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Super-resolution microscopy and single-protein tracking in live bacteria using a genetically encoded, photostable fluoromodule

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Abstract

Visualization of dynamic protein structures in live cells is crucial for understanding the mechanisms governing biological processes. Fluorescence microscopy is a sensitive tool for this purpose. In order to image proteins in live bacteria using fluorescence microscopy, one typically genetically fuses the protein of interest to a photostable fluorescent tag. Several labeling schemes are available to accomplish this. Particularly, hybrid tags that combine a fluorescent or fluorogenic dye with a genetically encoded protein (such as enzymatic labels) have been used successfully in multiple cell types. However, their use in bacteria has been limited due to challenges imposed by a complex bacterial cell wall. Here, we describe the use of a genetically encoded photostable fluoromodule that can be targeted to cytosolic and membrane proteins in the Gram negative bacterium *Caulobacter crescentus*. Additionally, we summarize methods to use this fluoromodule for single protein imaging and super-resolution microscopy using stimulated emission depletion.

Keywords

Fluorogenic; fluoromodule; bacteria; photostable; STED; single-protein tracking

INTRODUCTION

Our understanding of the inner workings of cells has greatly profited from the rapid developments in optical microscopy and imaging techniques. Among the different types of microscopes developed, the fluorescence microscope has contributed immensely to the field of cell biology. Fluorescence microscopy has single-molecule detection sensitivity, high specificity (due to specific chemical labeling or genetic encoding of fluorophores) and is relatively non-invasive, all of which make it a powerful tool for studying protein localization and dynamics in live cells. Despite these advantages, conventional epi-fluorescence microscopy can only resolve features that are about 300 nm or greater due to the diffraction limit of visible light. Much work uses immunofluorescent labeling, but potential artifacts from this method can be removed with fluorescent protein fusions (Harlow and Lane. 2008;Swulius et al. 2011;Slovak et al. 2005). Typical fluorescent protein labels that are a

few nanometers in size appear as a diffraction-limited point spread function (PSF) spanning ~300 nm or more of the sample field on a fluorescence microscope detector. This diffraction limit has been overcome through a variety of approaches over the last two decades, which are collectively termed super-resolution fluorescence microscopy. Briefly, super-resolution microscopy approaches comprise single-molecule localization based techniques (PALM, STORM, F-PALM) (Rust et al. 2006;Betzig et al. 2006;Hess et al. 2006;Moerner. 2007) as well as techniques that rely on shrinking the effective excitation PSF (stimulated emission depletion microscopy, STED) (Hell and Wichmann. 1994). Robust fluorophores are needed for both approaches. Additionally, for live cell imaging, these fluorophores should be genetically targeted and should not perturb the structure or function of the protein of interest.

Fluorescent proteins (FP) have greatly enhanced our ability to observe proteins inside living cells due to their specificity in labeling and ease of genetically fusing them to a protein of interest (Tsien. 1998). However, typical fluorescent proteins emit about ten-fold fewer photons compared to organic dyes, thus limiting their utility in super-resolution microscopy (Peterman et al. 1999). Therefore, approaches that can genetically target small organic fluorophores to proteins of interest can be useful for improved fluorescence images. For example, enzymatic labels such as SNAPTag and HaloTag have been extensively applied to image proteins in live cells (Los and Wood. 2007;Keppler et al. 2004). Such hybrid labels have the advantage of delivering almost any organic dye covalently to a protein of interest. Fluorogen activating peptides are a different class of hybrid tags that bind to a non-fluorescent small molecule in a non-covalent fashion and exhibit fluorescence. Malachite Green (MG) is one such fluorogen that is rotationally relaxed in its excited state in solution that results in its low quantum yield. However, upon binding to the cognate single chain variable fragment (scFv) known as dL5, MG exhibits an 18,000-fold fluorescence activation (Szent-Gyorgyi et al. 2008). The dL5-MG fluoromodule exhibits many advantages such as a far red emission, high photostability, a low picomolar binding affinity and an extremely slow off rate (Fig. 1) (Saurabh et al. 2015). dL5 fusions of proteins have been used in a variety of applications such as live cell super-localization microscopy, pH sensing, FRET sensing, single protein tracking and optogenetics (Yan and Bruchez. 2015;He et al. 2016). In order to target MG to proteins in different locations and to functionally diverse binding partners (such as Streptavidin), analogs of MG such as MG-ester (cell permeant), MG-B-tau (cell impermeant), MG-Biotin and polymeric MG (for targeting macromolecules) have been developed (Yan et al. 2015;Magenau et al. 2015;Saurabh et al. 2014).

Until recently, all reported applications of the dL5-MG system have been in eukaryotic cells (Saurabh and Bruchez. 2014). Bacteria are tiny organisms that provide us with excellent model systems to understand the mechanisms behind intricate biological processes. Yet they have not been rigorously studied using photostable fluorophores suitable for super-resolution microscopy and single particle tracking experiments. About 3–4 evenly dispersed fluorescent molecules are enough to fill up a typical bacterial cell (1 μm) in diffraction limited imaging. This makes single-molecule localization challenging and constantly demands improved methods to actively control the number of fluorophores in a given cell. Additionally, the complex architecture of the bacterial envelope makes specific delivery of organic fluorophores to intracellular proteins challenging. This is particularly true for Gram-negative bacteria, where the cell envelope is comprised of an outer S-layer, an outer

membrane, a peptidoglycan layer, and an inner membrane (Briegel et al. 2006). *Caulobacter crescentus* is a widely studied Gram-negative bacterium in which the localization patterns of many proteins have been documented (Shapiro et al. 2009). Several studies relying on super-localization approaches in *Caulobacter* have detailed the super-structures of diverse proteins such as the cytoskeletal proteins FtsZ, MreB, CreS, PopZ and members of the chromosome segregation machinery ParA and ParB (Gahlmann and Moerner. 2014;Ptacin et al. 2014). These studies have utilized fluorescent protein fusions to enable photo-activation and/or blinking as mechanisms for actively modulating the fluorophore concentration for single molecule localization based super-resolution approaches. Thus, most fluorophores used to tag proteins in *Caulobacter* to date lack the high photon output offered by fluoromodules such as dL5-MG. However, this advantage of dL5 comes at the expense of a short and relatively fast wash to remove non-specifically bound and activated MG from the bacterial membrane.

Further, few studies in bacteria have used STED as a super-resolution method, primarily due to a dearth of robust, genetically encoded fluorophores for targeting cytosolic proteins in bacteria (Jennings et al. 2011;Reimold et al. 2013;Klar et al. 2000). STED offers several advantages over super-localization techniques at the cost of an increase in microscope system complexity. First, typical STED images can be acquired in a few seconds to several minutes, which is faster than the several to tens of minutes necessary to acquire a super-localization data set. Second, while localization techniques require image analysis to reconstruct the super-resolved image, STED acquires the super-resolved image in real time. This aids in experimental troubleshooting and is extremely beneficial when the biology is dynamic and changes over the time scale of several minutes. Therefore, much remains to be obtained through STED microscopy combined with robust fluorophores that can be delivered beyond the complex bacterial membrane.

Recently, we reported the first application of the dL5-MG fluoromodule for super-resolution imaging in live *Caulobacter* cells (Saurabh et al. 2016). Here, we provide detailed protocols for labeling proteins in *Caulobacter crescentus* using the dL5-MG fluoromodule. We present the molecular cloning methods that can be used to genetically fuse dL5 to a protein of interest. Following this, we outline the labeling conditions for diffraction limited fluorescence imaging as well as single protein tracking in live cells. A great advantage of the dL5-MG fluoromodule is its lack of blinking combined with its utility in STED microscopy (Saurabh et al. 2016). We provide pointers to set up the STED microscope as well as outline methods to optimize the parameters for imaging dL5 using STED.

BASIC PROTOCOL 1

Cloning dL5 gene to your protein of interest and verification of the strain

The first step in obtaining a dL5 fused protein is to construct a bacterial strain expressing a dL5 fusion to the gene of interest. The precise design of the dL5 fusion and the corresponding expression strategy will be determined by the specific gene’s function and behavior. Generally, adapting strategies that have been successfully used to generate fusions of the gene to other tags (fluorescent, epitope, etc.) will yield favorable results. We advise creating an eYFP fusion (or other fluorescent tag suitable for super-resolution microscopy)

in parallel to the dL5 fusion. The eYFP fusion serves as an excellent control to pinpoint if any problems encountered (i.e. no fluorescent signal, loss of the gene's normal function, altered localization pattern) with strain design are related to dL5.

To reduce cell-to-cell variability in expression levels of the dL5 fusion protein, it is recommended to avoid plasmid based expression strategies and instead to create a strain harboring a genomic integration of the dL5 fusion. The following procedure details how to generate a *Caulobacter crescentus* strain carrying a C-terminal dL5 fusion to a gene at the native chromosomal locus. The specific design strategy will vary depending upon the bacterial species being tested and the protein of interest, but the general techniques will be applicable to the generation of most strains.

The protocol is divided into two steps: 1. Creating a plasmid containing the dL5 fusion to your gene of interest (Fig. 2A) and 2. integration of the plasmid into *Caulobacter crescentus* (Fig. 2B).

It is advantageous to use a standardized plasmid for creating a C-terminal eYFP fusion to a specific gene of interest – such as the pYFPC plasmid series used for *Caulobacter* (Thanbichler et al. 2007) – as the parent vector. First, replace the eYFP coding sequence with the dL5 coding sequence via any molecular cloning technique; we prefer Gibson assembly to generate the reported constructs (Saurabh et al. 2016). Following this, insert ~500 base pairs of genomic DNA sequence immediately upstream of the gene and the gene's ORF in frame with either the eYFP or dL5 sequence. Transform the resulting plasmids into a *Caulobacter* strain containing a deletion of the gene you are interested in. The plasmid will integrate into the genome within the 500 base pairs upstream of the gene. The final strain will express the gene of interest from its native promoter with an eYFP or dL5 C-terminal fusion, and with the fusion protein being the only allele present within the cell.

