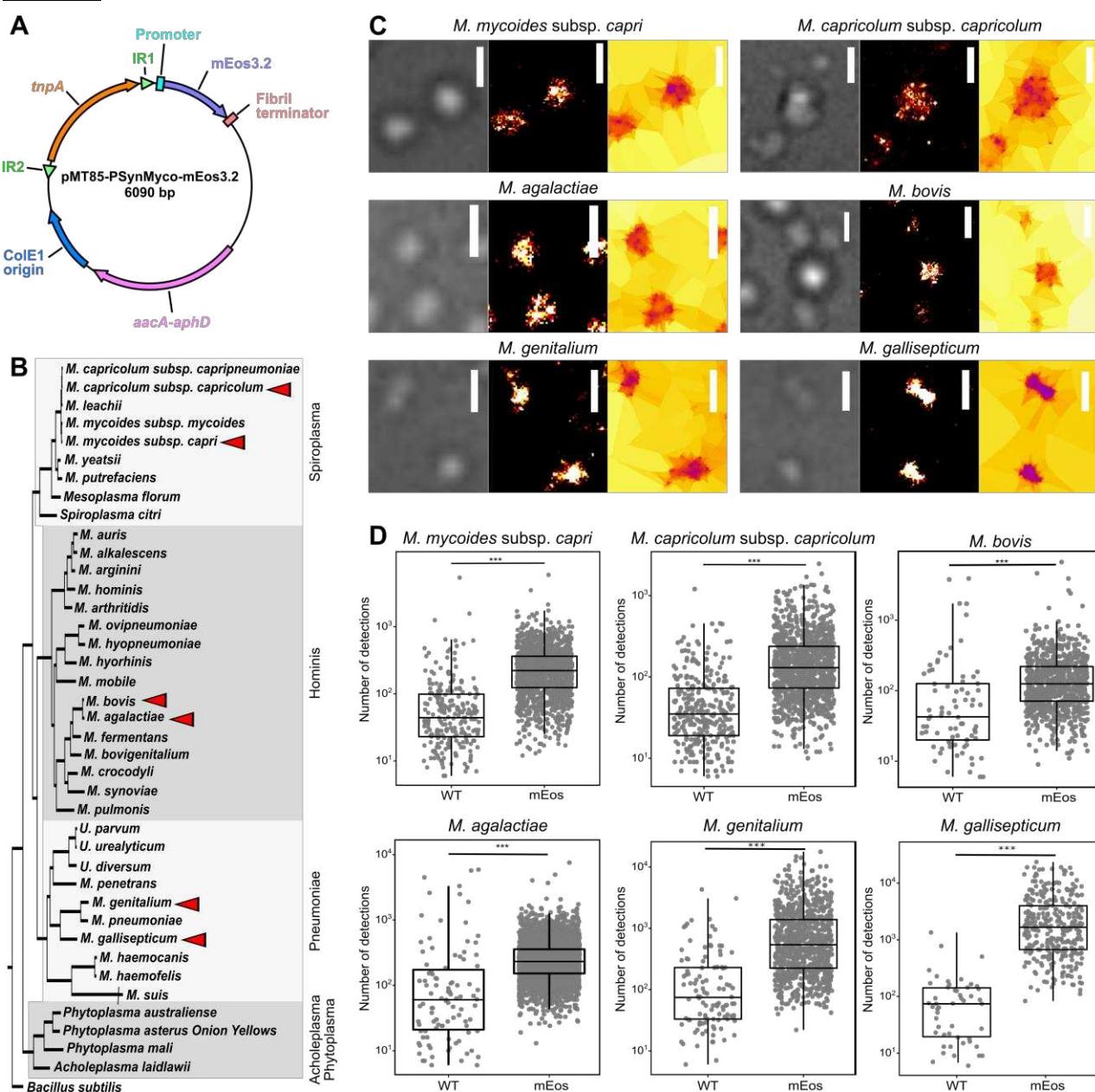


Figure 1: Assessing the functionality of the photo-convertible fluorescent protein mEos3.2 in multiple *Mycoplasma* species. **A.** Map of the plasmid pMT85-PSynMyc-mEos3.2. The main genetic components of the plasmid are indicated (IR: Inverted Repeat, *tnpA*: transposase, *aacA-aphD*: gentamycin resistance). **B.** Distribution of the *Mycoplasma* species used in this study. A phylogenetic tree of representative *Mollicutes* species was inferred using the maximum-likelihood method from the concatenated multiple alignments of 79 proteins encoded by genes present at one copy in each genome (adapted from Grosjean *et al.* 2014). The main phylogenetic groups are indicated by grey boxes. The six species transformed with pMT85-PSynMyc-mEos3.2 are identified by a red arrow. **C.** Sample images of mycoplasma cells expressing the mEos3.2 in their cytoplasm. For each of the six species, cells transformed with the plasmid

pMT85-PSynMyco-mEos3.2 were imaged using PALM. A representative subset of the field of view is given (from left to right: contrast-phase image, super-resolved reconstruction at 40 nm pixels, Tesseler segmentation of the localizations). Scale bar: 1 μ m. **D.** Quantification of the PALM signal intensity. For each of the six species, both wild-type (WT) and pMT85-PSynMyco-mEos3.2 transformed cells (mEos) were imaged by PALM and the data collected from a single representative field of view (512x512 pixels; pixel size: 0.16 μ m) were analyzed. The dot-plot presents the number of detections measured in each object segmented by Tesseler (equivalent to a cell), on top of which a boxplot showing the median, interquartile range, minimum and maximum is overlaid. Statistics tests (Mann Whitney) were performed to compare the two conditions WT and mEos (***: p-value < 1.10⁻¹⁰).

