Metabolic biorthogonal labeling and dSTORM imaging of peptidoglycan synthesis in Streptococcus pneumoniae

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HAL Id: hal-03510706

https://hal.science/hal-03510706

Submitted on 4 Jan 2022

Protocol

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Highlights

Peptidoglycan can be
labeled with a
clickable D-Ala-D-Ala
dipeptide

The labeled
peptidoglycan can be
conjugated to a
clickable Alexa Fluor
647 dye

Fluorescently labeled
peptidoglycan can be
observed at 30-nm
resolution by
dSTORM

Fluorescence microscopy is a method of choice for studying peptidoglycan assembly, but it
presents two major challenges: the peptidoglycan must be labeled with a probe that will not
perturb the physiological process, and the spatial resolution must reach the nanometer scale to
reveal fine details of the synthesis process. This protocol meets both challenges by combining
biorthogonal metabolic labeling of peptidoglycan in *Streptococcus pneumoniae* with super-
resolution fluorescence microscopy (dSTORM), also providing cues to adapt it to other bacteria.

STAR Protocols

Protocol

Metabolic biorthogonal labeling and dSTORM imaging of peptidoglycan synthesis in *Streptococcus pneumoniae*

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SUMMARY

Fluorescence microscopy is a method of choice for studying peptidoglycan assembly, but it presents two major challenges: the peptidoglycan must be labeled with a probe that will not perturb the physiological process, and the spatial resolution must reach the nanometer scale to reveal fine details of the synthesis process. This protocol meets both challenges by combining biorthogonal metabolic labeling of peptidoglycan in *Streptococcus pneumoniae* with super-resolution fluorescence microscopy (dSTORM), also providing cues to adapt it to other bacteria.

For complete details on the use and execution of this protocol, please refer to Trouve et al. (2021).

BEFORE YOU BEGIN

This protocol describes the steps for fluorescent labeling of peptidoglycan (PG) in *Streptococcus pneumoniae (S. pneumoniae)*. This strategy is based on the metabolic incorporation of a biorthogonal probe (an azido-D-alanyl-D-alanine) that will result in the labeling of the fourth amino acid of the peptide chain of the PG (Figure 1) (Trouve et al., 2021). The azido group will be conjugated to a fluorescent dye coupled to a strained alkyne through copper-free click chemistry. As PG is the common component of all bacterial cell walls, this labeling strategy has great chances to be successfully employed in other bacteria following the optimization of various critical steps that are highlighted along the protocol and in Figure 2.

This protocol also describes how to prepare samples of pneumococcal cells for dSTORM (direct Stochastic Optical Reconstruction Microscopy). dSTORM is a single-molecule localization microscopy method, which also requires optimization steps that are described in the text and in Figure 2. To adapt this protocol to a new bacterial species, you should first check that PG labeling results in a strong specific fluorescent signal by conventional fluorescence microscopy before proceeding to dSTORM (see Figure 2 for a flowchart presenting various options to optimize key points of the protocol). Importantly, since PG is synthesized in regions whose dimensions are in the nanometer scale, optimal data acquisition will be achieved using conventional and STORM fluorescence microscopes equipped with a × 100 objective.

Prepare frozen glycerol stocks of bacterial cells
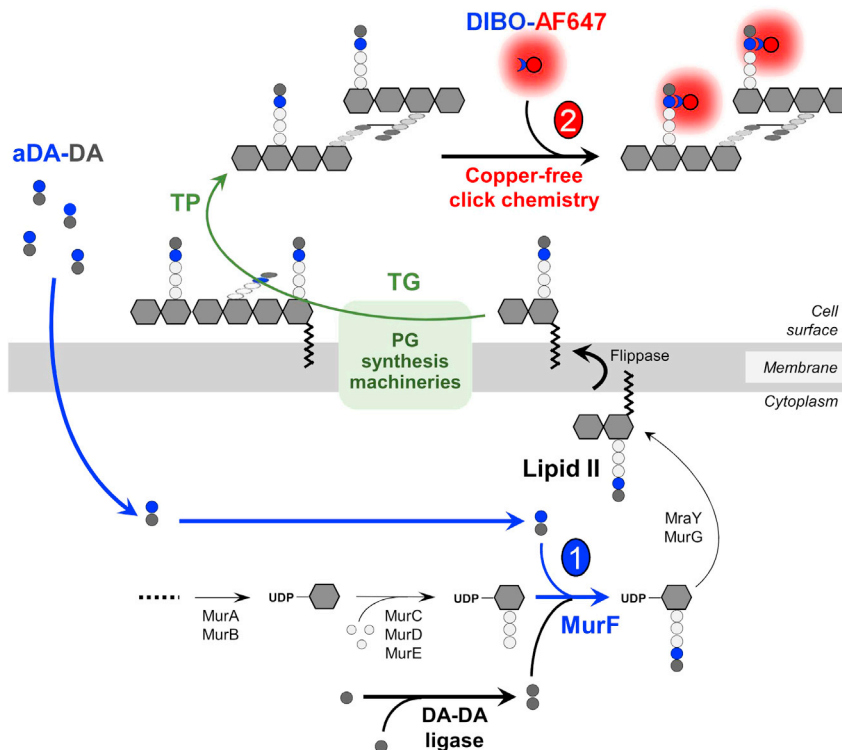
⊙ Timing: 1 day

Figure 1. Schematic representation of metabolic incorporation of the aDA-DA probe into the PG

The azido-D-Ala-D-Ala (aDA-DA) probe is incorporated into the peptide chain of the PG precursor (lipid II) by the MurF ligase (1). Following transfer of lipid II to the extracellular side of the membrane, the PG synthesis machineries polymerize the PG precursor into glycan chains through their transglycosylase (TG) activity, and crosslink the nascent chains into the existing PG through their transpeptidase (TP) activity. The peptide chains carrying an azido-D-Ala are eventually covalently linked to an AF647 dye conjugated to a DIBO group, through copper-free click chemistry (2). Adapted from Trouve et al. (2021)

S. pneumoniae growth is influenced by storage conditions and culture media. To compare data acquired on different cell cultures, it is thus important to prepare enough glycerol stocks for the whole study.

1. Grow pneumococcal cells until exponential phase
   a. In a 15-mL Falcon tube, inoculate 10 mL of TH (Todd Hewitt) medium with 10 μL of a frozen glycerol stock (−80°C) of *S. pneumoniae* cells.
   b. Incubate the culture at 30°C or 37°C in a static CO_2 incubator.

   Note: Pneumococcal cells are facultative anaerobes but they grow better in the absence of oxygen. Cells will just take longer to grow at 30°C compared to 37°C.

   c. Stop the culture when the OD (optical density) at 600 nm (OD_{600nm}) reaches about 0.3.

   Note: Pneumococcal cells are in the exponential growth phase between OD_{600nm} 0.2–0.6 and usually start to lyse after OD_{600nm} reaches 0.8. A standard generation time for a wild-type *S. pneumoniae* strain is about 30 min.

2. Make glycerol stocks of pneumococcal cells
   a. Centrifuge the cell culture at 4,000×*g* for 10 min at 22°C.
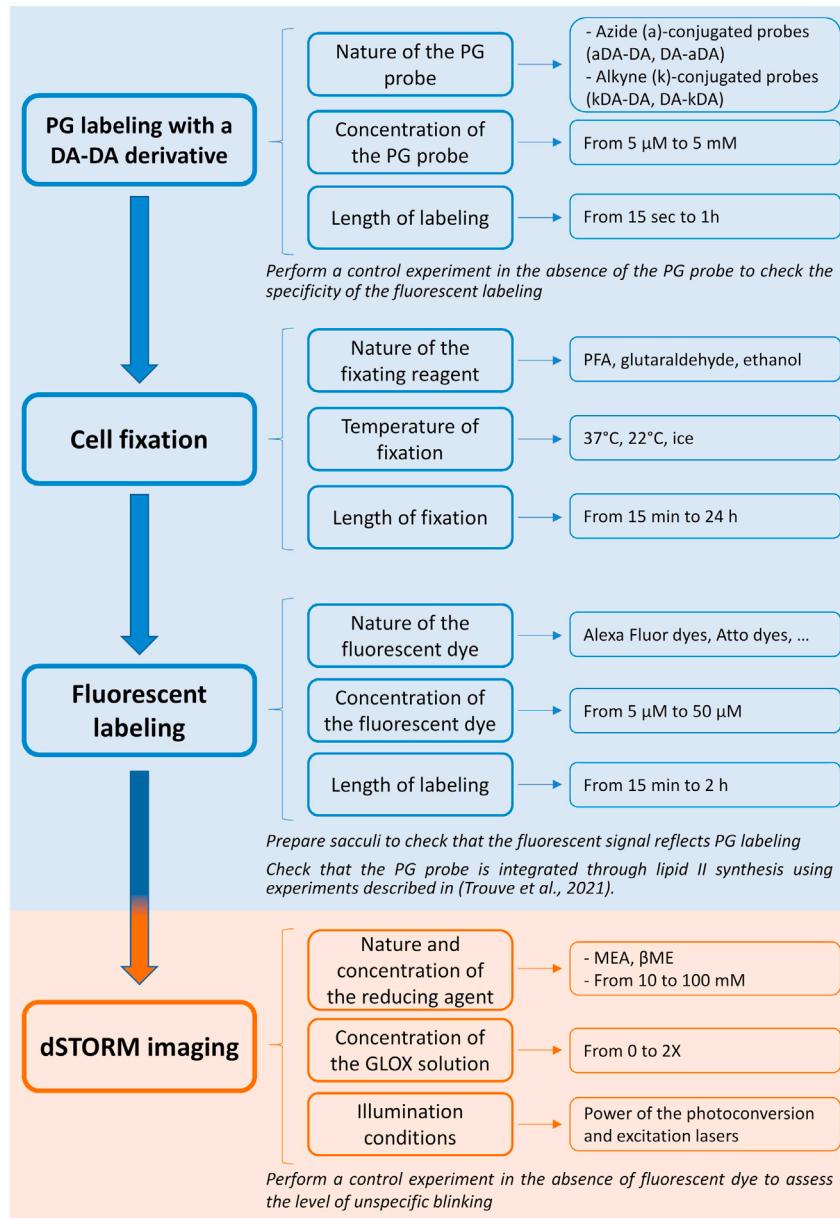
Figure 2. Flowchart of the optimization steps required for fluorescent PG labeling
Various options are proposed (right panels) to optimize key points (middle panels) of the peptidoglycan (PG) labeling, cell fixation, fluorescent labeling and dSTORM imaging processes. The blue color indicates steps for which conventional fluorescence microscopy should be used to assess the signal-to-noise ratio. Once these steps are optimized, dSTORM imaging conditions can in turn be adjusted.