It is important to note that this specific strategy will only work for non-essential genes. In order to tag an essential gene, one needs to engineer a vector that can be transformed into a *Caulobacter* strain that is wild-type for your specific gene of interest. There are many strategies to tag essential genes, including orthogonal expression, creating merodiploid strains, and cloning just the 3' 500 base pairs of the gene of interest into the fusion vector. Similarly, the specific design of the plasmid can be adjusted to make N-terminal dL5 fusions. One should strive for a final strain that does not perturb the native function of the protein, is as close to a wild-type background as possible, and does not hinder the ability to collect and interpret experimental data.

Materials

Reagents and solutions: QIAprep MiniPrep Kit (Qiagen)

FastDigest Restriction Enzymes (Fermentas)

Oligonucleotides (Integrated DNA Technologies)

Phusion DNA Polymerase (New England Biolabs)

Agarose

TAE Buffer (see recipe)

QIAquick Gel Extraction Kit (Qiagen)

Gibson Assembly Master Mix (New England Biolabs)

Competent *E. coli* DH5α cells

Luria Broth (see recipe)

Luria Broth + selective antibiotic plates

PYE (see recipe)

PYE + selective antibiotic plates

Appropriate antibiotics

Materials (Equipment): Thermocycler

Culture Tubes

Incubator

Electrophoresis machine

Gel imaging dock

Sequencing facility

Tabletop centrifuge

0.1 cm electroporation cuvettes

Electroporation machine

Recipes

TAE Buffer: 40 mM Tris Acetate

1 mM EDTA

Luria Broth: 10 g Tryptone

5 g Yeast Extract

5 g NaCl

Bring to 1L with water

Autoclave for 30 minutes

PYE: 2 g Bacto-peptone

2 g Yeast extract

0.3 g $MgSO_4 \cdot 7H_20$

0.1 g $CaCl_2$

Bring to 1L with water

Autoclave for 30 minutes

Protocol steps

Part I – Constructing C-terminal dL5 and eYFP fusion plasmids

1. Prepare a mini-prep of pYFPC-X (Thanbichler et al. 2007). X determines the resistance marker that will be present in the final strain. Elute in 47 μL H_2O.
2. Add 5 μL of 10× digest buffer to the pYFPC-X preparation. Add 1 μL each of NcoI and NheI and incubate at 37°C for one hour.
3. Amplify the dL5 coding sequence using the oligonucleotides 5’ tcaccggtcggccaccatggca**CAGGCCGTCGTTACCCAAGAACC** 3’ and 5’ atcccccgggctgcagctagtta**GGAGAGGACGGTCAGCTGGG** 3’ (bold letters in the oligo sequences highlight where the primer binds to dL5). The rest of the sequence contains “overhangs” to assemble the insert into the digested plasmid via Gibson assembly. Use any high-fidelity polymerase. Run the polymerase chain reaction (PCR) at an annealing temperature of 60°C. The PCR product will be 720 base pairs in size.
4. Run the digested pYFPC-X and PCR reaction from step 3 on a 1% agarose gel. Both reactions should produce discrete single bands (~3 kB for the digested plasmid). Carefully excise the bands from the gel and purify with any commercial gel extraction kit.
5. Quantify the vector backbone and PCR product by running 5 μL of each on a 1% agarose gel. Measure the intensities of each band. You can use the ratio of these intensities to the intensity of a band within the standard ladder containing a known amount of DNA to calculate the concentrations of the DNA pieces.
6. Set up two Gibson reactions. We have successfully utilized both commercially available and homemade Gibson master mixes. To each Gibson reaction, add ~100 ng of vector backbone. To one reaction, add a 3:1 molar excess of insert DNA to vector backbone. To the other reaction, add the same volume of H_2O that corresponds to the insert DNA volume. Set up the reactions on ice. Incubate at 50°C for one hour. Store at 4°C after the reaction is completed.

7 Add 2–10 μL of each Gibson reaction to an unthawed aliquot of competent *E. coli* DH5α cells. Incubate on ice for 30 minutes.

8 Heat shock the cells at 42°C for 45 seconds.

9 Add 1 mL of LB to each transformation tube. Incubate at 37°C for 1–4 hours.

10 Spread 200 μL of cells onto LB+ selective antibiotic plates. Incubate at 37°C overnight.

11 The number of colonies on the plate of cells transformed with Gibson mixture containing the insert DNA should be at least ~2× more than the number of colonies transformed with Gibson mixture lacking the insert DNA. Inoculate 2× 5 mL of LB+ selective antibiotic with a single colony and grow overnight at 37°C.

12 Perform a miniprep of the two overnight cultures. Sequence each plasmid with oligonucleotide 5’ ttatataagctttcgcgagacg 3’. Ensure that dL5 was inserted in the correct place with no mutations. This is pDL5C-X

13 Design oligonucleotides to amplify the ORF of the gene of interest plus 500 base pairs of upstream genomic sequence. For the forward primer, add the sequence 5’ cgcgagacgtccaattgcat 3’ to the 5’ end of the target binding sequence. For the reverse primer, add the sequence 5’ tgacgcgtaacgttcgaatt 3’ to the 5’ end the target binding sequence. These create the overhangs needed to assemble the insert DNA into the vector backbone. Remember to NOT include the native stop codon for the gene of interest. Amplify the insert using 1 μL of saturated, liquid *Caulobacter* culture as a template.

14 Digest both pYFPC-X and pDL5C-X with NdeI and EcoRI as described in steps 1 and 2.

15 Perform the Gibson assembly described in steps 4–12. This time you will be generating two plasmids: C-terminal fusions of the gene to both eYFP and dL5. Make sure that the final resulting plasmids are fused in frame to the tag and that there are no mutations present within the entire coding sequence.

Part II – Transformation of Caulobacter

16 Grow up 2× 5 mL cultures in PYE of a *Caulobacter crescentus* strain containing a deletion in your gene of interest.

17 Spin down all of the 5 mL culture and wash twice with H_2O. You will have two separate tubes containing cell pellets.

18 Resuspend the cell pellets in 80 μL of H_2O. Transfer to a 0.1 cm electroporation cuvette. Add 10 μL of a miniprep of the eYFP/dL5 fusion plasmid.

19 Subject the cells to electroporation by pulsing at 400 ohms, 25 μFD, 1.75 kV. Pulse time should be ~4–8 seconds.

20 Add 1 mL of PYE to electroporated cells. Incubate at 28°C for 4 hours.

21 Spread 200 µL of the recovery onto PYE+ selective antibiotic plates. Pellet the rest if the recovered cells and resuspend them in 100 µL of PYE. Spread the entire 100 µL culture on to a second PYE+ selective antibiotic plates. Incubate at 28°C for three days.

22 Streak individual colonies from the plates onto fresh PYE+ selective antibiotic plates.

23 At this point you should have multiple colonies from each clonal isolate. Perform diagnostic PCR reactions to ensure that the genetic fusion of interest was integrated at the correct location within the genome.

24 Perform assays to ensure that the function of the gene of interest is not perturbed when fused to dL5 and/or eYFP. Use wild type and the deletion strain you integrated into as controls. Restoration of normal growth rates (measured using O.D. 600) and cellular morphologies when the gene of interest is fused to dL5 is a good indication that the gene's function is not perturbed (Fig. 5B). Western blots using antibodies recognizing the protein of interest are useful to ensure that the expression level of the protein is not changed and that the tag is not being cleaved off. These assays can be tailored to the specific function of the gene of interest.

BASIC PROTOCOL 2

Growth and labeling of bacterial cells for imaging

This basic protocol is outlined for labeling *Caulobacter* cells (Fig. 3). However, the workflow will apply to any other Gram negative bacteria in general and should be able to provide researchers with the optimal concentration and labeling time with MG-ester for a particular application. For the success of any live cell labeling, it is of utmost importance that the cells be in a healthy condition prior to labeling. Therefore, bacteria should be grown in optimal media before labeling. Secondly, the labeling process should be carried out when the bacteria is in the log or exponential phase of growth. This ensures that the dL5 fusion to the protein of interest has been fully expressed. There are three main parameters for labeling bacteria using the dL5-MG fluoromodule. The first parameter to optimize is the labeling concentration. In order to measure the fluorescence from the cells, one can use a fluorescence microscope or a fluorometer/ fluorescence plate reader. The aim of the measurement is to obtain a saturating concentration of MG-ester. This concentration will depend on the copy number of the protein and the accessibility of the dye to dL5 fused to the protein of interest. Once the saturating labeling concentration is obtained, optimize the labeling time and washing steps. The aim will be to keep the total labeling time shorter than the cell division time. However, this requirement is not stringent and will depend on the application. In summary, the labeling steps are general and can be optimized for any Gram negative and possibly Gram positive bacterial system, if one follows this protocol.

Materials

Reagents and solutions: PYE

M2G

Antibiotics

Agarose

MG-ester (the dye can be requested from Prof. Marcel Bruchez, Carnegie Mellon University or purchased from SharpEdge labs or Spectragenetics. Additionally, the dye can also be synthesized based on previous work (Szent-Gyorgyi et al. 2008).

Recipes

PYE: 2 g Bacto-peptone

2 g Yeast extract

0.3 g $MgSO_4 \cdot 7H_20$

0.1 g $CaCl_2$

Bring to 1L with water

Autoclave for 30 minutes

M2G: 6.1 mM Na2HPO4

3.9 mM KH2PO4

9.3 mM NH4Cl

0.5 mM MgSO4

10 μM FeSO4 (EDTA chelate)

0.5 mM CaCl2

0.2% glucose

Protocol steps

1. Grow the *Caulobacter* strains overnight from frozen stocks or from a single colony on a plate in nutrient rich PYE growth media supplemented with the appropriate amounts of antibiotics
2. The following day, back-dilute the cells in PYE or M2G to an $O.D._{600}$ of 0.1 and grow for 1–2 division cycles (90–180 mins).
3. Induce cells using the appropriate inducers (if needed). Typically, inducers are added at an $O.D._{600}$ of 0.25–0.3.
4. Once the cells reach an $O.D._{600}$ of 0.3–0.4 and/or induction is complete, aliquot 990 μL of cultured cells in a 1.5mL Eppendorf tube.