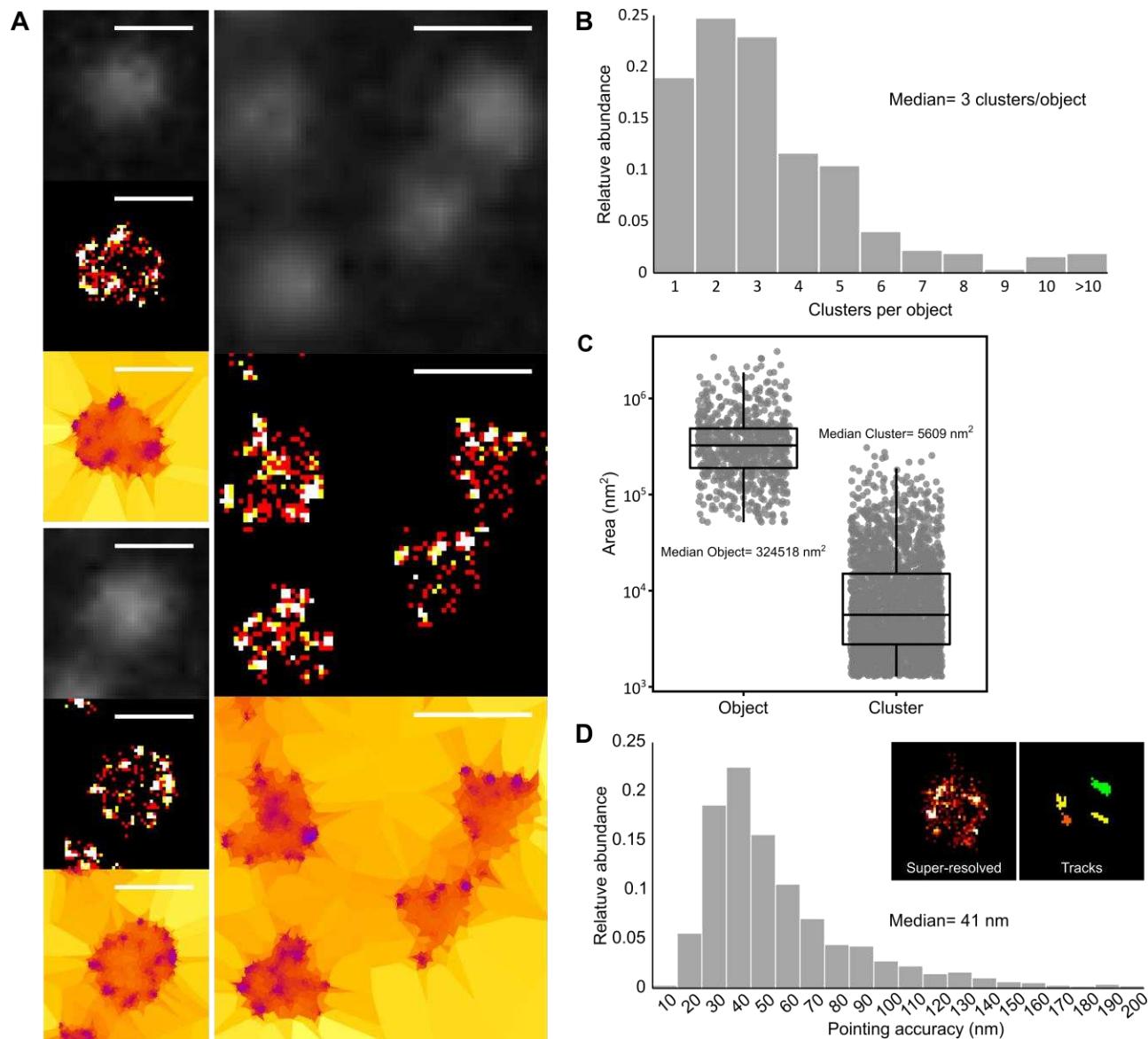


Figure 2: PALM imaging of an F-type ATPase in *Mycoplasma mycoides* subsp. *capri*. *Mmc* mEos3.2-0575 cells, expressing a mEos-fused variant of the β -subunit of the ATPase F₁-like domain, were imaged by PALM. In this *Mmc* mutant, the fluorescent fusion protein is expressed from the native genomic locus and replaces the wild-type variant. The data presented here correspond to a single representative field of view (512x512 pixels; pixel size: 160 nm). **A. Sample images of *Mmc* mEos3.2-0575 cells. For each field of view, the images correspond to: top: epifluorescence (diffraction-limited); middle: super-resolved reconstruction (40 nm pixel); bottom: Tesseler segmentation. Scale bar: 1 μ m. **B.** Tesseler clustering of the fluorescence signal. The number of clusters per Tesseler-segmented objects was computed. The bar graphs display the distribution of the number of clusters per object. **C.** Objects and clusters sizes. The dot plot presents the area (in nm²) of each object and cluster segmented by Tesseler, to which a boxplot showing the median, interquartile range, minimum and maximum is overlaid. The median value of each data set is indicated. **D.** Evaluation of the PALM imaging pointing accuracy. Inset: example of the tracks computed using PALM-Tracer and from which the MSD0 and pointing accuracies values are derived. The bar graphs**

display the distribution of the pointing accuracies derived from each track. The median value of the data set is indicated.