 b. Remove the supernatant and resuspend the cell pellet into fresh TH broth to reach an OD_{600nm} of 2, add glycerol (sterile stock solution at 80% (vol/vol)) to reach a final concentration of 17% and homogenize by inverting the cell sample several times.

 c. Make 50-μL aliquots of the mix in cryo-tubes and store the glycerol stocks at - 80°C.

Prepare stock solutions for CY medium (C medium + yeast extract)

◷ Timing: 1 day

3. Prepare and autoclave the "20X Pre-C" solution (See materials and equipment).
4. Prepare and autoclave the "Three-in-one salts" solution (See materials and equipment).
5. Prepare the "Supplements" solution (See materials and equipment) and sterilize it using a membrane filter with a pore size of 0.22 μm.
6. Prepare a biotin solution at 200 μg·mL^{-1} by dissolving 4 μg of biotin powder into 800 μL of hot ethanol and then adding 19.2 mL of 0.01 N HCl.
7. Prepare and filter sterilize the "Adams I" solution (See materials and equipment).
8. Prepare and filter sterilize the "Adams II" solution (See materials and equipment).
9. Prepare and filter sterilize the "Adams III" solution (See materials and equipment).
10. Prepare and autoclave the 1 M phosphate buffer.

Prepare stock solutions of peptidoglycan labeling reagents

⏱ Timing: 30 min

11. Prepare frozen aliquots of the aDA-DA (((*R*)-2-amino-3-azidopropanoyl)-D-alanine, also called azido-D-alanyl-D-alanine) peptidoglycan probe.
    a. Resuspend dessicated aDA-DA into 100% DMSO to obtain a 500 mM solution.
    b. Vortex extensively to dissolve all the powder.
    c. Dilute the 500-mM aDA-DA solution to 10 mM into commercial 1× PBS.
    d. Make 50-μL aliquots and store them at - 20°C (for up to 5 years).

Note: aDA-DA is not currently commercially available and should thus be obtained by chemical synthesis as described in (Trouve et al., 2021). Alternative DA-DA derivatives whose chemical synthesis has been published previously (Liechti et al., 2014; Lund et al., 2018) can be used instead of aDA-DA (see *Troubleshooting*, problem 1).

12. Prepare frozen aliquots of the DIBO-AF647 fluorescent dye.
    a. Resuspend the DIBO-AF647 powder into 100% DMSO to obtain a 10 mM solution.
    b. Vortex extensively to dissolve all the powder.
    c. Dilute the 10 mM DIBO-AF647 solution to 500 μM into commercial 1× PBS.
    d. Make 10-μL aliquots, protect them from light by wrapping the tubes with aluminum foil and store them at - 20°C (for up to 1 year).

Note: DIBO (dibenzocyclooctyne) is a strained alkyne reactive with azides via a copper-free click chemistry reaction. AF647 (Alexa Fluor 647) is one of the most common fluorophores for dSTORM but other blinking dyes might work as well and should be empirically determined by the user (see (Dempsey et al., 2011) for a review of STORM dyes). DIBO-AF647 is now commercialized as DBCO-AF647 (Jena Bioscience).

⚠ CRITICAL: Fluorescent dyes are sensitive to light and should be stored in the dark by wrapping the storage elements with aluminum foil.

Prepare stock solutions and glassware for dSTORM

⏱ Timing: 2 h

This step guides you to prepare a dSTORM buffer, which contains two main components: a reducing agent (here a thiol), which helps switching the excited fluorophore into a dark state, and an enzymatic oxygen scavenging system, which removes oxygen from the solution to avoid oxygen-induced photobleaching and decrease the rate of fluorophore transition to the fluorescent ON state. Upon optimal chemical conditions, the stochastic oxidoreduction of the fluorophore will allow its transition between the dark and the fluorescent states in the range of milliseconds to minutes. Upon optimal

illumination conditions, this random transition will allow blinking of the fluorophores. This step is optimized for stochastic oxidoreduction of the AF647 dye incorporated into the PG of *S. pneumoniae*; the composition of the dSTORM buffer should thus be modified to adapt the protocol when PG is labeled with another dye and/or in another bacterial species (see Figure 2 and the *Critical* point addressed below for the preparation of the dSTORM buffer, as well as (Dempsey et al., 2011) for the photophysical properties of various fluorescent dyes in different compositions of dSTORM buffer).

13. Prepare 1 M MEA (mercaptoethylamine) solution by dissolving 90 mg of cysteamine powder into about 1.1 mL of HCl 0.25 N and 70 μL of HCl 37% (w/w). Following complete dissolution of the cysteamine powder upon intensive vortexing, check that the pH is between 8.0 and 8.5 with a pH paper.

△ CRITICAL: The freshness of the MEA solution is critical for the reducing ability of the dSTORM buffer. Store at 4°C for up to one month and do not freeze.

14. Prepare the 10× GLOX solution (GLucose OXydase solution, see materials and equipment).

Note: The GLOX solution contains glucose oxidase and catalase enzymes, which allow oxygen scavenging from the dSTORM buffer. The glucose oxidase consumes oxygen while oxidizing glucose and the catalase consumes the hydrogen peroxide formed by this reaction to avoid toxicity problems.

15. Clean square and rectangular high-precision coverslips in a UV-ozone oven (UVOCS) for 20 min to bleach background fluorescence (see *Troubleshooting*, problem 2). Store the coverslips onto clean Kimwipes placed into a clean Petri dish.

Note: In our protocol we use a rectangular coverslip whose size is particularly well adapted to the sample holder of the microscope, but this coverslip might be replaced by a glass slide when using another microscope set up.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Streptococcus pneumoniae R800 *rpsL1* ; Str[R]	(Lefevre et al., 1979)	N/A
Chemicals, peptides, and recombinant proteins		
BHI (Brain Heart Infusion) broth	Sigma-Aldrich	Cat# 53286
((R)-2-amino-3-azidopropanoyl)-D-alanine (aDA-DA)	(Trouve et al., 2021)	N/A
Click-iT Alexa Fluor 647 DIBO Alkyne (DIBO-AF647)	Thermo Fisher Scientific	Cat# C20022
TRIS (tris(hydroxyméthyl)aminométhane)	Euromedex	Cat# 200923-A
PBS (Phosphate Buffered Saline)	Gibco (Life Technologies)	Cat# 14190-144
NaCl (sodium chloride)	Euromedex	Cat# 1112-A
KCl (potassium chloride)	Sigma-Aldrich	Cat# P9333
TCEP (Tris (3-caboxyethyl) phosphine hydrochloride)	Thermo Fisher Scientific	Cat# 20491
Glycerol	VWR	Cat# 24388.295
Cysteamine (MEA)	Sigma-Aldrich	Cat# 30070
Catalase from bovine liver	Sigma-Aldrich	Cat# C40
Glucose oxidase type VII from *Aspergillus niger*	Sigma-Aldrich	Cat# G2133
Paraformaldehyde (PFA)	Electron Microscopy Sciences	Cat# 15710