5. MG-ester is susceptible to carbinol formation at higher pH. Therefore, always store MG-ester in solid form (at room temperature) or in concentrated stocks (~100 μM or higher) prepared in Ethanol+5% Acetic acid that can be stored at 4°C.

6. Prior to each labeling experiment, measure the exact concentration of MG-ester in Ethanol+5% Acetic acid using an extinction coefficient of 91670 $M^{-1}cm^{-1}$ (Fig. 4). Prepare a 1 μM MG-ester solution in Ethanol+5% Acetic acid.

7. Add 10μL of 1 μM MG-ester to the tube containing cells. Note that this concentration will depend on your findings from the calibration experiment and the application (read critical parameter below).

8. In order to perform washes, incubate cells for 5 mins on a nutator or shaker to ensure uniform mixing of the dye.

9. Spin the cells down in a centrifuge at room temperature at 7000×g for 90s

10. You should see a cell pellet at the bottom of the tube. At this point aspirate the supernatant media from the tube and resuspend the cells in 1mL of M2G

11. Repeat steps 9–10 two times. Take care to resuspend the cells using a 1mL pipette rather than using a vortex. The cells tend to clump sometimes or lyse upon vigorous vortex.

12. After the final aspiration, resuspend the cells in 100–200 μL of M2G using a 200 μL pipette by pipetting up and down

13. Spot 1.0–1.5 μL of the above culture on an agarose pad

14. Let the pad dry for ~5 mins

15. Flip the pad over on a coverslip such that the side with the cells spotted faces the coverslip. This will be the imaging side where the microscope objective will make contact with the sample. Therefore, care must be taken to match the coverslip based on the microscope objective (working distance).

16. Finally, seal the coverslip using a glass slide and wax so that the agarose does not dry out while imaging.

BASIC PROTOCOL 3

Fluorescence microscopy setup

Based on the fluorescence spectra, it can be seen that the dL5-MG fluoromodule can be effectively excited using 630–650 nm lasers. It should be noted that Mercury arc lamps (such as HBO100) do not have a spectral line above 600 nm. Hence, they are not optimal for imaging the dL5-MG fluoromodule. When labeling a protein of interest, one must always compare its localization using fluorescence microscopy to previously known functional fusions with fluorescent proteins or other tags.

Materials—Fisherfinest™ Premium Plain Glass Microscope Slides

VWR Square Glass Coverslips, #1.5 Thickness, 22mm

Epi-fluorescence inverted microscope (IX71, Olympus)

Motorized stage (M-687 PILine, Physik Instrumente)

Circular polarizer

638 nm solid state laser (FiberTec II, Blue Sky Research).

Phase objective (UPlan FLN, 100×, 1.3 N.A., ph3, oil immersion, Olympus)

Super apochromat objective (UPlanSApo, 100×, 1.4 N.A. oil immersion, Olympus)

Dichroic mirror (ZT405/514/635rpc, Chroma Technologies)

Band pass emission filter (ET680/60m, Chroma Technologies)

EMCCD camera (iXon897 Ultra, Andor)

Protocol steps

1. In order to image bacteria on agarose pads for longer time scales, sealed chambers should be used. In order to make sealed chambers, prepare agarose pads by sandwiching 450 μL of molten agarose (in M2G) in between 2 coverslips (#1.5). Place these coverslips in a hydrated chamber so that they do not dry out as they solidify.
2. Once the agarose has solidified, cut out an approximately 1cm×1cm piece of the agarose pad and spot 1–1.5μL of cells on it. Allow the cells get adsorbed by the pad. This should take about ten minutes and one should not see any excess media on the pad where the cells were spotted. At the same time, start heating wax in a beaker.
3. Once the pad absorbs the cells, flip the pad such that the side with the cells is on a new coverslip. At this time, place the coverslip on a glass slide such that the pad is in between the glass slide and the coverslip with the cells still facing the coverslip.
4. Apply molten wax between the glass slide and the coverslip. As the wax solidifies it will create a seal for the agarose pad. Make sure that there are no openings and that wax seals every corner and edge.
5. Place the imaging chamber on the microscope such that the coverslip faces the objective.
6. Image using a 100× objective. For phase images use an objective with a phase ring. The same objectives can be used for diffraction limited imaging as well (Fig. 5). Note that objectives suitable for phase contrast have a lower N.A. compared to objectives that do not have a phase ring. Therefore, it is

recommended to use the highest possible N.A. objective for single molecule detection (Fig. 6).

7. Optimize the exposure time, laser intensity and EM gain (if using an EMCCD). In order to obtain the best images, focus on the sample in phase and then image them in the fluorescence channel with a low laser intensity (~500 W/cm^2 at the sample). Typical starting values for exposure times can be 50–100ms with EM gain at 100 for the camera used in our setup (see materials, above). Once fluorescence from cells can be seen, laser intensity, exposure time and EM gain should be further optimized to obtain the highest quality image.

Notes

Diffraction limited imaging and verification of cell viability—Before performing super-resolution microscopy on dL5-MG fluoromodule in live cells, it is imperative to test the strains and verify that they can be imaged optimally using a combination of labeling conditions and imaging settings (Fig. 5A). After optimizing these settings, images such as those in Fig 5B should be readily obtainable. Finally, in order to ensure that the dL5-MG fluoromodule is not causing any cytotoxicity or perturbing the protein of interest, one should perform a time lapse to ensure that the cells divide under imaging conditions (Fig 5C).

Single molecule imaging of strains—On account of its enhanced photostability, the dL5-MG fluoromodule is ideal for single protein tracking experiments. Single molecule labeling concentration should be ascertained through imaging. Once single molecule data has been acquired it can be analyzed through basic tracking codes available. The presence of a single molecule in each track should be confirmed by observing its intensity as a function of time. Single molecules will exhibit single step photobleaching (Fig. 6A and B). Further, since single molecule experiments require the ultimate level of sensitivity, it is important to ensure low backgrounds. Measure the signal to background ratio of a given strain and compare it to the reported values (Fig. 6 C and D).

BASIC PROTOCOL 4

STED imaging of live bacterial cells

The resolution enhancement achieved by STED microscopy relies crucially on the donut shaped depletion beam (Galiani et al. 2012). Specifically, STED requires a high quality donut achieved through precise optical engineering and alignment (Wu et al. 2015). Practically, forming a high quality donut beam necessitates specific microscope design parameters (Lau et al. 2012) and careful fine tuning of the alignment of the microscope, usually every time the microscope is used (Saurabh et al. 2016)(Fig. 7). Because of this, it is critical to have a calibration measurement to check the alignment and final resolution achieved by the microscope for any given experiment (Fig. 8).

Since the resolution enhancement increases as the intensity of the depletion laser increases, STED microscopy is limited to highly photostable and robust fluorophores that can withstand the high depletion laser intensities necessary to achieve the best resolution. One method to extend STED microscopy's application to slightly less photostable fluorophores is

fast scanning. By limiting the consecutive dwell time on a single pixel, irradiation of fluorophores that build up in the triplet state is reduced, thus reducing photobleaching (Donnert et al. 2006;Donnert et al. 2009). To build up sufficient signal for a high quality image, a line of pixels or full frame is scanned multiple times, allowing enough time for triplet states or other long lived states to relax back to the ground state between scans. Fast scanning can immensely improve image quality, and is crucial for obtaining good images with dL5 (Fig. 9).

Finally, because of the rigorous demands placed on the fluorophore during STED imaging, finding optimal imaging parameters is critical (i.e. excitation and depletion laser intensities, pixel size, and the scanning parameters). Here, we describe how to optimize parameters for two different STED microscopes. The first system is a home built dual-pulsed STED microscope which improves upon an earlier version that only operated in slow scanning mode. This slow scanning STED microscope was used to study the structure of centriole proteins in fixed eukaryotic cells (Lau et al. 2012;Lee et al. 2014). For the dual pulsed, fast scanning STED microscope, both the excitation and depletion lasers are pulsed. Thus, all of the depletion intensity occurs in the short period after excitation, ideally before an excited fluorophore has a chance to spontaneously emit. Fast scanning is achieved by a resonant mirror that oscillates at a fixed frequency, described in our recent work (Saurabh et al. 2016). Thus, the two scan parameters that are easily varied are the number of resonant mirror scans (summed per slow axis scan pixel) and the number of summed frames (i.e. complete images). The second system is a commercial time-gated continuous wave (CW) STED microscope. Here, the excitation laser is pulsed while the depletion laser is continuous. Thus, molecules in the excited state experience varying doses from the depletion laser depending on the amount of time they remain in the excited state. This means that a molecule that emits a photon at a later time experiences a higher depletion laser dose and thus contains higher resolution information. Time-gating allows one to achieve higher resolution with lower CW laser intensities by selecting these higher resolution photons (Vicidomini et al. 2011). In practice, increasing the gating time increases resolution at the expense of signal. This CW microscope allows the scan frequency to be modulated (altering the pixel dwell time), and the summing of pixel lines and frames.

Materials—Dual-pulsed STED microscope, including:

Titanium-sapphire mode-locked oscillator (~750 nm; 100 fs pulses at ~80 MHz)

Dispersive elements (e.g. glass rods and polarization preserving fiber)

Polarizers

Vortex phase plate

Pulsed diode laser (635 nm; <100 ps pulses electronically triggered from oscillator)

Polarizers

Dichroic mirrors (substrate >3 mm thickness)

Resonant mirror

Quarter wave plate (zero-order near 750 nm)

High-magnification, high NA objective lens (100×; >1.3 NA)

3D piezo nanopositioning stage

Pinhole

Emission filter

Lenses (achromatic doublets)

Mirrors (<λ/10 surface flatness)

Avalanche photo diode

Computer, FPGA, and software for image acquisition and analysis

Time-gated CW STED Microscope (Leica TCS SP8, Leica Microsystems)

CW depletion laser (592 nm)

White light laser (80 MHz, 510 nm)

HyD detector (operating in photon counting mode)

High-magnification, high NA objective lens (100×; 1.4 NA)

Single-molecule resolution sample

Dye molecule (e.g. ATTO 647N NHS-ester, Atto-Tec)

Nanopure water

Poly-L-lysine coated coverslip

Mowiol mounting solution

Protocol steps

Make Single-Molecule Test Sample

1 Dilute a dye sample (e.g. NHS-ester or an antibody-conjugate) to 10 nM in nanopure water and sonicate for 10 minutes to minimize aggregates.