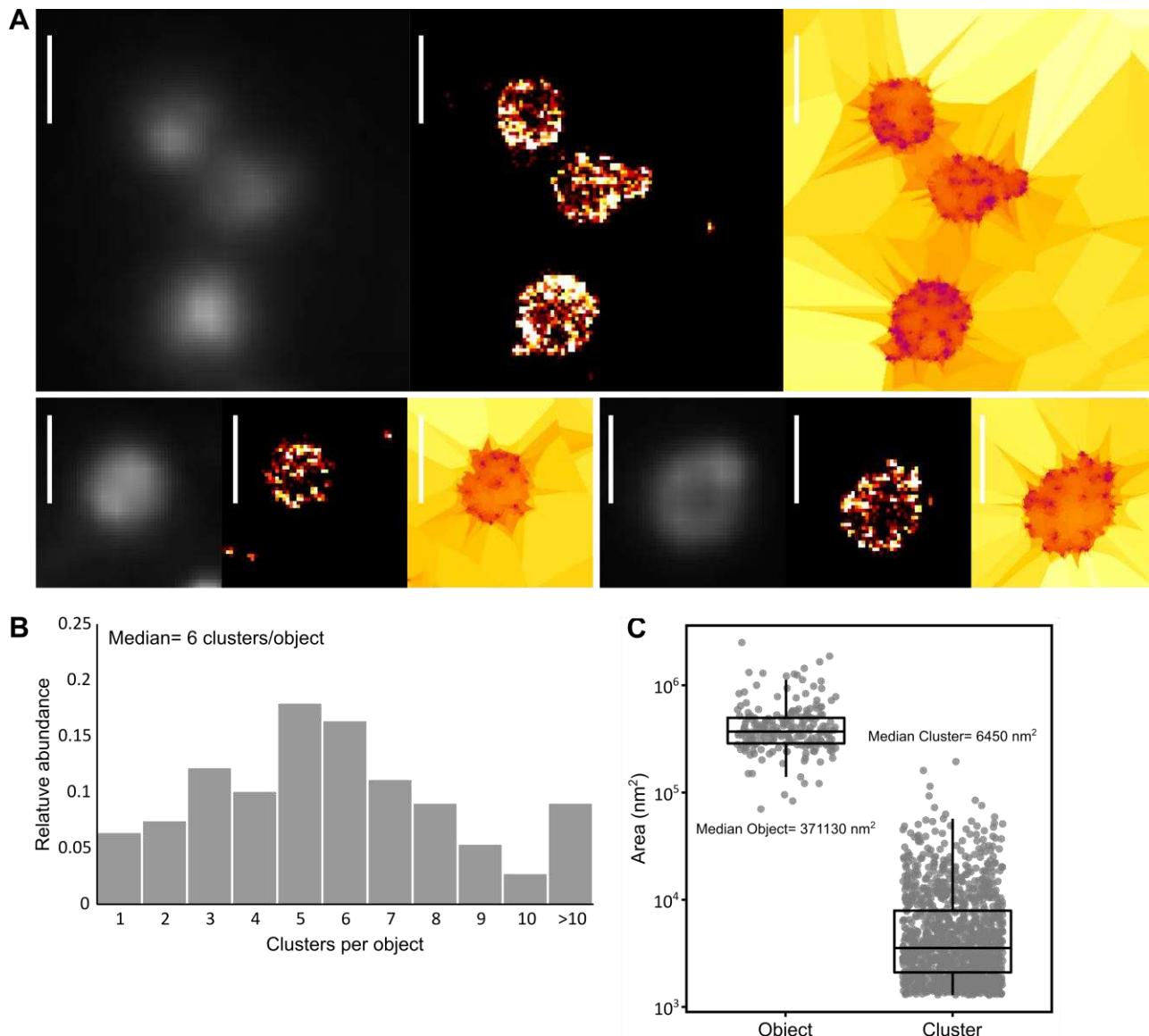


Figure 3: dSTORM imaging of an antibody-specific protease in *Mycoplasma mycoides* subsp. *capri*. *Mmc* 0582-HA cells expressing an HA-tag fused variant of the serine protease MIP₈₂ were immune-labeled and imaged by dSTORM. The tagged fusion protein is expressed from the native genomic locus and replaces the wild-type variant. The data presented here correspond to a single representative field of view (512x512 pixels; pixel size: 160 nm). **A.** Sample images of *Mmc* 0582-HA cells. For each field of view, the images correspond to: left: epifluorescence (diffraction-limited); middle: super-resolved reconstruction (40 nm pixel); right: Tesseler segmentation. Scale bar: 1 μ m. **B.** Tesseler clustering of the fluorescence signal. For each field of view, the number of clusters per Tesseler-segmented objects was computed. The bar graphs display the distribution of the number of clusters per object. **C.** Objects and clusters sizes. The dot plot presents the area (in nm²) of each object and cluster segmented by Tesseler, to which a boxplot showing the median, interquartile range, minimum and maximum is overlaid. The median value of each data set is indicated.

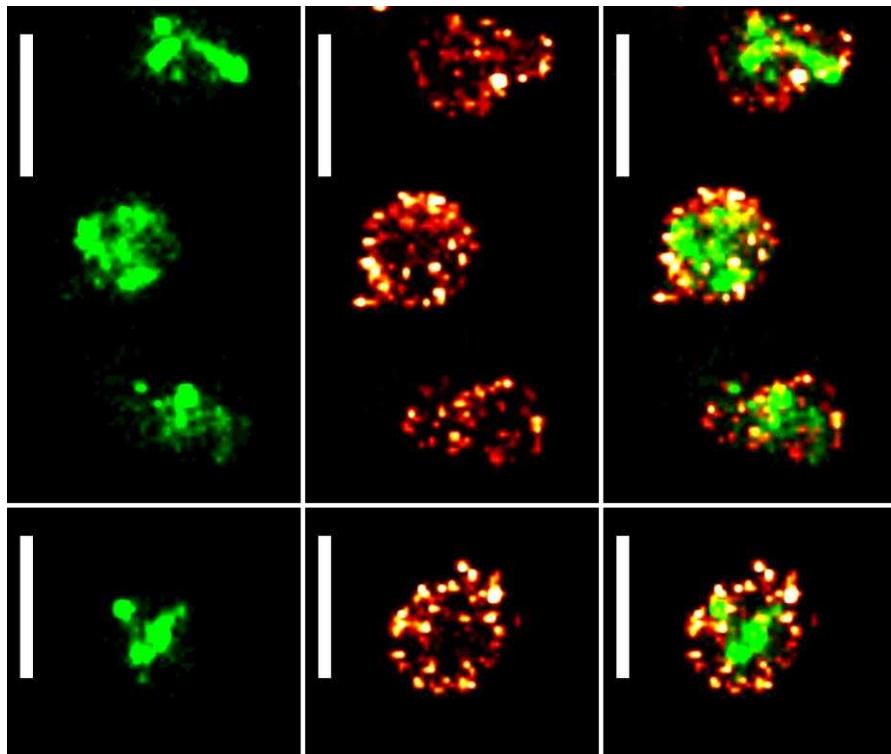


Figure 4: PALM/dSTORM two-colors imaging of *Mycoplasma mycoides* subsp. *capri*. Sample images of *Mmc* 0582-HA pMT85-PSynMyco-mEos3.2 cells, expressing both an HA-tag fused variant of the serine protease MIP₀₅₈₂ and the fluorescent protein mEos3.2. The tagged fusion protein is expressed from the native genomic locus and replaces the wild-type variant. The mEos3.2 is expressed from the transposon inserted at a random site in the bacterial chromosome. For each field of view, the images correspond to: left: reconstructed PALM image (40 nm pixel); middle: reconstructed dSTORM (40 nm pixel); right: overlay of the reconstructed PALM and dSTORM images. Scale bar: 1 μ m. All the images were sampled from the same coverslip and field of view.