(Continued on next page)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agarose D5	Euromedex	Ref# D5-E
RBS 25 solution	Sigma-Aldrich	Cat# 83460
Deposited data		
Raw phase contrast, bright field, diffraction-limited and dSTORM images	(Trouve et al., 2021)	https://zenodo.org/record/4573897#.YIl_ZKEpBPY
Software and algorithms		
Metamorph	Molecular Devices	https://www.moleculardevices.com/
Volocity	Quorum Technologies	https://quorumtechnologies.com/volocity
ImageJ	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
FIJI	(Schindelin et al., 2012)	https://imagej.net/Fiji
ThunderSTORM	(Ovesný et al., 2014)	https://zitmen.github.io/thunderstorm/
Other		
Precision cover glasses, No. 1.5H, 24 X 50 mm, 170 ± 5 mm	Marienfeld	Cat# 0107222
Precision cover glasses, No. 1.5H, 22 X 22 mm, 170 ± 5 μm	Marienfeld	Cat# 0107052
Autofluorescent pink and yellow plastic slides	CHROMA	Cat# 92001
Silicon glue Picodent Twinsil speed	Picodent	Cat# 1300 1002
TetraSpeck™ Microspheres	Thermo Fisher Scientific	Cat# T7279

MATERIALS AND EQUIPMENT

"20× Pre-C" solution		
Reagent	Final concentration	Amount
Anhydrous sodium acetate	$24 \, mg \cdot mL^{-1}$	12 g
Casamino acids	$100 \, mg \cdot mL^{-1}$	50 g
L-tryptophane	$100 \, \mu g \cdot mL^{-1}$	50 mg
L-cysteine	$1 \, mg \cdot mL^{-1}$	500 mg
NaOH (1–10 N)	n/a	Up to pH 7.5
ddH$_2$O	n/a	Up to 500 mL
Total	**n/a**	**500 mL**

Note: pH should first be grossly adjusted using 10 N NaOH, and then finely adjusted to pH 7.5 using 1 N NaOH. Sterilize by autoclaving. Stable for several months at 22°C.

"Three-in-one salts" solution		
Reagent	Final concentration	Amount
MnSO$_4$ (100 mM)	0.02 mM	20 μL
MgCl$_2$ (2 M)	500 mM	25 mL
CaCl$_2$ (100 mM)	5 mM	5 mL
ddH$_2$O	n/a	Up to 100 mL
Total	**n/a**	**100 mL**

Note: Sterilize by autoclaving. Stable for several months at 22°C.

"Supplements" solution

Reagent	Final concentration	Amount
"Three-in-one salts" solution	n/a	20 mL
D-glucose (20%)	5.6%	40 mL
D-sucrose (50%)	0.7%	2 mL
Adenosine (2 mg·mL^{-1})	0.6 mg·mL^{-1}	40 mL
Uridine (2 mg·mL^{-1})	0.6 mg·mL^{-1}	40 mL
Total	n/a	142 mL

Note: Sterilize by filtration. Stable for several months at 22°C.

"Adams I" solution

Reagent	Final concentration	Amount
Biotin (200 μg·mL^{-1})	15 μg·mL^{-1}	15 mL
Nicotinic acid	150 μg·mL^{-1}	30 mg
Pyridoxine HCl	175 μg·mL^{-1}	35 mg
Calcium panthoneate	600 μg·mL^{-1}	120 mg
Thiamine HCl	160 μg·mL^{-1}	32 mg
Riboflavin	70 μg·mL^{-1}	14 mg
ddH$_2$O	n/a	Up to 200 mL
Total	n/a	200 mL

Note: Stir the "Adams I" solution for 2 h before sterilization by filtration. Stable for several months when stored in the dark (vitamins are light sensitive) at 4°C.

"Adams II" solution

Reagent	Final concentration	Amount
FeSO$_4$ (7H$_2$O)	0.5 mg·mL^{-1}	500 mg
CuSO$_4$ (5H$_2$O)	0.5 mg·mL^{-1}	500 mg
ZnSO$_4$ (7H$_2$O)	0.5 mg·mL^{-1}	500 mg
MnCl$_2$ (4H$_2$O)	0.2 mg·mL^{-1}	200 mg
HCl (37%)	0.37%	10 mL
ddH$_2$O	n/a	Up to 1 mL
Total	n/a	1 L

Note: Sterilize by filtration. Stable for several months at 4°C.

"Adams III" solution

Reagent	Final concentration	Amount
Adams I	n/a	80 mL
Adams II	n/a	20 mL
L-asparagine	2 mg·mL^{-1}	1 g
Choline choride	200 μg·mL^{-1}	100 mg
CaCl$_2$ (100 mM)	160 μM	800 μL
ddH$_2$O	n/a	Up to 500 mL
Total	n/a	500 mL

Note: Sterilize by filtration. Stable for several months in the dark (vitamins are light sensitive) at 4°C.

1 M phosphate buffer

Reagent	Final concentration	Amount
KH_2PO_4 (1 M)	1 M	5.3 mL
K_2HPO_4 (1 M)	1 M	94.7 mL
Total	n/a	100 mL

Note: Sterilize by autoclaving. Stable for several months at 22°C. This buffer is not at pH 8.0 but brings the pH of the CY medium to 8.0.

10× GLOX solution

Reagent	Final concentration	Amount
Tris-HCl pH 7.5 (1 M)	100 mM	100 µL
KCl (1 M)	25 mM	25 µL
TCEP pH 7.0 (100 mM)	1 mM	10 µL
Glucose oxidase powder	5 mg·mL^{-1}	5 mg
Catalase (20 mg·mL^{-1})	0.4 mg·mL^{-1}	20 µL
Glycerol (100%)	50%	500 µL
ddH$_2$O	n/a	345 µL
Total	n/a	1 mL

Note: Centrifuge (10,000×*g*, 10 min, RT) and store the supernatant at - 20°C up to 2 years.

CY medium

Reagent	Final concentration	Amount
"20× Pre-C" solution	n/a	2 mL
"Supplements" solution	n/a	1.3 mL
L-glutamine (1 mg·mL^{-1})	0.02 mg·mL^{-1}	1 mL
"Adams III" solution	n/a	1 mL
Pyruvate (2%)	0.02%	0.5 mL
Phosphate buffer (1 M)	32 mM	1.5 mL
Yeast extract (5%)	0.1%	0.9 mL
ddH$_2$O	n/a	38 mL
Total	n/a	46.2 mL

Note: Growth of pneumococcal cells in BHI or CY media results in low fluorescence background. By contrast, TH (Todd-Hewitt) medium results in strong fluorescence background signal.

dSTORM buffer with GLOX

Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (500 mM), NaCl (170 mM)	75 mM (Tris), 25 mM (NaCl)	6 µL
Glucose (50%)	10%	8 µL
MEA (1 M)	100 mM	4 µL
GLOX solution (10×)	1×	4 µL
Tetraspeck beads	n/a	0.4 µL
ddH$_2$O	n/a	17.6 µL
Total	n/a	40 µL

△ CRITICAL: The composition of the dSTORM buffer provided here is optimized for *S. pneumoniae* PG labeled with AF647; it should be adjusted when the protocol is adapted to another fluorescent dye and/or another bacterial species (Figure 2). In particular, the nature and concentration of the reducing agent, which is the key point of the dSTORM buffer, should be optimized when using a different fluorescent dye. MEA or βME (β-mercaptoethanol) are most commonly used, at concentrations varying from 10 to 100 mM. The optimal concentration of the GLOX solution might also vary from 0 to 2×. See (Dempsey et al., 2011) for the photophysical characterization of various fluorescent dyes in different STORM buffers.

Note: Tetraspeck beads are used as fiducial markers to correct the drift when cells are mounted between two coverslips. When cells are mounted in microhole agarose pads, no fiducial marker is used and the drift is corrected using the cross correlation method.

dSTORM buffer without GLOX		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (500 mM), NaCl (170 mM)	75 mM (Tris), 25 mM (NaCl)	3 μL
Glucose (50%)	10% (w/vol)	4 μL
MEA (1 M)	100 mM	2 μL
ddH$_2$O	n/a	11 μL
Total	n/a	20 μL

STEP-BY-STEP METHOD DETAILS
Step I: Peptidoglycan labeling in growing cells

⏲ Timing: 1 day

In this step, *S. pneumoniae* cells in the exponential growth phase are grown for a short period (5 min, called the PULSE period) in the presence of the aDA-DA probe. During this incubation period, aDA-DA is incorporated into the PG precursor (called lipid II), leading to the labeling of the fourth position of the peptide chain with an azide group (see Figure 1 and (Trouve et al., 2021)). Following lipid II oligomerization and cross-linking of glycan chains, the new PG will then be labeled for a short time. Optionally, the labeled cells will be further grown in the absence of the probe to investigate the fate of the new PG and determine how it is remodeled over time (PULSE-CHASE experiment).

1. Prepare fresh CY medium.
2. Thaw a 50-μL aliquot of a frozen glycerol stock of pneumococcal cells.
3. Inoculate 1 mL of CY medium with the glycerol stock (starting OD$_{600nm}$ = 0.1) and incubate the culture for 90 min in a 15-mL Falcon tube placed at 37°C in a static CO$_2$ incubator.
4. Dilute the culture 1/10th by adding 9 mL of CY medium. Further incubate the culture at 37°C until the OD$_{600nm}$ reaches 0.3.
5. Dilute the culture 1/10th by mixing 1 mL of sample with 9 mL of CY medium (starting OD$_{600nm}$ = 0.03). Further incubate the culture at 37°C until the OD$_{600nm}$ reaches 0.3.