Ideally, the single-molecule sample should use the same fluorophore used in the cell sample. Sometimes, however, the fluorophore may not be robust enough to image single molecules. In this case, a molecule with similar photophysical characteristics and fluorescence spectrum should be used. For instance, Atto647N, STAR RED, or STAR 635P are spectrally similar to dL5-MG and can be used as a single molecule test sample prior to imaging dL5-MG. Alternatively, fluorescent beads with a diameter smaller than the expected resolution can be used.

2 Place 250 μL of dye sample onto Poly-L-lysine coated coverslip (18 × 18 mm) and incubate for 15 minutes at room temperature in the dark.

3 Wash the coverslip 3 times with nanopure water to remove unbound dye molecules.

4 Place 12 μL of Mowiol mounting solution on a clean slide. Gently lower poly-L-lysine coated coverslip onto mounting solution with the dye side facing down. Clean any mounting solution that may seep out using a paper towel.

5 Allow samples to set from 3 hours to overnight in the dark.

Align the STED microscope

6 Check the fine alignment of the STED microscope

Dual-pulsed STED Microscope

- **a** Remove the pinhole and emission filter.
- **b** Image the excitation and depletion PSFs through scatter from an 80 nm gold particle. This requires reducing the laser intensity, typically by adding neutral density (ND) filters.
- **c** Use these PSFs to fine tune the alignment of the STED microscope (Wu et al. 2015).
- **d** Realign the pinhole using scatter of the excitation from the gold particle.
- **e** Replace the emission filter.

Time-gated CW STED Microscope

- **f** Make sure to utilize any automated alignment features provided by the manufacturer prior to imaging.

Optimize imaging parameters on cell sample

7 Start with imaging parameters that provide high quality confocal images. Reduce the pixel size to Nyquist sample at the expected resolution (20–30 nm pixels). It is often necessary to increase the intensity of the excitation beam to compensate for the smaller pixel size and loss of photons due to the depletion beam.

8 Gradually increase the intensity of the depletion beam. Increase it as high as possible while still maintaining reasonable image quality. It may be necessary to simultaneously raise the excitation intensity as well.

If it is impossible to obtain a satisfactory image with sufficient signal, it may be necessary to increase the pixel size.

9 Modify the pixel dwell time and line/image summing. These parameters can have a large effect on STED image quality (Fig. 9).

Dual-pulsed STED Microscope

- **a** Because the pulsed microscope utilizes a resonant mirror for the fast scan axis, the pixel dwell time is controlled by the number of resonant mirror scans per slow axis pixel line.

b For certain fluorophores, decreasing the number of resonant mirror scans followed by summing multiple frames may enable further image improvements.

Time-gated CW STED Microscope

c Modify the scanning frequency to change the pixel dwell time.

d Certain fluorophores will benefit by summing lines and/or frames with faster pixel dwell times.

e Finally, increase the gating time (typically ~0.3 ns) to balance increased resolution with decreased signal.

10 Check the excitation and depletion laser intensities. If the depletion laser intensity is increased drastically, it may be necessary to repeat step 9.

Image the single molecule sample to check STED microscope resolution

11 Using the imaging parameters determined by the cell sample, image the single-molecule sample.

It may be necessary to alter some parameters to obtain images (e.g. dwell time, excitation laser intensity, and pixel size), especially if the single molecule sample contains a different fluorophore from that used for cellular imaging. However, altering the depletion laser intensity and gating time should be avoided to maintain the resolution.

12 Obtain images of ~100 single molecules for accurate resolution analysis. Be sure to screen images for molecules that visibly blink or bleach in the middle of the scan, as these will bias the determined resolution. Such blinking/bleaching molecules often appear as asymmetric Gaussians that are much thinner along the slow scan axis, rather than symmetric Gaussians.

13 Fit single molecules to a 2D symmetric Gaussian function, extracting the FWHM of ~50–100 molecules.

14 The mean FWHM of the distribution of single molecule FWHMs is a reasonable estimate of the microscope's resolution (Fig. 8).

Image cell sample

15 Image a newly prepared cell sample (prepared as in Basic Protocol 3 above) using the imaging conditions determined previously (Fig. 10). Ensure multiple fields of view across several samples with many cells to validate structures.

REAGENTS AND SOLUTIONS

Mowiol Mounting Solution

25% w/v glycerol (Sigma-Aldrich)

10% w/v Mowiol 4–88 (Polysciences)

2.5% w/v 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma-Aldrich)0.01% w/v p-phenylenediamine (PPD; Sigma-Aldrich)

0.1M Tris buffer pH 6.8

Atto 647N NHS-ester (Atto Tech)

COMMENTARY

Background Information

The idea of specifically targeting an organic fluorogen to a protein of interest was first demonstrated by Tsien and coworkers through the use of FlAsH tags (Griffin et al. 1998). Today, there are many enzymatically active tags that can target any small molecule with the right reactive handle to a protein of interest. The key advantage of the enzymatic labeling scheme is the availability of a wide variety of dyes for targeting. However, there are several disadvantages to these approaches such as a high labeling concentration that contributes to background, temperature sensitive reactions, and the need for multiple washing steps that extend the labeling time. Further, delivery of dyes with the specific reactive handles (HaloTag, SNAPTag) inside bacteria is often challenging.

Fluorogen activating peptides, and particularly the dL5-MG fluoromodule, circumvent many of these problems. MG is available as several cell permeable and cell impermeant derivatives. This makes dL5 a robust fluoromodule that can be directed and imaged on the cell surface or inside the cell. Further, the dL5-MG fluoromodule has a very high affinity (K_d = 6 pM) and an extremely slow off rate. Short labeling times can be achieved with minimal washing and low concentration of the fluorogen. Additionally, the binding reaction is equally efficient at room temperature and 37°C.

Prior to this work, the dL5 scFv has been used across various eukaryotic cell types. Here, we use the cell permeability of MG-ester for targeting intracellular proteins in *Caulobacter crescentus*. Since MG-ester activates in the *Caulobacter* cell membrane, brief washes are needed to remove the non-cytosolic population of the fluorogen. However, this small disadvantage is offset by the remarkable photostability of the fluoromodule (2× more photons compared to Alexa 647 and 5× more photons than eYFP), which enables single protein tracking for a long time scale. Further, the utility of dL5-MG for STED microscopy makes it an excellent tool for super-resolution imaging. There have been a limited number of examples of STED imaging in bacteria due to a small number of FPs suitable for STED and challenges in intracellular delivery of optimal organic STED fluorophores. Additionally, we have found that under typical imaging conditions used for STED, one can only obtain a single STED scan from eYFP with a reasonable signal. The dL5-MG fluoromodule solves this issue and can be an excellent tool for performing STED imaging in live bacteria in the future.

Critical Parameters

Any small molecule labeling relies on the fine balance between the molecule concentration, incubation time and washing steps (Fig. 5A). It is of utmost importance to optimize in these parameters for every experiment. The aspects that govern these three parameters are protein

copy number, protein accessibility and the type of experiment (ensemble / single molecule). In order to find the optimal labeling concentration, perform a range finding experiment where the cells are incubated with a series of dye concentrations (typically, 1 nM, 10 nM, 100 nM and 1 μM). Use a relatively longer incubation time such as ~30 mins to 1 hour. Use 2× washes to begin with but if the background is higher, consider more washes. For single molecule measurements, typically 10–100pM MG-ester should be used to label cells (incubation times and washing steps remaining the same). There is always a trade-off between labeling concentration, labeling duration and number of washing steps. Careful attention must be paid to optimize these parameters prior to critical experiments.

Troubleshooting

The troubleshooting tips are noted in table 1 below.

Anticipated Results

dL5-MG labeling is very reliable across *Caulobacter crescentus* and reproducible results can be obtained for a variety of tagged proteins. The combination of dL5-MG with an optimal STED setup can result in at least a four-fold higher resolution compared to diffraction limited microscopy. Typical fluorescence images should be similar to those shown here, for a comparable protocol.

Time Considerations

The time considerations for molecular cloning methods and validation of constructs will differ depending on the case. Labeling with dL5-MG is more time efficient compared to enzymatic tagging schemes on account of the higher binding affinity. Labeling times of under ten minutes can be consistently achieved with *Caulobacter*. Diffraction limited fluorescence imaging and single molecule imaging will also depend on the experiment at hand. STED images can be acquired between 10 seconds and several minutes, depending on label density and the field of view. Importantly, this is faster than the several minutes to tens of minutes necessary to acquire single molecule localization images, and no image reconstruction is necessary.

Acknowledgments

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Significance Statement

Fluorescence imaging in bacteria has relied extensively on fluorescent proteins for the last two decades as a useful alternative to immunofluorescence. While fluorescent proteins are easy to use, their utility for super-resolution microscopy in bacteria is limited due to their low photon output. Hybrid labels such as fluorogen activating peptides have been optimized for high photostability but have not been utilized in bacteria, until recently by our lab. Here, we report these methods in order to enable researchers to use this photostable and robust fluoromodule to image proteins in live bacteria. Further, we outline protocols to perform STED microscopy using this fluoromodule. The fluoromodule provides enhanced spatio-temporal resolution and promises to improve our understanding of bacterial structure/ function control systems.