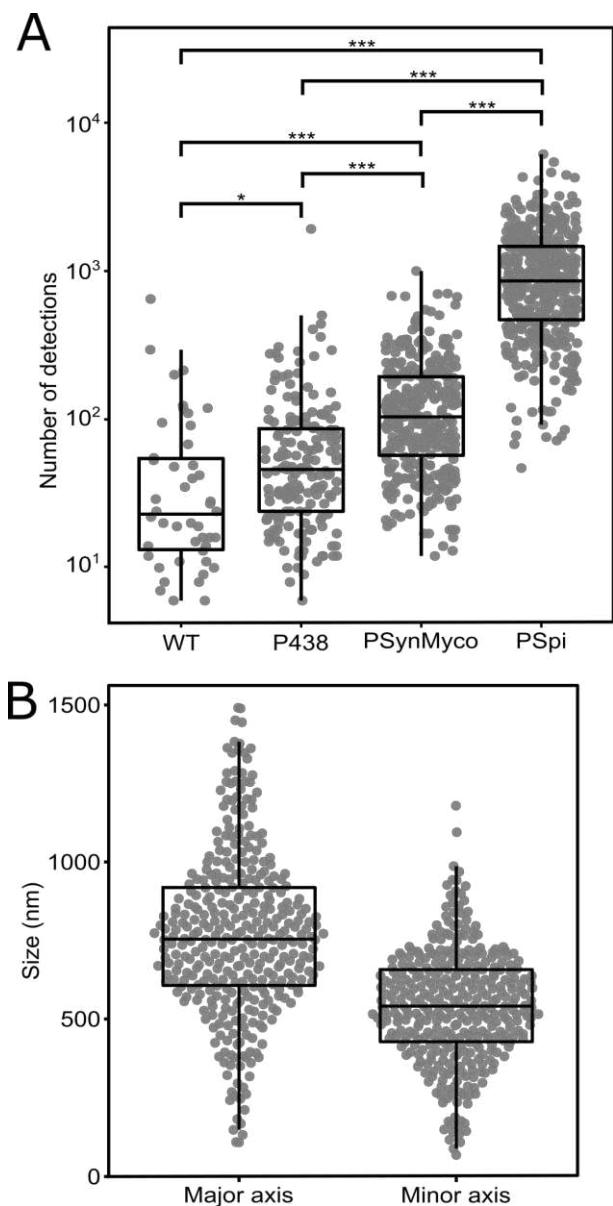
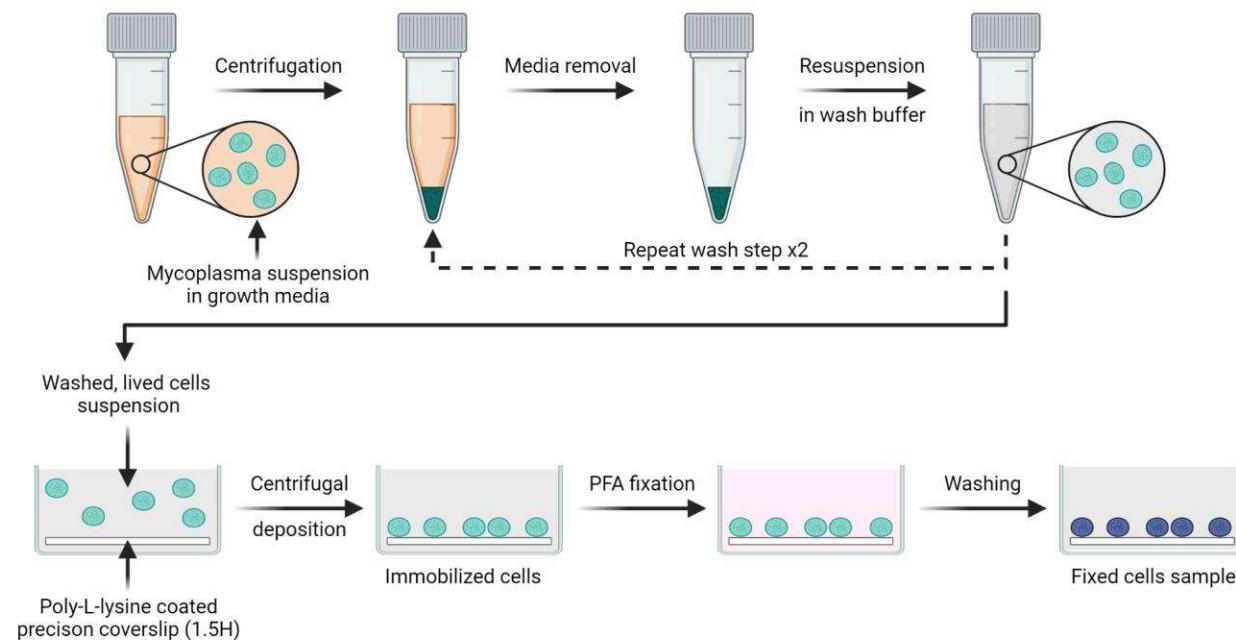


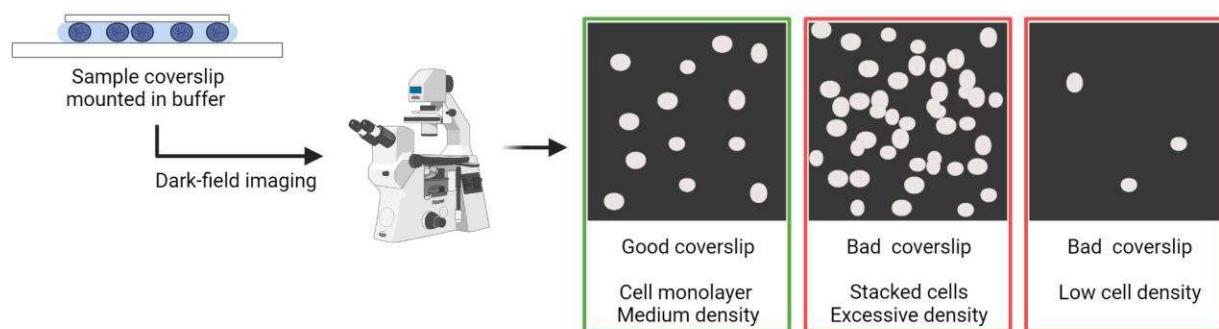
Figure 5: Evaluation of promoter strength and cell size through PALM imaging in *Mmc*. **A.** Comparison of promoter strength by PALM imaging. *Mmc* cells, either wild-type (WT) or transformed with the plasmids pMT85-P438-mEos3.2 (P438), pMT85-PSynMyc-mEos3.2 (PSynMyc) or pMT85-PSpi-mEos3.2 (PSpi) were imaged by PALM. For each strain, the data collected from a single representative field of view (512x512 pixels; pixel size: 0.16 μ m) were analyzed. The dot-plot presents the number of detections measured in each object segmented by Tesseler (equivalent to a cell), on top of which a boxplot showing the median, interquartile range, minimum and maximum is overlaid. Statistics tests (Mann Whitney) were performed to compare the four strains (*: p-value <0.05; ***: p-value < 1.10⁻¹⁰). **B.** Deriving cell size data from PALM images. *Mmc* cells transformed with the plasmid pMT85-PSpi-mEos3.2 were imaged by PALM. The data collected from a single representative field of view (512x512 pixels; pixel size: 0.16 μ m) were analyzed. The dot-plot presents the dimensions (in nm) of the major axis and the minor axis of each

object segmented by Tesseler (Figure S7), on top of which a boxplot showing the median, interquartile range, minimum and maximum is overlaid.