   Note: These dilutions allow one to reach a steady state of growth, ensuring that all cells are actively dividing.

6. Pre-warm fresh CY medium, aDA-DA solution and PFA at 37°C.
7. Centrifuge (4,000×*g*, 15 min, 22°C) the culture and resuspend the cell pellet into 1/25th volume of CY medium (see *Troubleshooting*, problem 1).
8. Mix 200 μL of the cell sample with 50 μL of 10 mM aDA-DA (final concentration of 2 mM).

9. Incubate cells for 5 min at 37°C in a water bath (labeling PULSE period).

   Note: Incubation of cells with 2 mM aDA-DA for 5 min provides optimal signal-to-noise ratio, temporal and spatial resolution in *S. pneumoniae*. However, the optimal aDA-DA concentration and duration of the PULSE period might vary in other bacteria (see *Troubleshooting*, problem 1).

10. Fix cells by adding 260 μL of PBS-PFA 4% (the final PFA concentration will be 2%) to the 250-μL sample. Incubate for 12–18 h on ice at 4°C (see *Troubleshooting*, problems 2 and 5).

   △ CRITICAL: Steps 7–10 should be performed as fast and as precisely as possible to get the maximal temporal resolution and efficiency of aDA-DA incorporation.

   Optional: For a PULSE-CHASE experiment aiming at investigating the fate of the labeled PG, add the following steps between steps 9 and 10:

   a. Centrifuge (4,000×*g*, 5 min, 22°C) the cells and resuspend the pellet into 250 μL of pre-warmed CY medium.
   b. Incubate for 15 min at 37°C in a water bath (CHASE period).

   △ CRITICAL: To perform comparable PULSE and PULSE-CHASE experiments on the same bacterial culture, double the culture volume for the PULSE labeling step, and split the sample in two at the end of the labeling period. One half of the labeled sample will be fixed directly and the other half will be used for the CHASE step (incubation in the absence of the aDA-DA probe) before fixation.

   ⊪ Pause Point: Fixed cells can be stored on ice (in an ice bucket placed at 4°C) for up to 2 days before fluorescent tagging.

Step II: Fluorescent tagging of labeled peptidoglycan

   ⊙ Timing: 90 min

In this step, a covalent bond will form through a copper-free click chemistry reaction between the azide group incorporated into the PG peptides and the DIBO group carried by the AF647 fluorescent dye.

11. Centrifuge the fixed sample (7,000×*g*, 5 min, 22°C) and resuspend the cell pellet into 150 μL of PBS 1× and 10 μL of 500 μM DIBO-AF647 (final DIBO-AF647 concentration of about 30 μM) (see *Troubleshooting*, problem 3).
12. Incubate for 1 h at 22°C (CLICK reaction) in the dark (to preserve the fluorescence of AF647), and add 1 mL of 1× PBS.
13. Wash twice by centrifuging (7,000×*g*, 5 min, 22°C) the cell sample and resuspending the cell pellet into 1 mL of 1× PBS.

   Note: When adapting this protocol to a new bacterial species, when using another PG probe or fluorescent dye, many factors need to be optimized. For each optimization step, the fluorescent signal-to-noise ratio can be evaluated by conventional fluorescence microscopy before proceeding to dSTORM data collection. Figure 2 provides a flowchart for this optimization process.

14. During the two washes, prepare the dSTORM buffer (see *Troubleshooting*, problem 4).

Note: For an experiment in which cells will be mounted between two coverslips, add Tetra-speck fiducial markers in the dSTORM buffer. For an experiment in which cells will be mounted in a microhole agarose pad, do not add any fiducial marker in the dSTORM buffer.

15. Centrifuge the cell sample (7,000×g, 5 min, 22°C) and resuspend the pellet into 15 μL of dSTORM buffer.

Note: Depending on the density of the cell suspension, add more STORM buffers to reach the desired cell density on the microscopy field (about 200 *S. pneumoniae* cells for a 32.2 × 32.2 μm field, see Figure 6).

Optional: Instead of step 14, to prepare sacculi from labeled cells and check incorporation of aDA-DA into the PG, add 300 μL of 8% sodium dodecyl sulfate (SDS) and incubate for 1 h at 100°C. Wash 5 times with 1 mL of 1× PBS. Then incubate with 20 μg·mL^{-1} DNAse and 20 μg·mL^{-1} RNAse in 100 mM Tris-HCl pH 7.5, 20 mM MgCl$_2$ (2 h, 37°C) on a rotating wheel. After addition of 2 mM CaCl$_2$ and 200 μg·mL^{-1} proteinase K, incubate sacculi for 12–18 h at 37°C on a rotating wheel. Finally, wash the sacculi 3 times with 1× PBS before imaging by conventional fluorescence microscopy.

Step III: Mounting of cells for dSTORM imaging

⏲ Timing: 20–30 min

This step describes how to mount pneumococcal cells to observe them along their longitudinal or radial axis. It will need to be adapted for each particular bacterial species and shape, because their morphological features will influence the way they lie on the microscopy coverslips or enter microholes.

16. Load 8 μL of cell suspension onto a rectangular UV-treated microscopy coverslip.
17. Place a square coverslip onto the cell drop and place a Kimtech wipe on top.
18. Apply a 2.3-kg weight onto the coverslip for 15 min to allow the cells to settle on the microscopy slide (see *Troubleshooting*, problems 2 and 5).

Note: Place and remove the weight by applying a precise vertical movement to prevent slipping of the coverslip.

19. Seal with transparent nail polish.

Optional: The nail polish can be replaced by twinsil silicon glue.

Optional: Instead of steps 16 to 19, to orient the cells vertically relative to the microscopy coverslip, mount the cells in a microhole agarose pad by applying the following steps:

a. Load 300 μL of 2% (w/vol) agarose solution onto a rectangular UV-treated microscopy coverslip.
b. Place a micropillar device (Söderström et al., 2018) horizontally on top of the agarose drop and wait for about 30 min until the agarose has solidified.
c. Remove the micropillar device as vertically as possible and cut the excess of agarose around the microholes.

Note: The microhole agarose pad should be prepared about 40 min before the end of the fluorescent tagging step (step 12).

d. Prepare the dSTORM buffer without the GLOX solution.

e. Load 20 μL of dSTORM buffer without GLOX on the agarose pad, incubate for 10 min at 22°C and remove excess buffer.

Note: As the agarose pad contains water, step *e* is essential to replace water with the dSTORM buffer.

f. Load 15 μL of cell suspension onto the agarose pad and apply a slow circular movement to spread the cell suspension all over the agarose pad.
g. Let the agarose pad sit for 10 min at 22°C.
h. Wash twice with 10 μL of dSTORM buffer (containing GLOX) to remove bacteria that have not entered the microholes. Do not flush into the microholes but rather apply a horizontal flow at the surface of the agarose pad.
i. Place a square UV-treated coverslip on top of the agarose pad.
j. Immobilize the coverslip with two pieces of tape.

Note: The microhole agarose pad is too thick to be sealed with nail polish or silicon glue.

Step IV: dSTORM data acquisition

© Timing: 20 min

This step describes how dSTORM data can be acquired on labeled pneumococcal cells observed at 25°C with a home-built PALM/STORM setup. This setup is based on an Olympus IX81 inverted microscope equipped with a diode-pumped solid-state laser at 405 nm (CrystaLaser) and a diode 643 nm laser (Toptica Photonics). To achieve wide-field illumination, circularly polarized laser beams are focused to the back focal plane of a × 100 1.49-numerical-aperture oil immersion apochromatic objective lens (Olympus). The laser illumination intensities are tuned by an acousto-optic tunable filter (AOTF; AA Opto Electronic). Fluorescence images are acquired with an Evolve 512 back-illuminated EMCCD camera (Photometrics) controlled by the MetaMorph software (Molecular Devices). Commercial STORM systems can also be purchased from companies such as Abbelight or Nikon. The optimal imaging conditions will depend on the fluorescent dye, the composition of the dSTORM buffer and the bacterial species that is observed. Experimental cues to optimize the acquisition of dSTORM data are given in Figure 2.