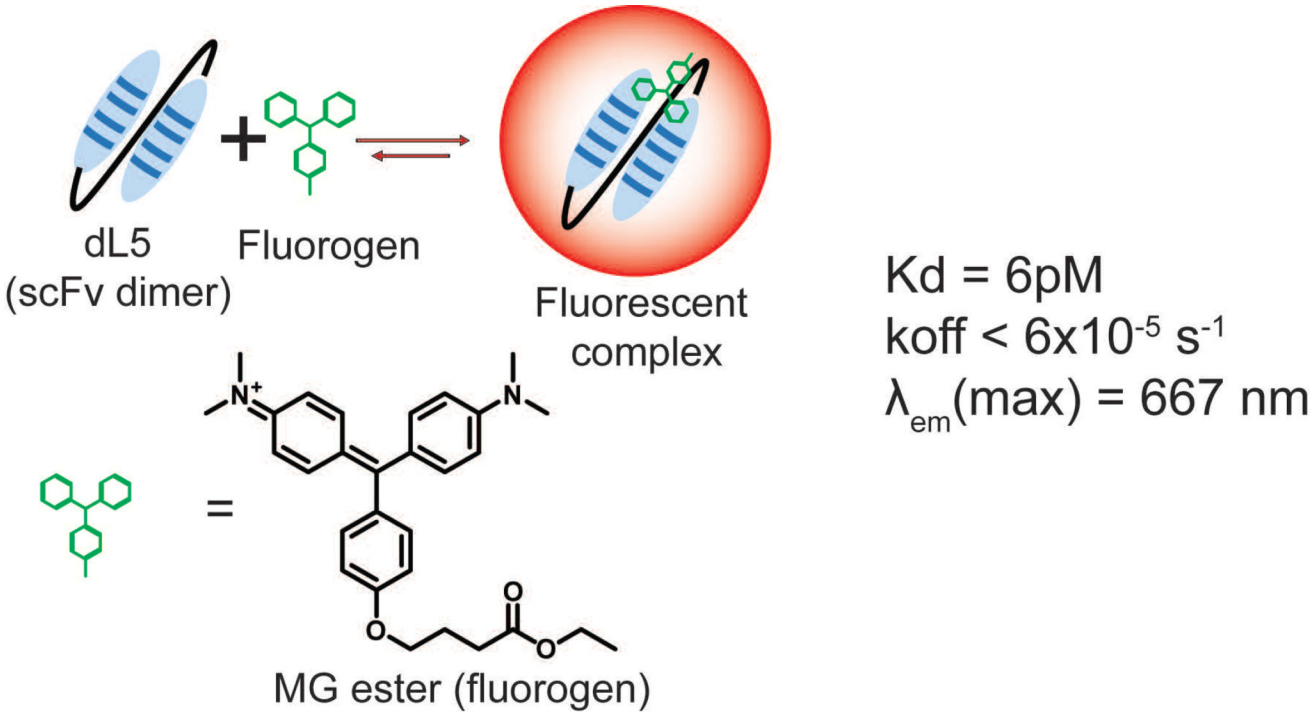

Figure 1.

Schematic showing the binding reaction between dL5 and MG-ester. Structure of MG-ester is also shown. Relevant physical parameters of the complex are noted on the right(Saurabh et al. 2016). Adapted with permission from Saurabh *et al, J. Am. Chem. Soc.*, **2016**, *138* (33), pp 10398–10401. Copyright (2016) American Chemical Society.

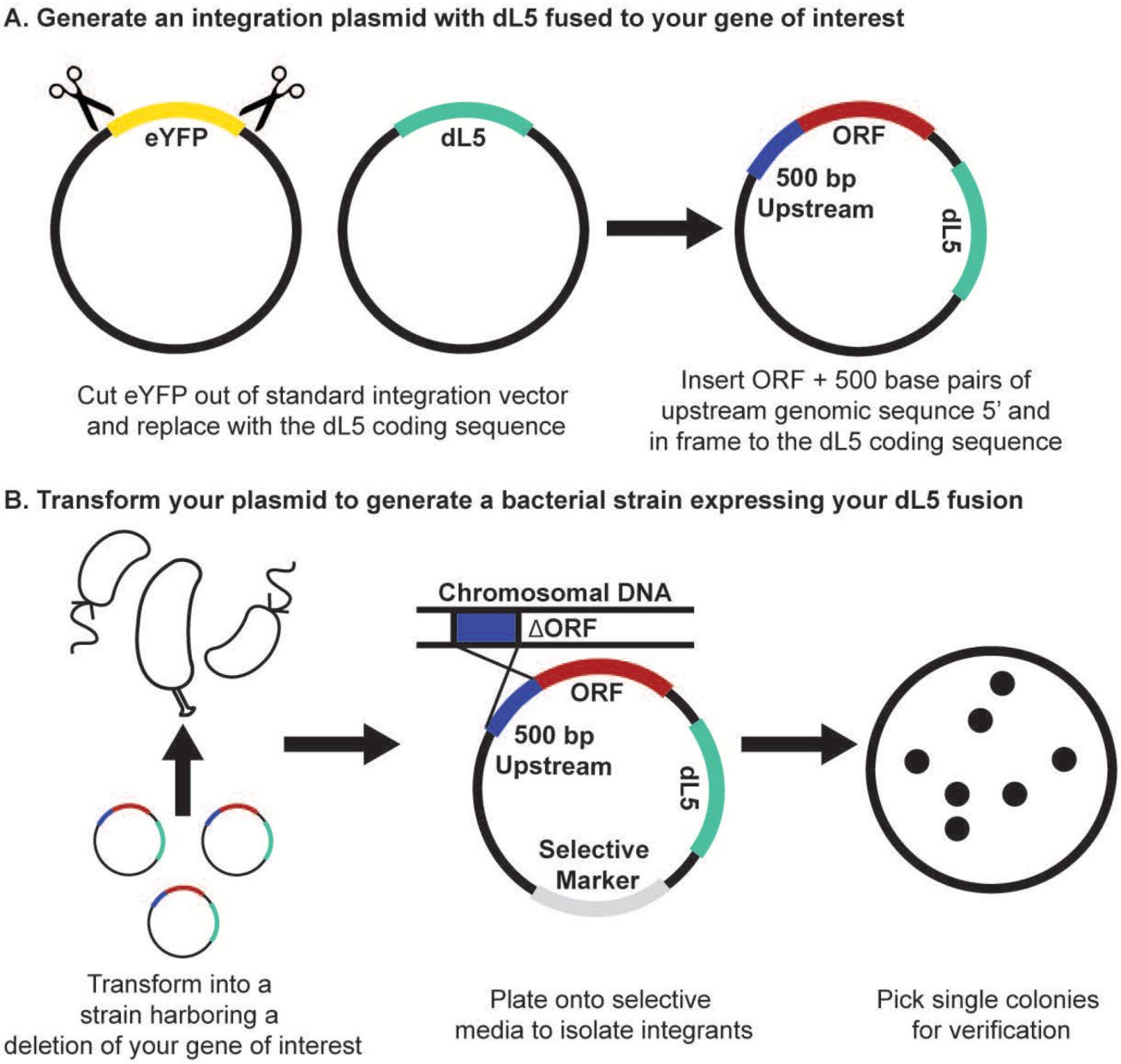

Figure 2.
Cloning steps to generate dL5 fusions to a general protein of interest.

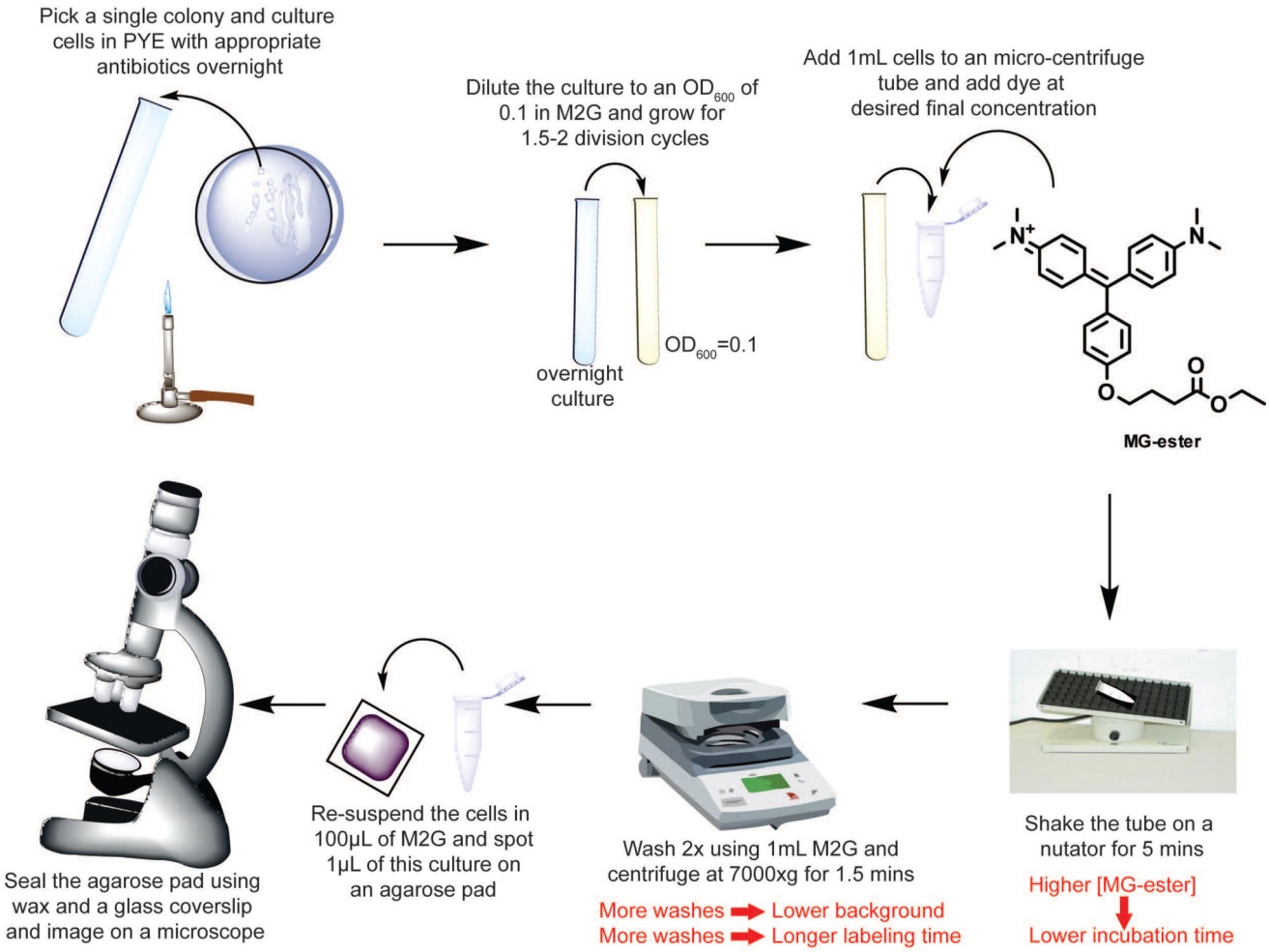

Figure 3.