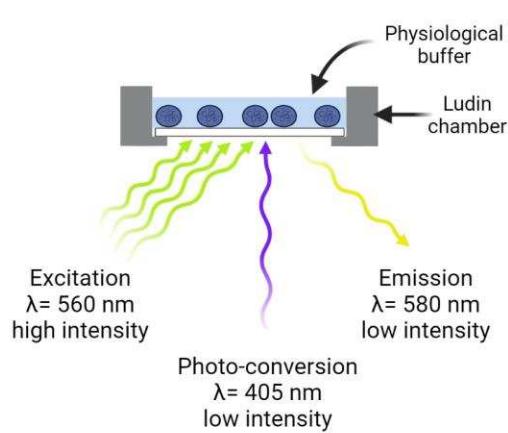
A. Mycoplasma cell deposition



B. Coverslip quality control



C. PALM imaging



D. dSTORM imaging

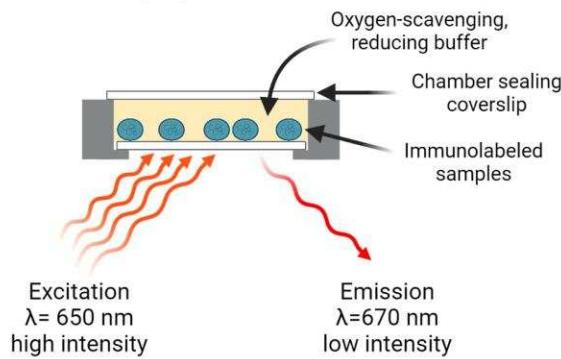


Figure S1: Schematic overview of the SMLM samples preparation process. **A.** Mycoplasma cells deposition and fixation on coverslips. Cells grown in culture media are harvested and washed, then deposited on poly-L-lysine coated coverslips using centrifugation to force the cells to sediment. After PFA fixation, the coverslips can be further processed by immune-labeling or used directly. **B.** Quality control of the sample coverslips. In order to check the proper deposition of cells on the coverslip, and to gauge the cell density, coverslips are mounted on glass slides (cells facing the glass) using a mounting buffer. Dark-field imaging is then performed to assess the samples. **C.** Schematic overview of the PALM imaging process. The coverslip is mounted in a Ludin chamber, filled with PBS buffer. Imaging is performed by providing continuous excitation illumination at medium intensity while simultaneously illuminating the sample with low-intensity ultra-violet light to photo-convert the fluorescent protein. **D.** Schematic overview of the dSTORM imaging process. The coverslip is mounted in dSTORM imaging buffer, in a sealed Ludin chamber. Imaging is performed by providing continuous excitation illumination at high intensity in order to send the fluorophores to their dark state.

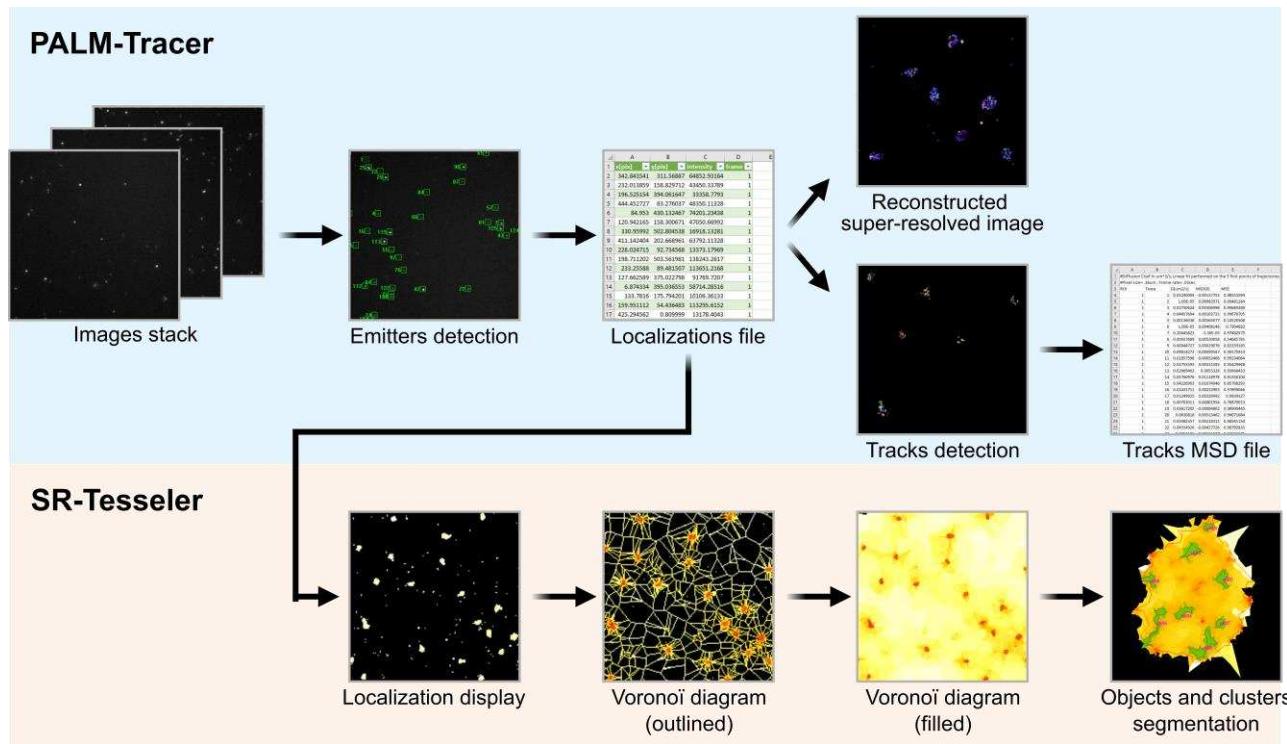


Figure S2. Schematic overview of the SMLM imaging data processing. Images stacks collected during data acquisition are processed using the Metamorph plug-in PALM-Tracer. Emitters are detected on each frame, and their localizations are computed by Gaussian fitting, yielding a localization file indicating for each emitter an x and a y localization value, an intensity value, and a frame number. This localization file is then used to reconstruct a super-resolved image, and to create emitters tracks from which the MSD values are derived. SR-Tesseler is used in parallel to analyze the localization file. First, a Voronoi diagram is computed, based on which objects (red outline) and clusters (green fill) are automatically segmented based on set density, detection and size threshold parameters.