20. Place an autofluorescent pink slide on top of the objective and set up the 643-nm laser at low intensity; orient the laser vertically relative to the slide (epifluorescence illumination) and center it in the camera field of view.
21. Place an autofluorescent yellow slide on top of the objective and set up the 405-nm laser at low intensity, and center the laser in the camera field of view to overlap with the 643-nm laser.
22. Place the sample with the rectangular coverslip oriented toward the objective.
23. Find a field in which cells are concentrated enough but not overlapping and acquire a bright field image.
24. Acquire a diffraction-limited fluorescence image of the bacterial field:
    a. Set up the 643-nm laser to 0.5% of intensity ($8.5 \text{ W} \cdot \text{cm}^{-2}$).
    b. Adjust the focus.
    c. Acquire a diffraction-limited image.
25. Acquire the dSTORM image stream:
    a. Progressively increase the 643-nm laser intensity from 0.5 to 100% (from $8.5 \text{ W} \cdot \text{cm}^{-2}$ to $1.7 \text{ kW} \cdot \text{cm}^{-2}$, within about 1 min) to convert the fluorophores into their dark state while preserving the chromophore integrity (see *Troubleshooting*, problem 4).
    b. When the fluorophores start blinking (see *Troubleshooting*, problem 4), acquire the image stream using 100% intensity of the 643-nm laser and 50 ms of exposure time and EM gain of 200.
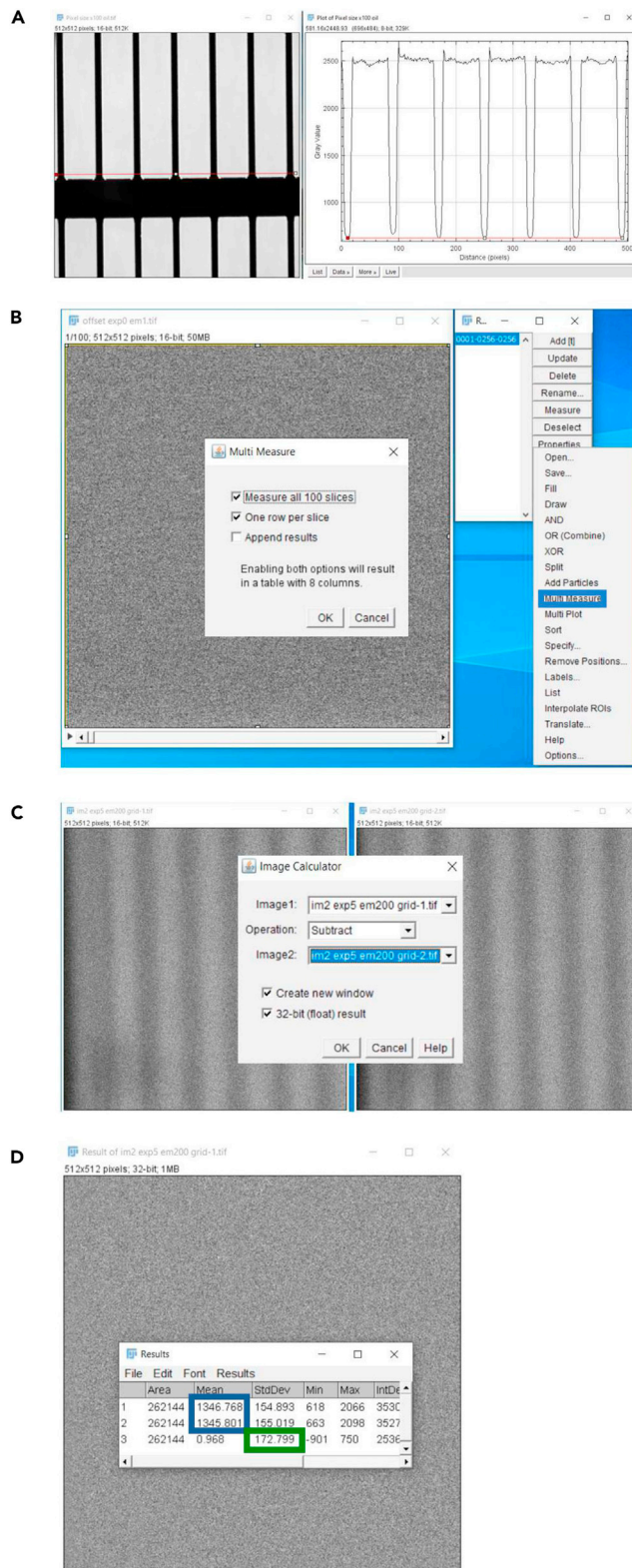
Figure 3. Measurement and calculation of camera parameters
(A) Image of a microscope ruler taken with a 100× oil objective (left panel) and its intensity profile used to calculate a pixel size value (right panel).

Figure 3. Continued

(B) Screenshots accompanying the procedure to determine a camera Base level.

(C) Two unfocused images of a grid taken in the same conditions in order to calculate the photon conversion factor.

(D) The result of image subtraction (images from panel C, whose mean values are shown in the blue box) and measured standard deviation of the signal (green box).

Note: the AF647 fluorophores should turn off rapidly and start blinking before reaching 100% of laser intensity (1.7 kW·cm^{-2}). When the fluorophore blinking frequency starts decreasing, progressively increase the intensity of the 405-nm laser from 0.1 to 3% (0.05–1.5 W·cm^{-2}), to maintain a constant blinking frequency.

Note: Adjust the exposure time so that the ON time of each fluorophore covers a single frame, and the EM gain to cover the dynamic range of the camera (to avoid saturation or under sampling of the signal).

Note: The first time you perform the experiment, you can analyze the acquired dSTORM frames using the NanoJ-SQUIRREL plugin in order to plot the resolution versus the number of frames. The optimal number of frames for future experiments can be estimated from the plateau phase of the curve.

Note: Before placing a new sample, clean the left-over immersion oil on the objective and apply a new drop of oil.

Step V: dSTORM data processing

⏱ Timing: 40 min

This part of the protocol describes the raw data fitting process, as well as rendering and post-processing of the final super-resolution images using the ThunderSTORM plugin of ImageJ. The first step here is to fill in the camera parameters in the Camera setup window (Figure 4B). It is done to calibrate acquired images by converting pixels to nanometers and intensity grey levels (ADUs) to photoelectrons. The parameters such as Photoelectrons per A/D counts (also known as CCD sensitivity or Camera gain, not to mix with the EM gain), Base level (Bias or Camera Offset), and Quantum efficiency can be found in the Certificate of Camera Performance or in the Camera Datasheet that are normally sent with a camera. Alternatively, they can be measured following, for example, the protocol from Photometrics (https://www.photometrics.com/learn/white-papers → "Camera Test Protocol") which is presented below. It is especially recommended to periodically control the EM gain amplification, which is known to degrade with time depending on the usage of an EMCCD camera.

The formula used in ThunderSTORM to convert the image signal from grey levels (ADUs) to photoelectrons is the following:

$$\text{Signal [e}^-] = (\text{Signal [ADUs]} - \text{Base level [ADUs]}) * \frac{\text{Gain [e}^-\text{/ADUs]}}{\text{EM Gain}}$$

In order to get the number of detected photons, this value should be further divided by the camera Quantum efficiency:

$$\text{Signal [photons]} = \text{Signal [e}^-] / \text{Quantum efficiency [e}^-\text{/photons]}$$

Note: The Quantum efficiency parameter is wavelength specific, so check the emission spectrum of your dye of choice before referring to the graph. This parameter is not presented in every version of ThunderSTORM plugin.

For a practical purpose, the ratio of gains can be represented as a single parameter called the photon conversion factor and calculated by a single point mean variance test (see step 28).

26. Calculate the pixel size of the camera as follows (Figure 3A):
    a. Take a brightfield image of a calibration target slide (e.g., R1L3S2P from Thorlabs).
    b. In ImageJ select the "Straight line" tool and plot a cross-section profile ("Analyze" → "Plot profile").
    c. Measure the distance between the furthest visible lines of the ruler; divide the known distance (provided by the manufacturer of the calibration slide) in micrometers by the measured value in pixels.

27. Calculate the base level of the camera as follows (Figure 3B):
    a. Prevent any light from entering the camera (e.g., by closing a camera shutter).
    b. Set exposure time to 0 ms (if you get an error message, set it to 1 ms) and record a stack of 100 images.

    Note: Go to "Analyze" → "Set Measurements" and check that the "Mean grey value" parameter is selected.

    c. In ImageJ, calculate the mean intensity value for each frame (in grey levels). For this, make an ROI of the image size, add it to ROI manager ("Analyze" → "Tools" → "ROI manager" → "Add"), then select your ROI from the list and click "More" → "Multi Measure".
    d. Calculate the base level as the mean of the 100 obtained values by clicking "Results" → "Summarize" in the *Results* window.

28. Calculate the photon conversion factor of your camera as follows:
    a. Take two images using the same light level, with 5 ms exposure time and EM Gain used for the dSTORM data collection (Figure 3C).
    b. In ImageJ, calculate the mean intensity of each image ("Analyze" → "Measure") and then average the two values (Figure 3D, blue box).
    c. Using the "Image Calculator" tool ("Process" → "Image Calculator"), subtract one image from another. Both boxes should be checked: create new window and 32-bit (float) result (Figure 3C).

    Note: Go to "Analyze" → "Set Measurements" and check that the "Standard deviation" parameter is selected.

    d. Measure the standard deviation of the resulting image ("Analyze" → "Measure") (Figure 3D, green box).
    e. Calculate the variance from the standard deviation:

    $$Var = \frac{StdDev^2}{2}$$

    f. Calculate the photon conversion factor (PCF) using values from steps 27, 28b and 28e:

    $$PCF = \frac{Average_{1,2} - Base\ level)}{Var}$$

    g. Repeat previous steps with a few more exposure times (e.g., 10, 20, 40 ms) to ensure that the PCF factor does not vary with different light levels.