Steps for growth and labeling *Caulobacter* cells expressing dL5 fusions.

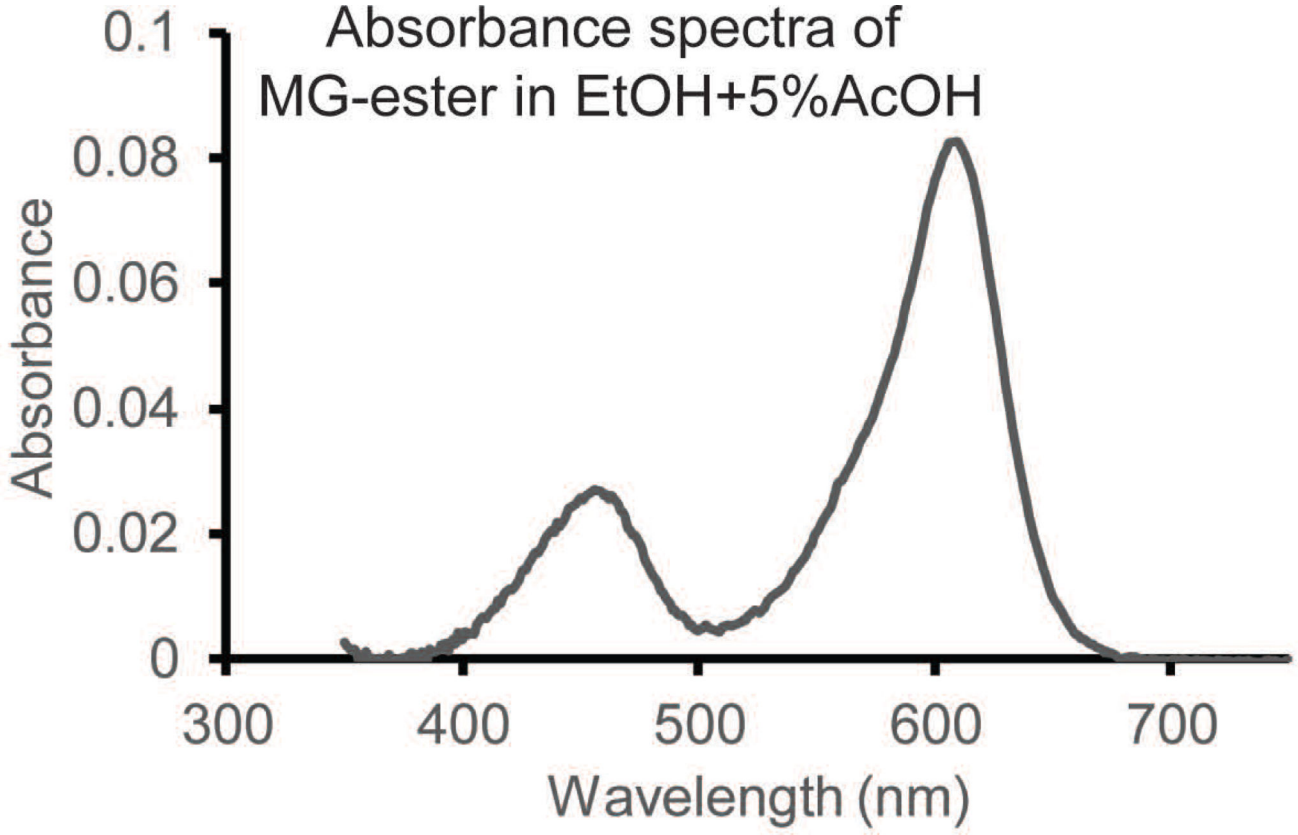

Figure 4.

Absorbance spectra of MG-ester measured in a solution of Ethanol + 5% Acetic acid. The sample has an O.D. at 608 nm of 0.082 which corresponds to a concentration of 890 nM.

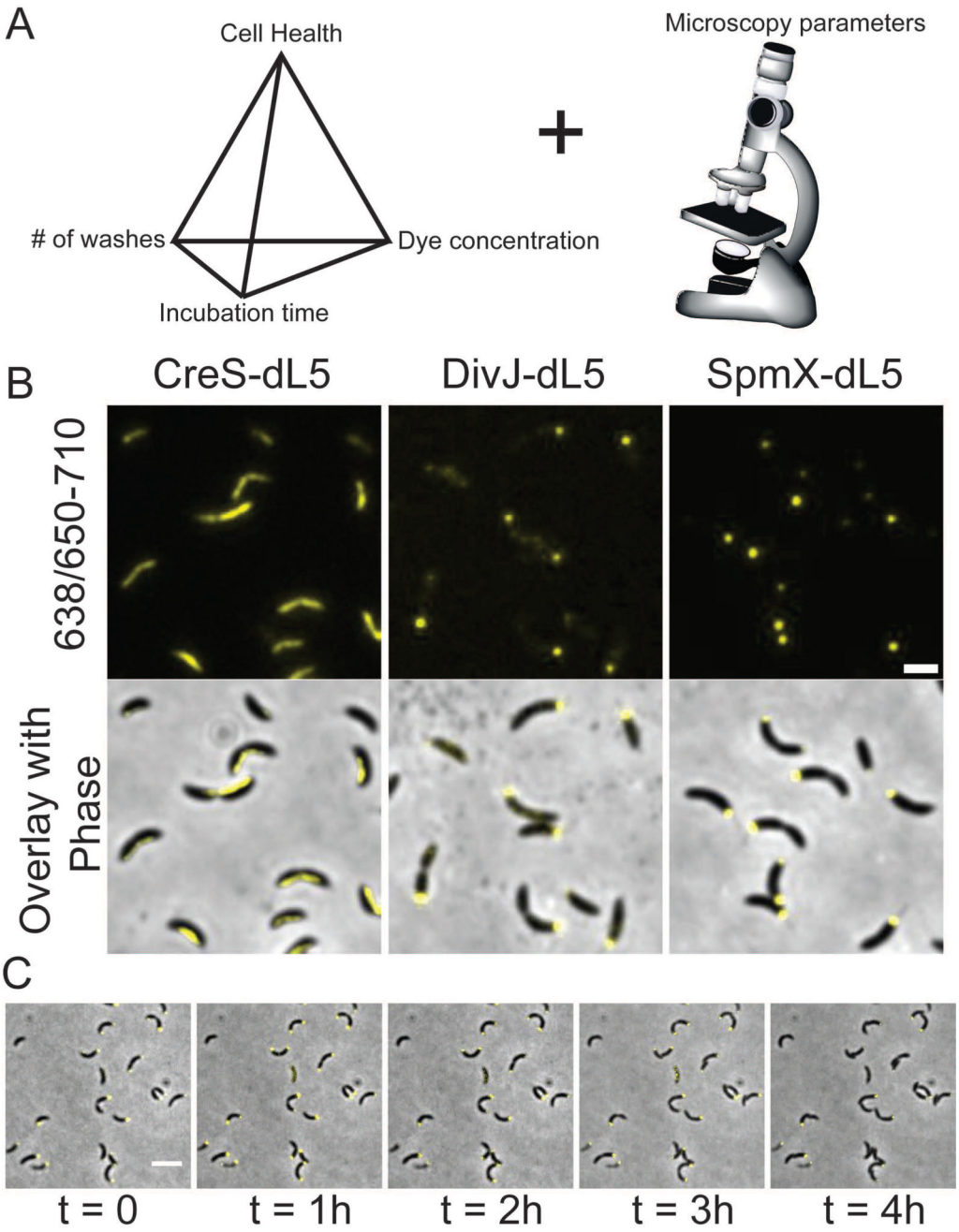

Figure 5.

A. Optimal cell labeling depends on a fine balance between cell health, dye concentration, dye incubation time and number of washes as shown by this tetrahedron. Additionally, high quality images require an optimization of microscopy parameters. B. Diffraction limited widefield fluorescence microscope images of *Caulobacter* cells expressing dL5 fused to CreS, DivJ and SpmX, respectively. Scale bar is 1 μm. C. Time lapse micrographs showing an overlay of phase contrast and fluorescence channels for cells expressing SpmX-dL5 to demonstrate that the cells are growing and dividing normally under these imaging conditions

(Saurabh et al. 2016). Adapted with permission from Saurabh *et al, J. Am. Chem. Soc.*, **2016**, *138* (33), pp 10398–10401. Copyright (2016) American Chemical Society.