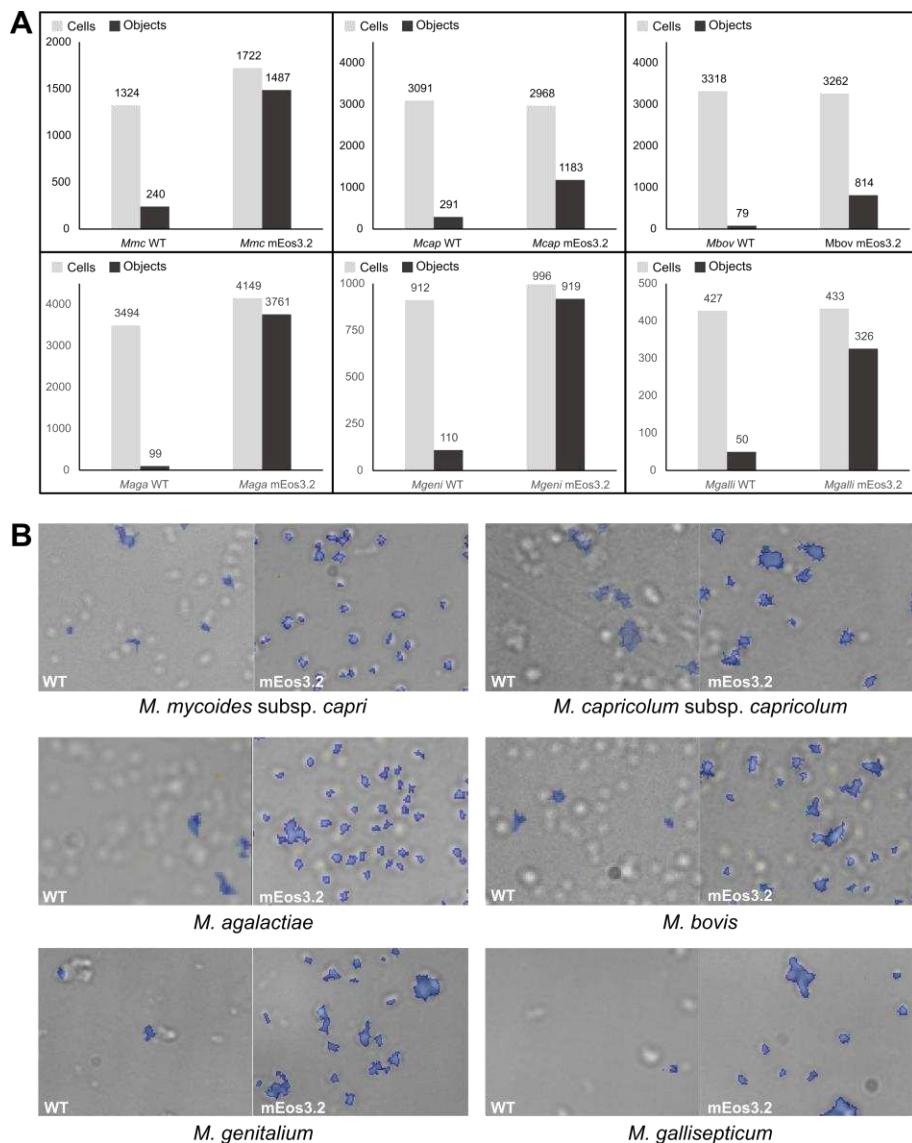


Figure S3: Assessing the functionality of the photo-convertible fluorescent protein mEos3.2 in multiple *Mycoplasma* species. Six mycoplasma species were transformed with the plasmid pMT85-PSynMyco-mEos3.2 and imaged by PALM (see Figure 1). The data presented here correspond to a single representative field of view (512x512 pixels; pixel size: 160 nm). **A.** Comparison of the number of cells and Tesseler segmented objects in the field of view. The bar graph indicates the number of objects segmented by Tesseler (equivalent to a cell) compared to the number of actual cells found in the imaged field. **B.** Localization of the Tesseler objects compared to the mycoplasma cells. Sample images show the overlay of a contrast-phase image and of the objects segmented by Tesseler (blue) in the same field.

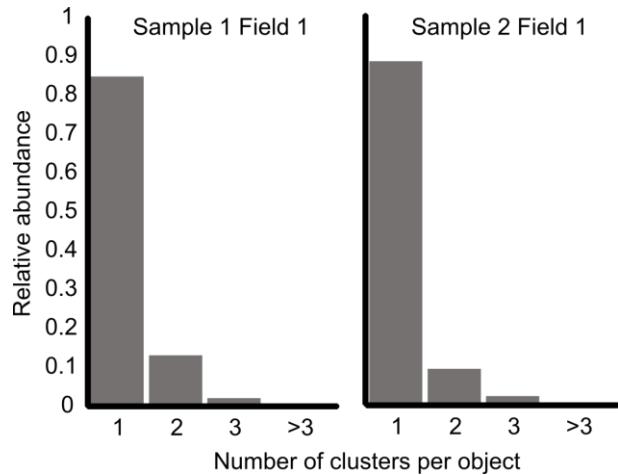


Figure S4: Tesseler clustering of mEos3.2 localizations in the cytoplasm of *Mycoplasma mycoides* subsp. *capri*. *Mmc* pMT85-PSynMyco-mEos3.2 cells were imaged using PALM, and Tesseler clustering of the fluorescence signal was performed. For each dataset, the number of clusters per Tesseler-segmented object was computed. The bar graphs display the distribution of the number of clusters per object. The data presented here correspond to two fields of view (512x512 pixels; pixel size: 160 nm), taken from two independent coverslips.