    Note: As mentioned above, the PCF factor replaces the ratio of gains and should be entered into the field "Photoelectrons per A/D counts" of the *Camera setup* window in ThunderSTORM, whereas the "EM Gain" should be set to 1, in order to not double count the real value.
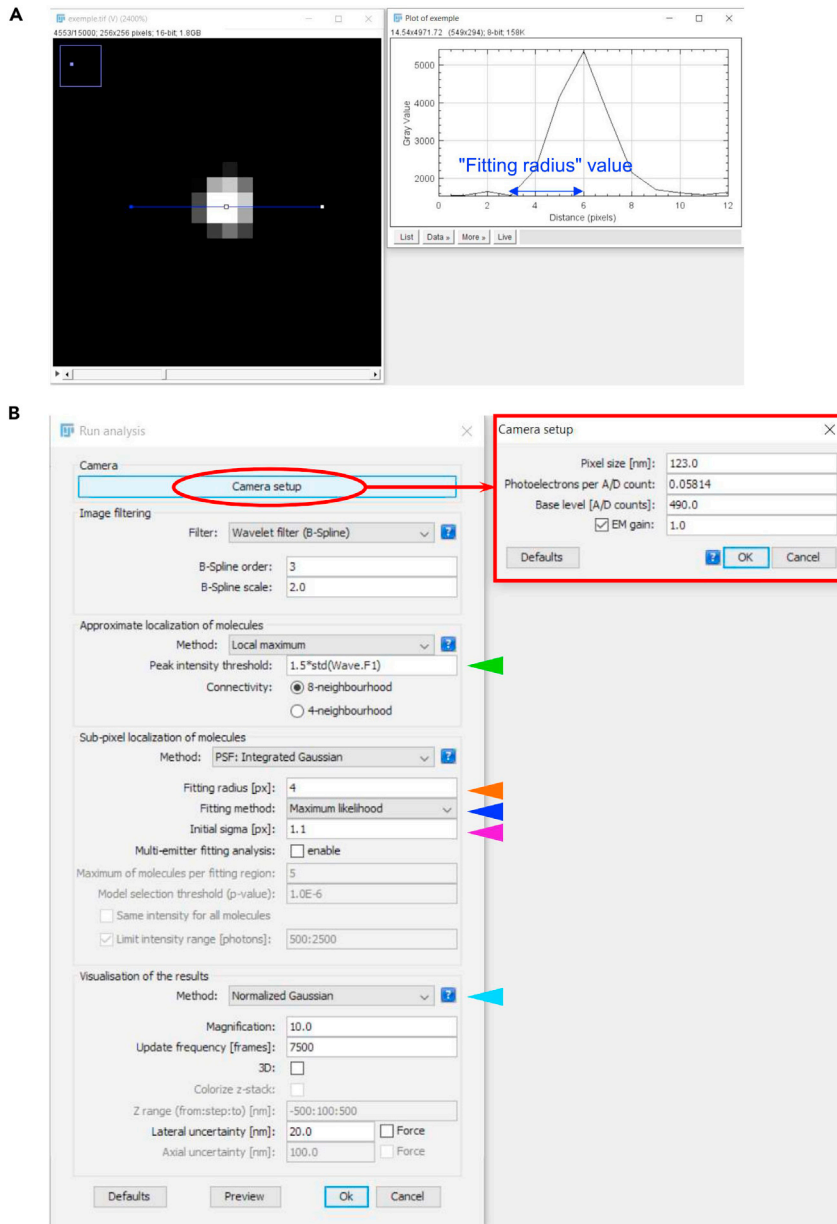
Figure 4. Running parameters for dSTORM data processing using ThunderSTORM

(A) Representative image of a single fluorescent spot across which a line has been drawn to plot the point spread function. On the right panel, the Fitting radius value is measured as the distance between the minimum and maximum value of the point spread function.

(B) Snapshot of a *Run analysis* window from ThunderSTORM, showing various parameters (arrowheads of different colors are mentioned along the protocol) that must be entered to process data.

29. To determine the fitting radius, open the image stream under ImageJ and measure the distance between the maximum and minimum values of a point spread function. For this, draw a line across a single fluorescent spot using the "Straight line" tool, and plot its intensity against pixels ("Analyze" → "Plot profile") (Figure 4A).

30. Process the dSTORM image stream using the ThunderSTORM ImageJ/FIJI plugin as follows:

Note: A complete guide to dSTORM data processing using ThunderSTORM can be found in (Ovesný et al., 2014). Here, the protocol only aims at providing basic directions for this.

a. Launch the ThunderSTORM plugin under FIJI.
b. Open the *Camera set up* window and specify the "Pixel size", "Photoelectron count", "Base level" and "EM Gain" values (Figure 4B, red box) found in the certificate of camera performance or equivalent document, or otherwise determined in steps 26–28.
c. For the "Peak intensity threshold" (Figure 4B, green arrowhead), start with a default value and adjust if needed (between 0.5 and 2) to correct for false positive (threshold is too low, noise is detected) or false negative localizations (threshold is too high and some of real localization are not detected).
d. In the "Sub-pixel localization of molecules" parameters, specify the "Fitting radius" value (Figure 4B, orange arrowhead) determined in step 29. Decimal values of the fitting radius must be rounded up.
e. Specify "Maximum likelihood" as the "Fitting method" (Figure 4B, dark blue arrowhead).

Note: "Maximum likelihood" is the most precise fitting method in the list, however, it is more resource demanding and requires longer time for fitting.

f. Enter an "Initial sigma" value (Figure 4B, violet arrowhead) approximated to the third of the "Fitting radius" value.

Note: The "Initial sigma" value will be optimized after a first run of localization.

g. Select "Normalized Gaussian" as the visualization method (Figure 4B, light blue arrowhead).

Note: We use the "Normalized Gaussian" method because it allows displaying the calculated localization precision for each molecule.

h. Run data analysis.
i. Using the "Plot histogram" tab (Figure 5A, red ellipse) in the ThunderSTORM result page, plot the "Sigma" parameter (Figure 5A, violet box). Calculate the "Initial sigma" value as the ratio between the "Mean" value of the sigma distribution (Figure 5B, violet arrowhead) and the "Pixel size" value determined in step 26.
j. Enter the new "Initial sigma" value in the "Sub-pixel localization of molecules" parameters (Figure 4B, violet arrowhead) and run the analysis again.

Note: This step is only performed once, unless the fluorophore and microscope are modified.

k. Using the "Plot histogram" tab (Figure 5A, red circle) in the *ThunderSTORM result* window, plot the "Uncertainty_xy" value (Figure 5A, green box). Note the localization precision as the "Mean" value of the uncertainty distribution (Figure 5B, green arrowhead).
l. If you have used fiducial markers during the data acquisition, correct the drift by choosing "Fiducial markers" (Figure 5A, dark blue circle) in the "Drift correction" tab (Figure 5A, orange ellipse). Otherwise, use the "Cross correlation" method (Figure 5A, light blue ellipse) to correct the drift.

Note: In our experiments, we use fiducial markers when cells are trapped between two coverslips, and corrected the drift using the cross correlation method when cells are trapped in microholes. The cross correlation algorithm works especially well with regular structures and a high number of detected molecules. When working with fiducial markers, make sure to
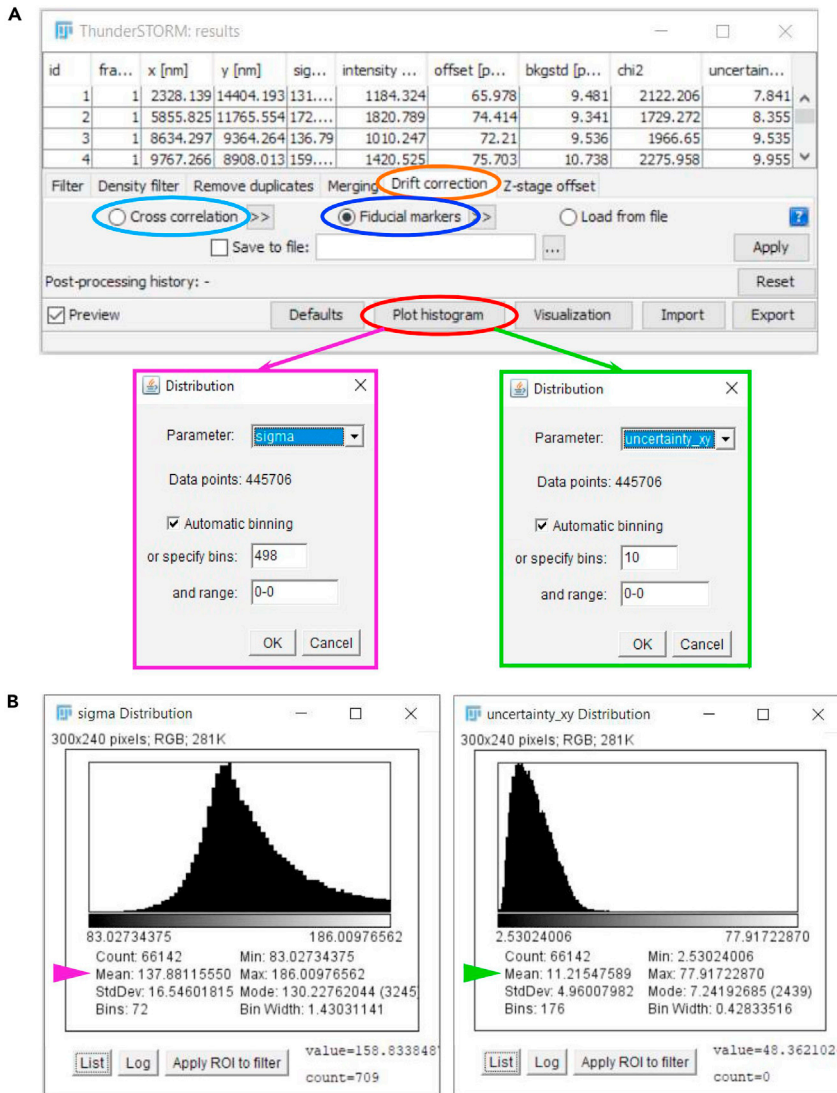
Figure 5. Refinement of dSTORM data processing using ThunderSTORM
(A) Snapshot of a *ThunderSTORM results* window showing various options (ellipse of different colors are mentioned along the protocol) to refine the reconstruction of the dSTORM image.
(B) Snapshots of histogram distributions used to determine the "Sigma" value (pink arrowhead) and the localization precision (green arrowhead).