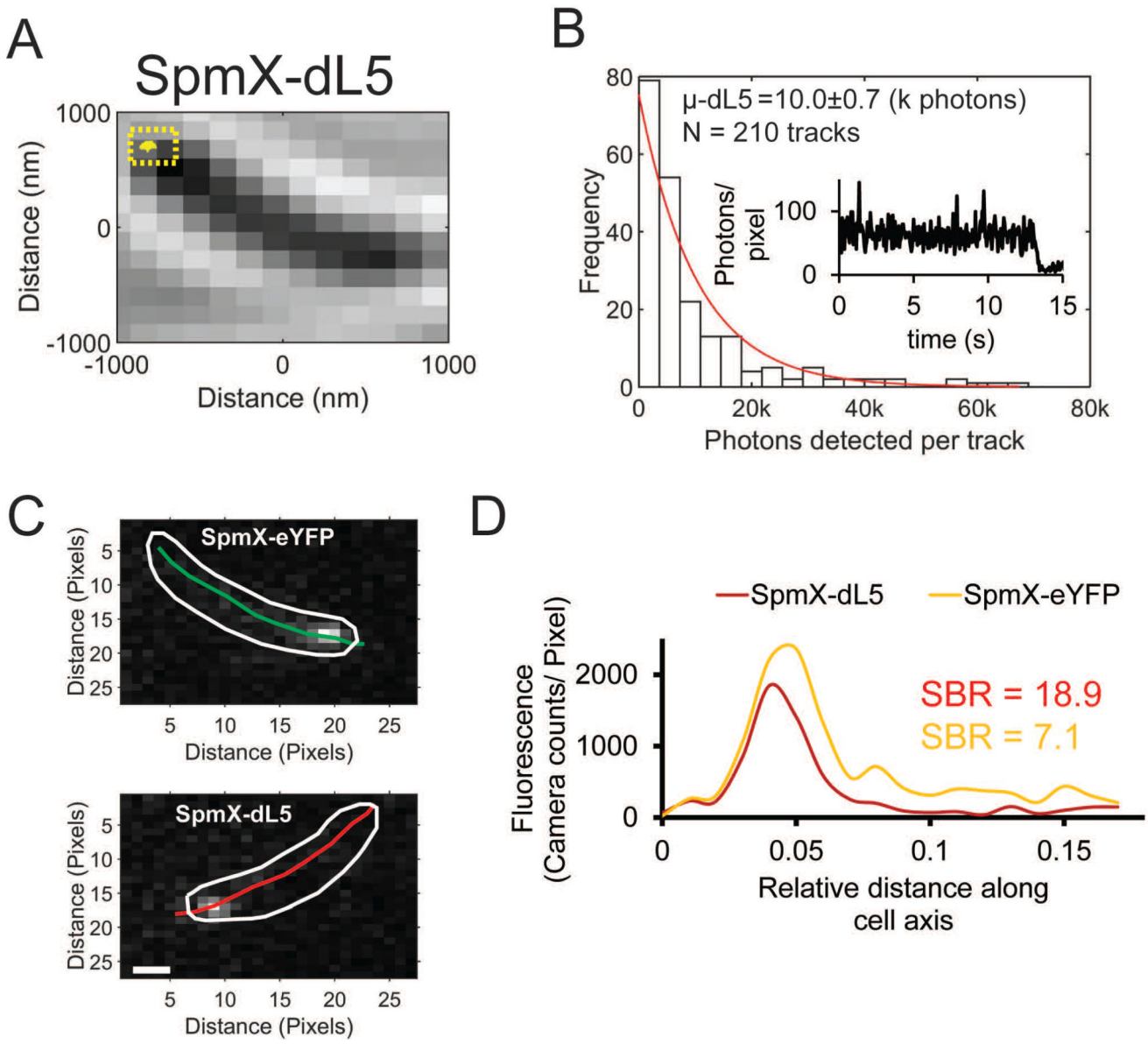

Figure 6.

A. Typical single molecule trace for SpmX-dL5 overlaid with reverse contrast white light images of a *Caulobacter* cell. B. Distribution of photons detected per track for 210 tracks observed in *Caulobacter* cells expressing SpmX-dL5. Inset shows a typical trace from a single molecule track. Single step photobleaching confirms the presence of a single molecule. C and D. Typical single molecules of SpmX-eYFP and SpmX-dL5 and a comparison of signal to background in the respective cells (Saurabh et al. 2016). Adapted with permission from Saurabh *et al, J. Am. Chem. Soc.*, **2016**, *138* (33), pp 10398–10401. Copyright (2016) American Chemical Society.

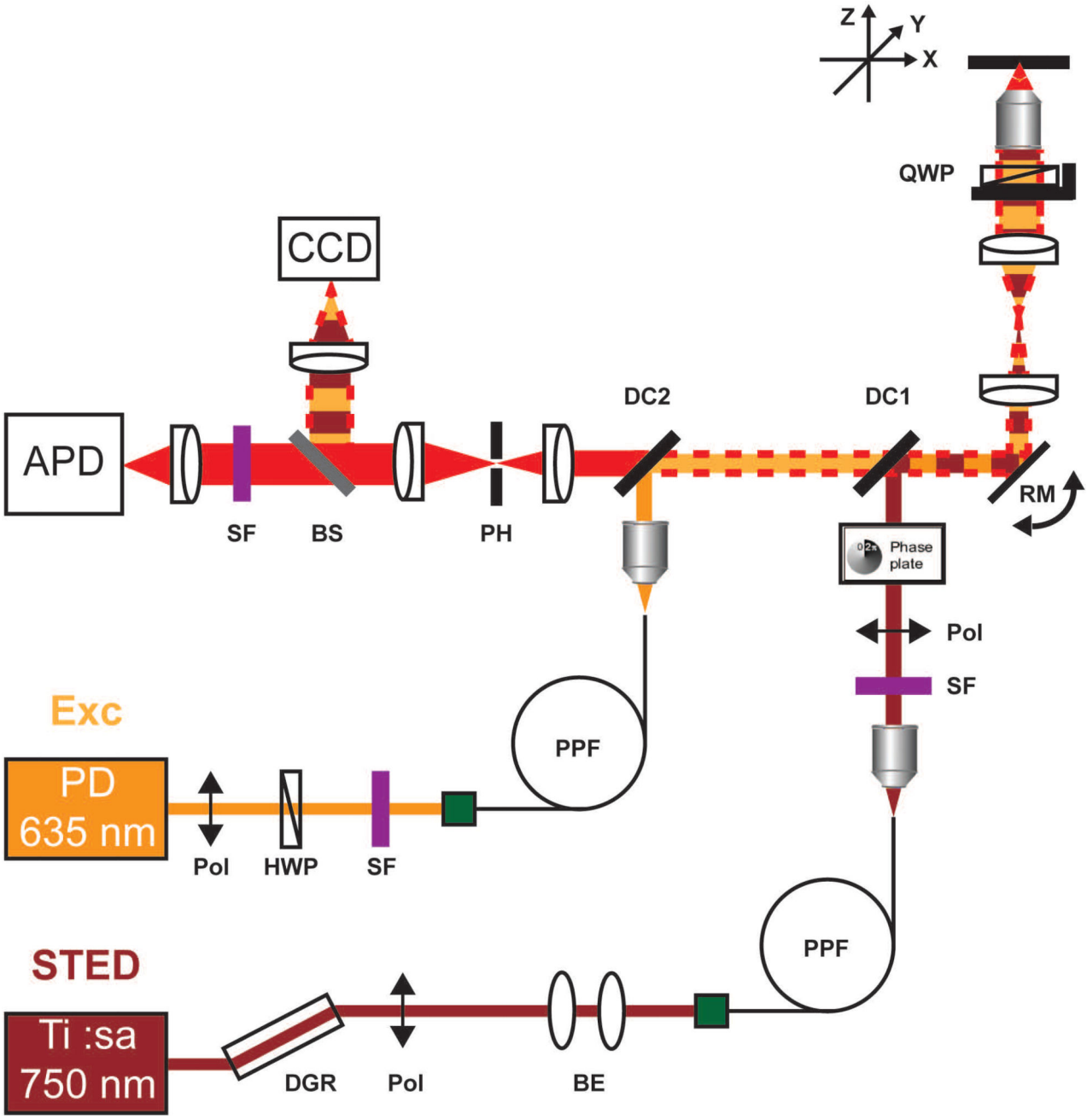

Figure 7.

Fast-Scanning Pulsed STED Setup. APD, avalanche photo diode; CCD, charge-coupled device; DC, dichroic mirror; QWP, quarter-wave plate; RM, resonant mirror; PH, pinhole; BS, beam splitter; SF, spectral filter; Pol, polarizer; PPF, polarization-preserving fiber; VPP, vortex phase plate; BE, beam expander; HWP, half-wave plate; DGR, dispersive glass rods; PD, pulsed diode laser; Ti:Sa, Titanium:sapphire mode-locked oscillator (Saurabh et al. 2016). Reprinted with permission from Saurabh *et al, J. Am. Chem. Soc.*, **2016**, *138* (33), pp 10398–10401. Copyright (2016) American Chemical Society.

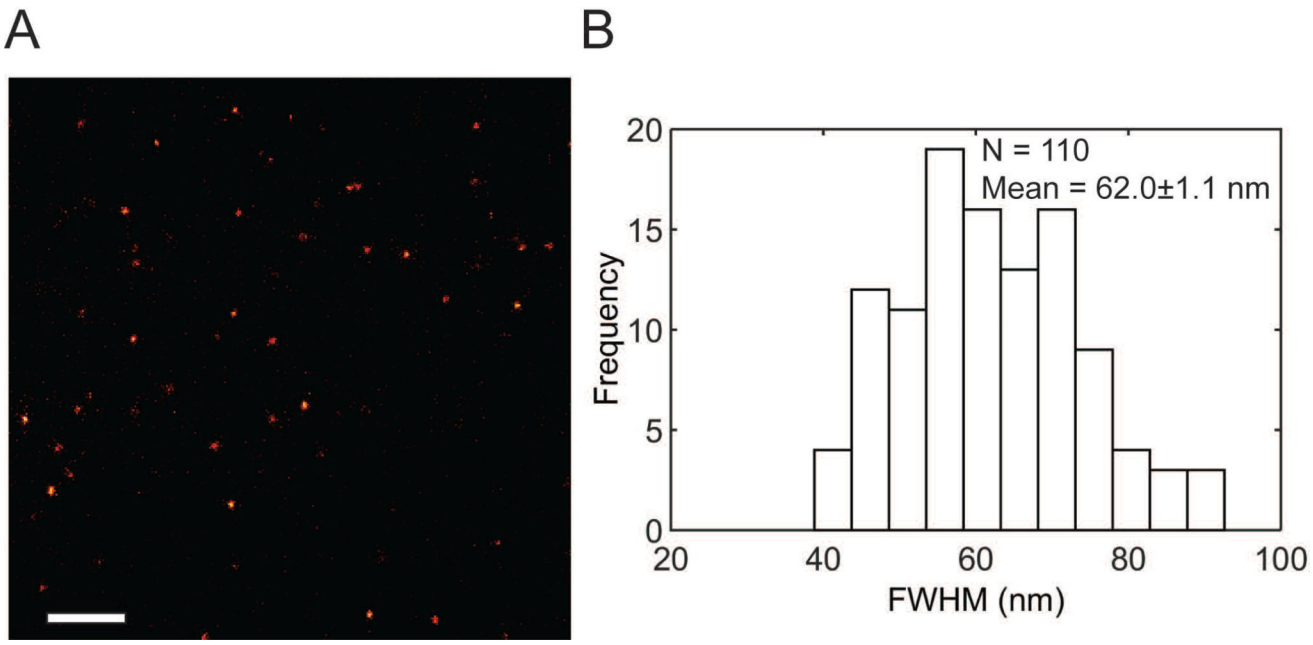

Figure 8.