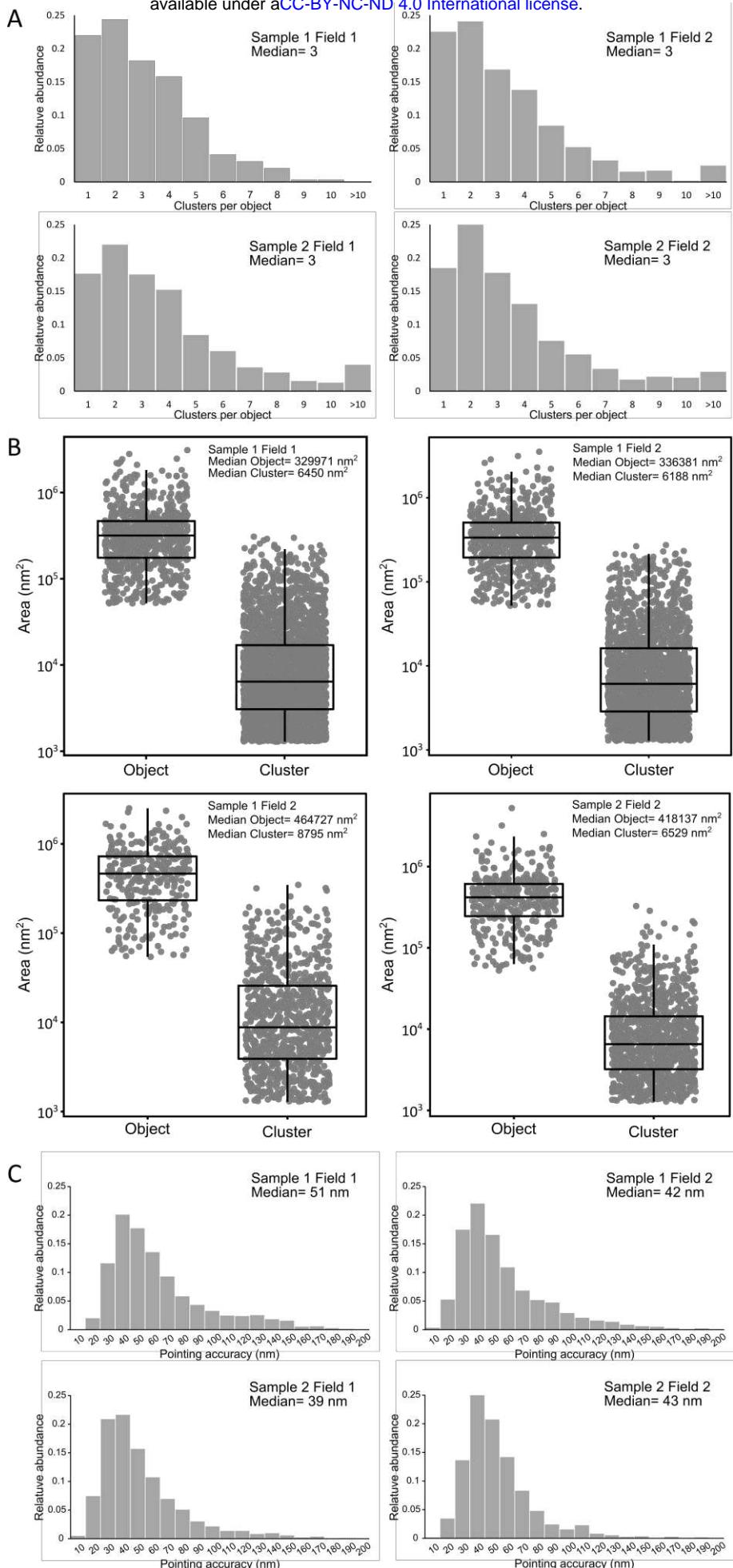


Figure S5: Replicates of the PALM imaging of an F-type ATPase in *Mycoplasma mycoides* subsp. *capri*. *Mmc* 0575-mEos3.2 cells, expressing a mEos-fused variant of the β -subunit of the ATPase F_1 -like domain, were imaged by PALM (see Figure 2). The data presented here correspond to four fields of view (512x512 pixels; pixel size: 160 nm) taken from two independent coverslips. **A.** Tesseler clustering of the fluorescence signal. The number of clusters per Tesseler-segmented objects was computed. The bar graphs display the distribution of the number of clusters per object. **B.** Objects and clusters sizes. The dot plot presents the area (in nm²) of each object and cluster segmented by Tesseler, to which a boxplot showing the median, interquartile range, minimum and maximum is overlaid. The median value of each data set is indicated. **B.** Evaluation of the PALM imaging pointing accuracy. The bar graphs display the distribution of the pointing accuracy derived from each track. The median value of each data set is indicated.