have few of them in the field of view and check that they all display the same drift, indicating that there is no freely moving fiducial marker.

m. Generate the final reconstructed dSTORM image through the "Visualization" tab.

Note: Other post-processing analyses can be run through the localization table tabs to improve the quality of the final image. For example, multi-emitters can be filtered out by their Sigma value using the "Filter" tab, background noise can be removed using the "Density filter" tab, or multiple localizations of the same molecule can be combined together with the "Merging" function.
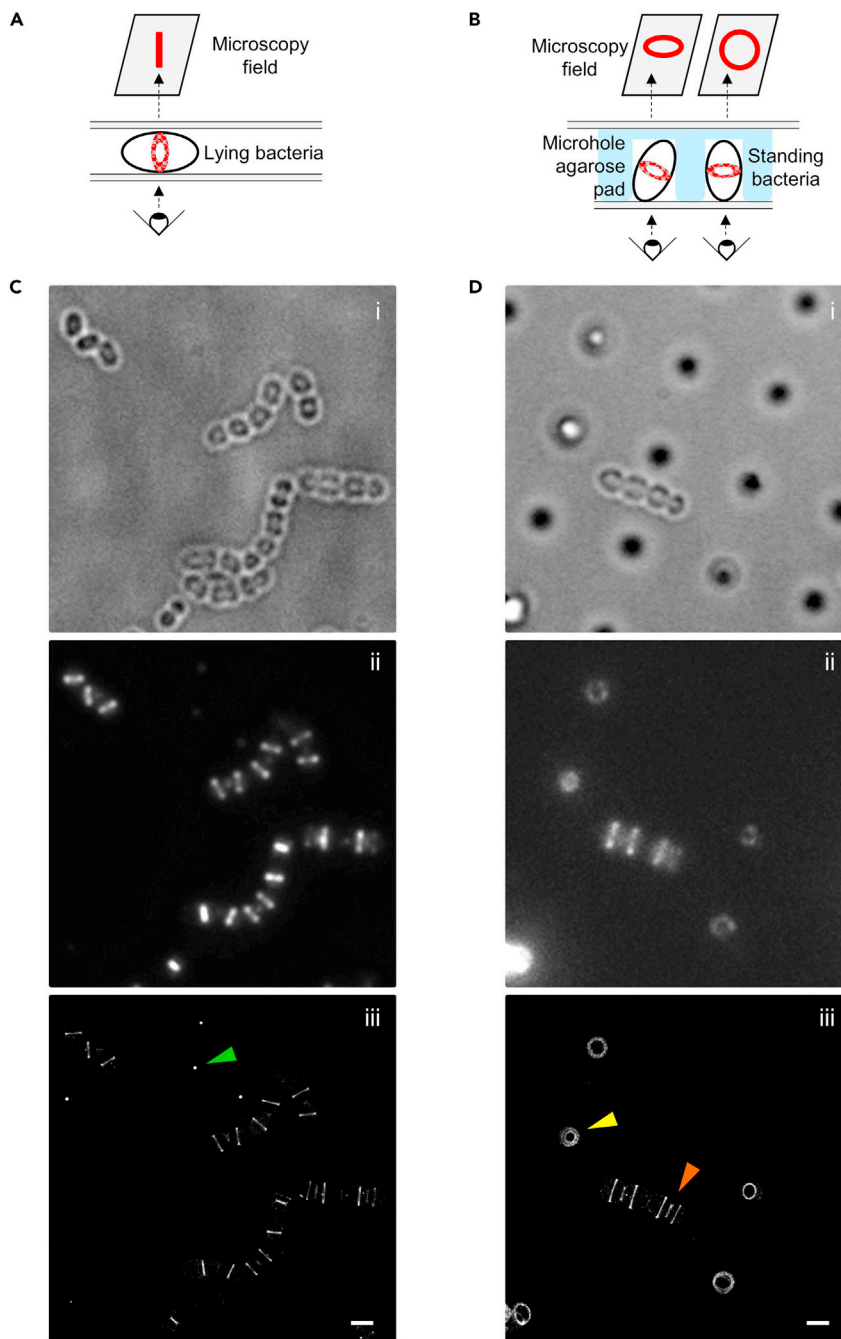
Figure 6. Observation of PG-labeled pneumococcal cells by low-resolution fluorescence microscopy and super-resolution dSTORM

(A) Schematics showing a cell mounted between two coverslips, allowing a side view of the labeling pattern. In this orientation, the 2D projection of the annular labeled regions results in band-like patterns.

(B) Schematics showing cells trapped in a microhole agarose pad, allowing an elliptic or annular top view of the labeling.

(C and D) Large fields of *S. pneumoniae* D39$_{\Delta cps}$ cells labeled with aDA-DA and DIBO-AF647. Cell samples are mounted between two coverslips (C) or using a microhole agarose pad (D). The green arrowhead points at a Tetraspeck bead, the yellow arrowhead points at a cell trapped in a microhole, the orange arrowhead points at a cell lying on the agarose pad. Bright field (i), diffraction-limited (ii) and reconstructed dSTORM (iii) images are shown. Scale bar, 1 μm.

EXPECTED OUTCOMES

Bright field, diffraction-limited and dSTORM reconstructed images obtained from PG-labeled cells, oriented horizontally or vertically relative to the microscopy coverslip, are shown in Figure 6. In our experiments, successful dSTORM data acquisition provided localization precisions up to 12 nm and spatial resolutions up to 30 nm. In the reconstructed dSTORM images, cells are at different stages of the cell cycle. The labeling patterns can be classified along the cell cycle and analyzed as described in (Trouve et al., 2021).

LIMITATIONS

This PG labeling and dSTORM imaging protocol should be easily reproducible in *Streptococcus pneumoniae*. In other bacteria, its success will depend on the ability of the aDA-DA probe to enter the cell and to be incorporated into the lipid II precursor. Unspecific fluorescent signal might also be observed. To overcome these putative issues, alternatives to several crucial labeling aspects are provided in the Troubleshooting section and Figure 2, including the nature of the PG probe and fluorescent dye.

To get satisfying dSTORM signals, the illumination lasers need to be sufficiently powerful. In our experiments, the dSTORM data were acquired on a home-built PALM/STORM microscope with laser powers described in the protocol. The localization precision and the spatial resolution of the reconstructed images will reflect the overall quality of the dSTORM data. The localization precision will mainly depend on the photon output (the higher the better). The spatial resolution on the other hand will depend on various parameters, including the duty cycle of the fluorophore (the ratio between the duration of the ON state versus the OFF state of fluorophores, which influences the spatial and temporal separation of fluorescence emission profiles and thus the identification of single emitters), the switching cycle (the number of ON/OFF state cycles before photobleaching), the contrast ratio (signal-to-noise ratio, the highest the better) and the labeling density (based on the Nyquist criterion, the spatial resolution will be half the sampling rate). A didactic guide regarding key aspects of STORM and addressing these points can be found at https://www.microscopyu.com/techniques/super-resolution/single-molecule-super-resolution-imaging as well as in (Choquet et al., 2021; Sahl et al., 2017; van de Linde et al., 2011; Vangindertael et al., 2018). The parameters that affect the resolution are strongly dependent on the environment of the fluorophores, including the chemical composition of the dSTORM buffer and the nature of the molecule to which the fluorophore is attached.