A. Typical field of view showing single Atto647N molecules embedded in Mowiol. Scale bar is 1 μm. B. Distribution of full width at half maximum for 110 individual molecules demonstrating a resolution of 62.0 ± 1.1 nm.

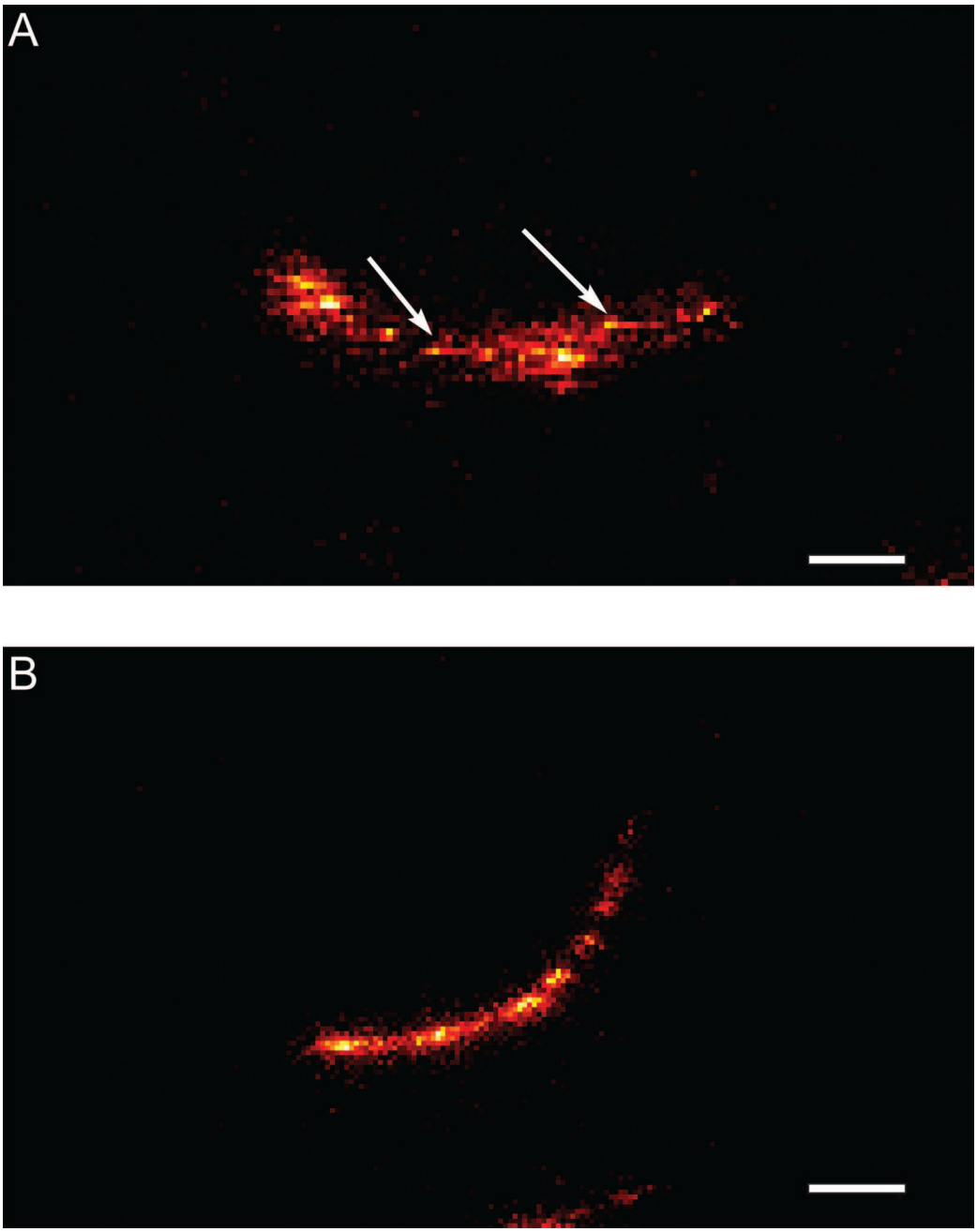

Figure 9.

A. STED image of a *Caulobacter* cell expressing CreS-dL5 using slow scanning parameters. Arrows point to regions where we see evidence of blinking or bleaching during the slow scan B. STED image of another *Caulobacter* cell expressing CreS-dL5 using fast scanning parameters. Fast scanning gets rid of regions of blinking or premature bleaching. Scale bars are 500 nm.

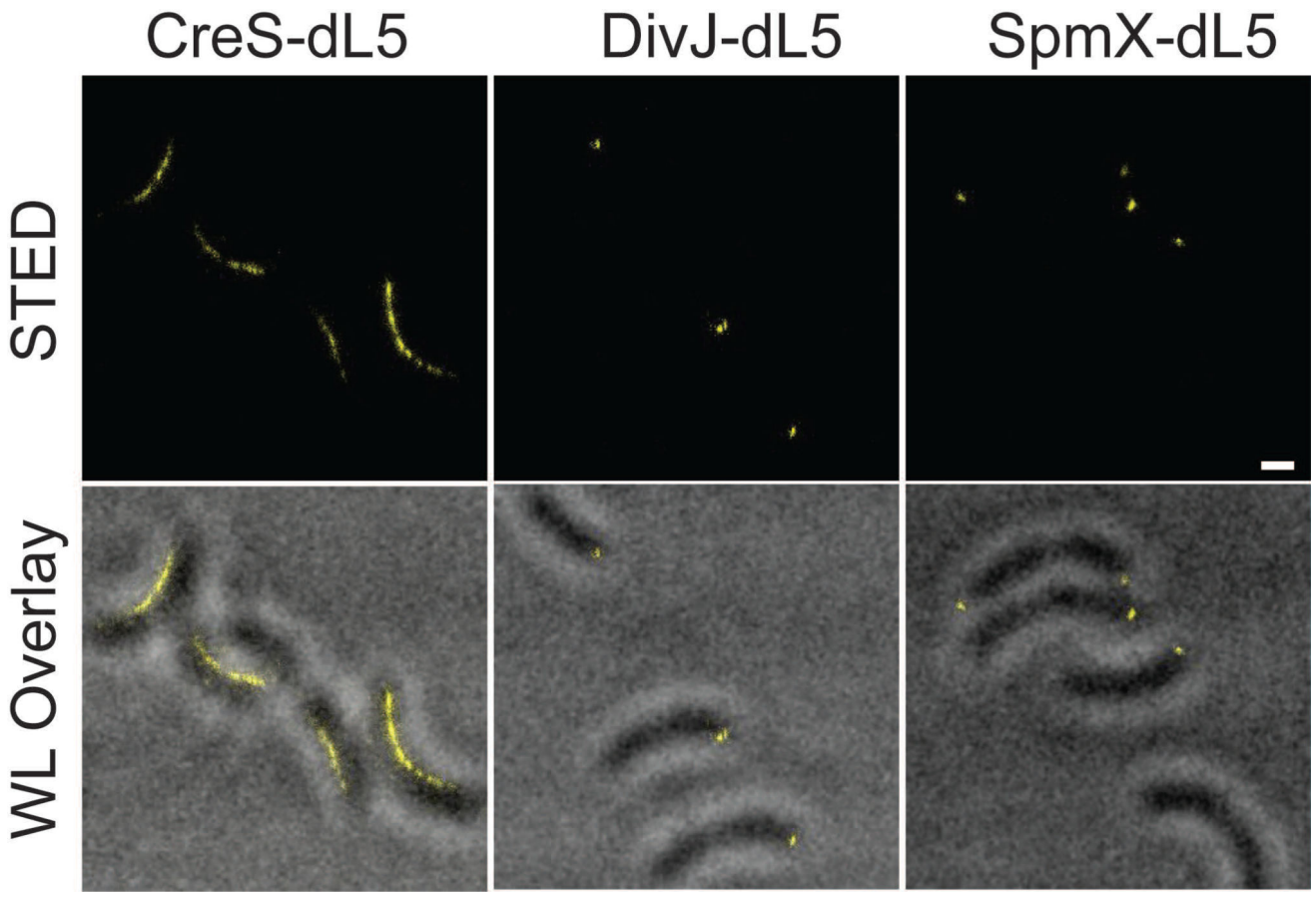

Figure 10.

Fast scanning STED images of *Caulobacter* cells expressing dL5 fused to CreS, DivJ and SpmX, respectively. Scale bar is 500 nm (Saurabh et al. 2016). Adapted with permission from Saurabh *et al, J. Am. Chem. Soc.*, **2016**, *138* (33), pp 10398–10401. Copyright (2016) American Chemical Society.

Table 1

Troubleshooting tips

PROBLEM	POSSIBLE CAUSE	SOLUTION
FLUORESCENCE BACKGROUND IS HIGH	Cells may have inherently high background	Test cells without MG-ester for inherent background
		Perform more washes
	MG-ester is activated in the cell wall/ periplasm	Perform more washes
FLUORESCENCE SIGNAL IS LOW	Expression of the protein may be low	Verify that the protein is expressed through western blots
	Microscope settings	Ensure that the microscope is optimized for imaging the fluoromodule. Mercury lamps do not excite dL5-MG fluoromodule efficiently
	Possibly MG-ester is at a lower concentration than needed	Verify the concentration through absorbance at 608 nm
POOR RESOLUTION ENHANCEMENT IN STED IMAGING	Low depletion laser intensity	Increase the depletion laser intensity/optimize imaging parameters to accommodate higher depletion laser intensity
	Detection of early, low resolution photons (CW STED only)	Increase gating time
	Poor depletion PSF	Check alignment by imaging gold beads