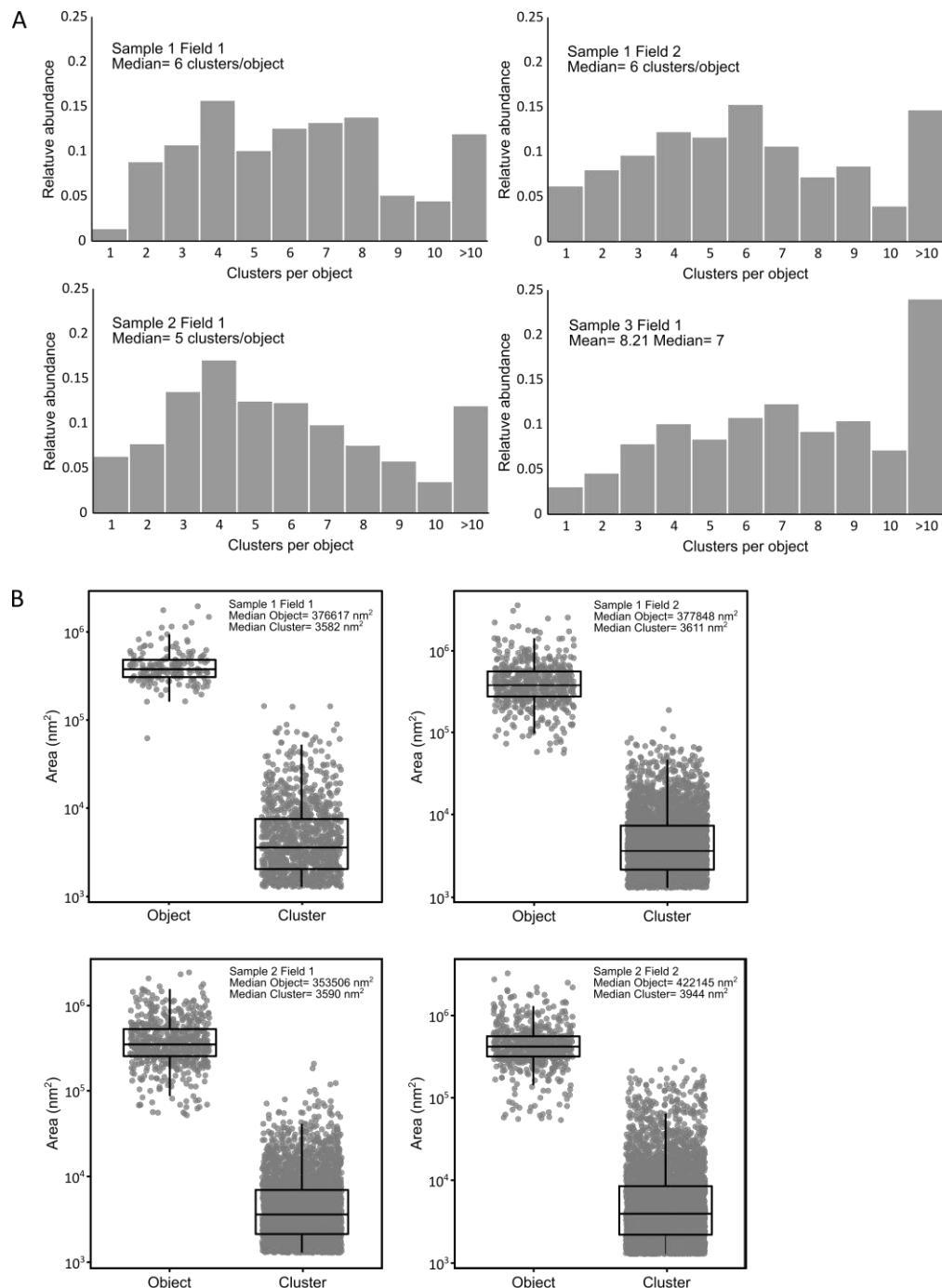


Figure S6: Replicates of the dSTORM imaging of an antibody-specific protease in *Mycoplasma mycoides* subsp. *capri*. *Mmc* 0582-HA cells expressing an HA-tag fused variant of the serine protease MIP₀₅₈₂ were immune-labeled and imaged by dSTORM (see Figure 3). The data presented here correspond to four fields of view (512x512 pixels; pixel size: 160 nm) taken from two independent coverslips. A. Tesseler clustering of the fluorescence signal. For each field of view, the number of clusters per Tesseler-segmented objects was computed. The bar graphs display the distribution of the number of clusters per object. B. Objects and clusters sizes. The dot plot presents the area (in nm²) of each object and cluster

segmented by Tesseler, to which a boxplot showing the median, interquartile range, minimum and maximum is overlaid. The median value of each data set is indicated.

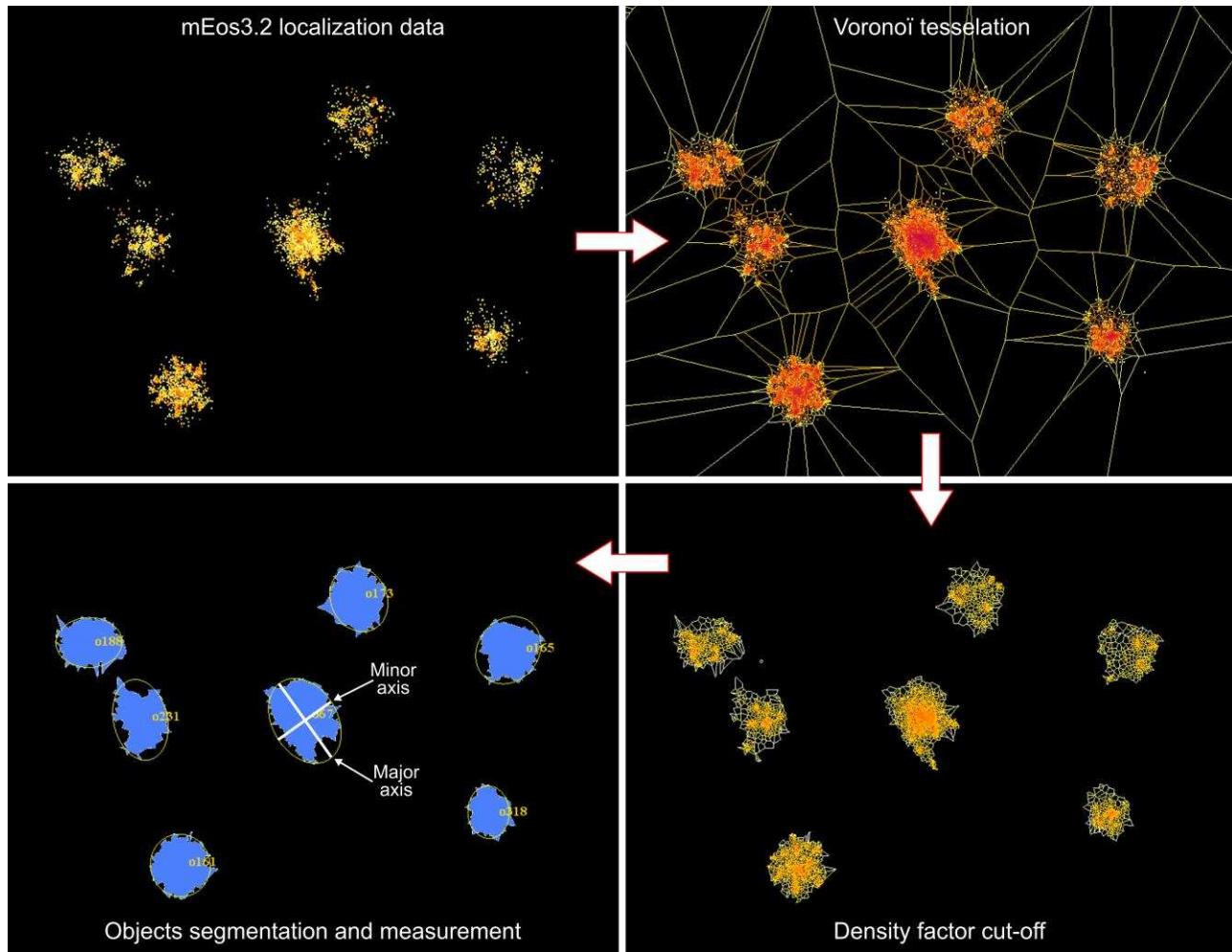


Figure S7: Schematic overview of the cell size estimation from PALM imaging data. Top-left: localization data collected from *Mmc* cells expressing the mEos3.2 as a soluble monomer in the cytoplasm are displayed in Tesseler. Top-right: Voronoï tessellation of the localizations (no density cut-off). Bottom-right: Voronoï tessellation of the localizations is performed again after setting a density factor cut-off (here: 1) in order to filter the signal coming from outside the cells. Bottom-left: Tesseler objects are segmented and their dimensions (major axis and minor axis) are computed.