Various points in this protocol (complemented by "Note" or "Troubleshooting" appendages, and presented in Figure 2) will thus require optimization to get the best resolution.

TROUBLESHOOTING

Problem 1

No fluorescent signal is detected when labeled cells are observed by conventional fluorescence microscopy. This issue might have several causes that are addressed below.

Potential solution 1

In our protocol, cells are concentrated before incubation with aDA-DA (step 7). This concentration allows reducing the volume of the cell suspension to save some aDA-DA probe, whose availability is currently very limited. In these conditions, *S. pneumoniae* continues to grow for a short while (about 15 min) and incorporate new PG but other bacteria might stop growing. In this case, the maximum concentration above which the bacteria stop synthesizing PG (and thus stop incorporating aDA-DA) will have to be determined.

Potential solution 2

In our protocol, we used a dipeptide conjugated to azide at its N-terminus (aDA-DA) to label the fourth amino acid of the PG pentapeptide chain through lipid II synthesis (steps 8–9) (Trouve et al., 2021). This probe is incorporated intracellularly into the PG precursor by the MurF ligase (Figure 1) (Kuru et al., 2019). Absence or poor labeling in some bacterial species might thus come from aDA-DA not entering the cell and/or not being efficiently incorporated by MurF. Alternative approaches include the use of different dipeptide analogues (step 1 of the *Preparation of PG labeling reagents* section and Figure 2), which might cross the cell envelope more efficiently, and/or constitute better substrates for MurF.

Alternative clickable dipeptides have already been used in other bacterial species and/or reviewed, including DA-DA conjugated to azide at their C-terminus (DA-aDA), or DA-DA dipeptides carrying a N-terminal or C-terminal alkyne (Liechti et al., 2014; Lund et al., 2018; Siegrist et al., 2015). The concentration of the PG probe and the incubation time (steps 8–9) that will provide the best signal-to-noise ratio (including for the aDA-DA probe) will have to be determined empirically for each bacterial species (see Figure 2).

Finally, it is important to note that in our protocol, the use of a DIBO-conjugated dye allows a strain-promoted alkyne-azide cycloaddition, or in other words a click chemistry reaction that does not require the use of a catalyzer. When using unstrained alkynes, the reaction with azide will require copper catalysis. The concentration of the clickable fluorescent dye and the duration of the click reaction (steps 11–12) that will provide the best signal-to-noise ratio (including for DIBO-AF647) will have to be determined empirically for each bacterial species (see Figure 2).

Problem 2

Background fluorescent signal is observed on the microscopy coverslip.

Potential solution 1

Fluorescence signals that do not co-localize with bacteria might result from dirt present on the coverslips. In this case, you should first increase the incubation time in the UV oven (step 3 of the *Preparation of stock solutions and glassware for dSTORM* section).

Alternatively, you might try to clean the coverslips using 100% acetone and/or 1M KOH combined with sonication.

Another possibility is to incubate the coverslips for 10 min at 50°C–70°C in RBS 25 solution with agitation, followed by 2 washes with ddH_2O for 10 min with agitation.

A last alternative is to place coverslips for 12–18 h in a methanol:hydrochloric acid (50:50 vol/vol) mixture while stirring; repeatedly rinse with ddH_2O until the neutral pH is reached, dry in a laminar flow cell culture hood and finally clean under UV for 30 min.

Potential solution 2

Background fluorescent signals might also result from leftovers of DIBO-AF647 in the cell suspension, or in other words from the presence of dye that is not incorporated into PG. This issue might be resolved by performing additional washes (step 13) before resuspending cells into the dSTORM buffer.

Potential solution 3

Background fluorescence might also result from cell lysis.

In this case, you should optimize cell fixation (step 10) by increasing the amount of PFA, fixing with glutaraldehyde and/or ethanol, or by fixing at higher temperature (see Figure 2).

Cell lysis might also arise from mechanical stress exerted during the sample mounting (steps 16 to 18). Make sure that you apply the weight vertically when you mount the sample between two coverslips. Bacteria other than *S. pneumoniae* might not bear the pressure applied on the coverslip. In this case, you should exert less weight onto the coverslip. An alternative to this is to use agarose pads (See step 19, optional).

Problem 3

In *S. pneumoniae*, fluorescent labeling and fluorophore blinking should be largely enriched at midcell and equatorial positions as observed in Figure 4 and (Trouve et al., 2021). In other bacteria, if fluorescent signal and/or blinking is observed on the whole cell surface, one should first determine whether or not it results from PG synthesis and labeling. One way to test this is to perform the labeling experiment in the absence of the aDA-DA probe. If in such condition fluorescent signal is still observed, it might either result from i) unspecific binding of the DIBO-AF647 dye at the surface of the cell, ii) fluorescence of growth medium components that transiently bind the cell surface (see an example in *Deinococcus radiodurans* (Floc'h et al., 2018)), or iii) fluorescence of intrinsic cell surface components. The second and third cases should be tested by performing the experiment in the absence of the DIBO-AF647 dye (see Figure 2).

Potential solution 1

If background signal comes from fluorescence of growth medium components or reagents used in this protocol, it might be eliminated by testing other growth media, by further washes (step 13) or by storing all the reagents in glassware (plasticware is a source of unspecific fluorescence, see (Floc'h et al., 2018)).

Potential solution 2

If background signal comes from unspecific binding of DIBO-AF647, it might be reduced by decreasing the concentration of the dye (step 11), by reducing the length of the click reaction (step 12), and/or by performing additional washes to eliminate all unbound DIBO-AF647 molecules (step 13).

Potential solution 3

In all cases, a possible alternative is to use a different fluorescent dye (step 11), which will have less affinity for the cell surface and/or whose spectral properties will differ from those of the fluorescent cell surface component or reagent. Alternatively, you can use unstrained alkyne-AF647 instead of DIBO-AF647, or use an unstrained alkyne-conjugated DA-DA in combination with an azide-conjugated fluorescent dye. In these cases, the alkyne-azide reaction will require copper catalysis.

Problem 4

Fluorescent labeling is observed but fluorophores take very long to be sent into the dark state and/or they do not blink.

This problem usually arises when too much oxygen is present in the dSTORM buffer, it will result in photobleaching of the fluorophores upon illumination. It can also happen if the illumination power is too low and/or if the dSTORM buffer is not reducing enough to send the fluorophores in the dark state.

Potential solution 1

If you do not work at 100% of intensity for the excitation laser (643-nm laser in this experiment), you should increase the intensity until you observe regular blinking of the fluorophores (step 25). If you work at 100% intensity of the laser, check the power density of the laser to make sure that it is in between 0.5 and 10 kW·cm^{-2}. Alternatively, you can reduce the beam size to increase the power density. Otherwise, you should consider buying a more powerful laser.

Potential solution 2

To increase the reducing ability of the dSTORM buffer, make fresh MEA, and/or increase the MEA concentration (step 14). βME can also be used as an alternative reducing agent.

Potential solution 3

To remove more oxygen from the dSTORM buffer, increase the concentration of the GLOX enzymes (step 14). You can also try a buffer degassing procedure or use the commercially available Everspark buffer (Idylle).

Problem 5

A sample drift is observed on the reconstructed dSTORM image.

To try to identify the source of the drift, you should first check whether it displays a specific pattern.

Potential solution 1

If the drift does not display any pattern, the cells might not be well fixed and thus still able to move. In this case, you should optimize cell fixation (step 10) by increasing the amount of PFA or by fixing it at higher temperature. Alternatively, cells can also move if they are not well immobilized between the two coverslips. In this case, you should apply the weight for a longer time (step 18), and/or apply a heavier weight.

Potential solution 2

Alternatively, random drift might also arise from vibrations of the microscopy room (floor, walls) during data collection (step 25). If not already the case, place the microscope onto an anti-vibration platform.

Potential solution 3

If the drift displays some pattern (e.g., if it is oriented along a single axis), the problem might be related to the orientation of the microscope stage. In this case, contact the microscope provider to solve the problem.

Potential solution 4

Finally, temperature fluctuations can also induce a drift during data collection (step 25). If possible, you should work with a thermostated cage.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Cecile Morlot (cecile.morlot@ibs.fr).

Materials availability

As of today, the aDA-DA probe used in our protocol is not commercially available and should be obtained through collaboration with a chemist who has expertise in preparing the compound as described in (Trouve et al., 2021).

Data and code availability

The datasets generated in the study associated with this protocol (Trouve et al., 2021) are available on the Zenodo platform (https://zenodo.org/record/4573897#.Yll_ZKEpBPY).

AUTHOR CONTRIBUTIONS

Conceptualization, J.T., O.G., C.M.; Formal analysis, J.T., O.G.; Investigation, J.T., O.G.; Methodology, J.T., O.G., C.M.; Validation, J.T., O.G., C.M.; Visualization J.T., O.G., C.M.; Writing - original draft, J.T., O.G., C.M.; Writing - review and editing, J.T., O.G., C.M.; Supervision, C.M.; Project administration, C.M; Funding acquisition, C.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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