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Real-time microscopy and physical perturbation of bacterial pili using maleimide-conjugated molecules

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Abstract

Bacteria use surface-exposed, proteinaceous fibers called pili for diverse behaviors including horizontal gene transfer, surface sensing, motility, and pathogenicity. Visualization of these nanomachines and their activity in live cells has proved challenging largely due to their small size. Here, we describe a broadly applicable method for labeling and imaging pili and other surface exposed nanomachines in live cells. This technique uses a combination of genetics and epifluorescence microscopy in which a cysteine substitution is made in the major pilin subunit for subsequent labeling with thiol-reactive maleimide dyes. Large maleimide-conjugated molecules can also be used to physically impede the dynamic activity of nanomachines. We describe parameters for selecting cysteine substitution positions, optimized labeling conditions for imaging pilus fibers, and methods for impeding pilus activity. Visualization of extracellular nanomachines using this approach can provide a more comprehensive understanding of the role played by these structures in distinct bacterial behaviors.

Introduction

Bacterial surface-exposed nanomachines including pili, secretion systems, and flagella play an essential role in several diverse processes across distantly related bacterial species. These structures have been difficult to fully characterize since the most common techniques for their visualization such as immunofluorescence or electron microscopy disrupt their activity or require cell fixation and cannot be used to interrogate their dynamic activity. Although valuable, these end-point assays provide only a limited snapshot view of nanomachine action. This limited perspective has been especially problematic in the study of type IV pili and how these small fibers participate in diverse behaviors since dynamic activity mediated by polymerization and depolymerization of pilin subunits is thought to be important for their function. Recent development of a technique involving cysteine substitution within the major structural subunit of surface-exposed nanomachines has allowed direct observation of

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Author contributions

C.K.E. and Y.V.B conceived the study. C.K.E. and T.N.D. performed the experiments. C.K.E., A.B.D., and Y.V.B. analyzed the data. C.K.E. wrote the manuscript with help from A.B.D. and Y.V.B.

Competing interests

The authors declare no competing interests.

Data availability

The data that support this study are available from the corresponding author upon request.

dynamic activity in real time, thereby increasing our understanding of their function in diverse processes and the underlying mechanisms of their synthesis[1–7]. While this technique was originally applied to the labeling of flagella[1], here we focus on our application of this technique to the type IV pili and discuss how it may be expanded to other surface-exposed nanomachines.

In this protocol, we describe methods for labeling exposed nanomachines (Fig. 1), starting with how to select residues for cysteine replacement (Fig. 2) and provide insight into optimizing labeling protocols for the greatest likelihood of success. We detail how to label pili in different systems and describe how to perturb their dynamic activities using a similar method.

Applications

Cysteine substitutions coupled with labeling by maleimide-conjugated compounds have been used to visualize both flagella and pili in diverse bacterial species. They have been used to address several outstanding questions on how bacteria assemble external nanomachines and use them to interact with their environments. For example, labeling of flagella has bolstered our understanding of how bacteria regulate motility[1], flagellar assembly[6], and flagellar length[3,7]. Labeling of the type IVa competence pili in *Vibrio cholerae* has clarified how bacteria use pili to take up environmental DNA that can be used to acquire new genes including those encoding antibiotic resistance or virulence factors[4]. Labeling of the type IVc tad pili of *Caulobacter crescentus* has revealed that these structures exhibit both extension and retraction, despite lacking a pilus retraction ATPase PilT orthologue, and that this retraction is important for sensing surface contact[2].

Mutating the pilus retraction ATPase PilT in systems that possess this component often results in hyperpiliation and disrupts normal extension and retraction dynamics, however these mutations have also been shown to alter transcription profiles of cells[8]. Blocking retraction through steric hindrance by bulky compounds as described below allows for temporal control of the disruption of pilus retraction in live cells and allows for direct assessment of the role for pilus retraction in bacterial behaviors in the context of genetically intact systems. Type IV pili are distantly related to other surface-exposed structures including type IV and type II secretion systems, competence pili of Gram-positive organisms and the archaellum (used for swimming motility) of archaea, which are all understudied due to limitations in methods to visualize their activities in real-time. Likewise, flagella are related to the type III secretion system involved in the secretion of virulence proteins[9]. Due to the homology between these systems, this labeling method could be applied to studying these and other surface-exposed nanomachines.

Strengths and limitations of existing protocols

The most common technique for visualizing pili is electron microscopy in one of its varying forms including transmission electron microscopy, scanning electron microscopy, and cryo-electron microscopy. While the use of traditional electron microscopy dates to the 1930’s and allows for visualization of small structures at the nanoscale, this technique requires

harsh treatment of cells that often shears structures off the cell surface, hindering comprehensive analysis of their length and number while providing only a limited temporal view of pili in the context of bacterial behavior. Employment of cryo-electron microscopy largely mitigates the issue of cell surface shearing and provides high-resolution three-dimensional structural models of nanomachine complexes[10–12]; however, this method still requires cell fixation by freezing and requires numerous images of structures averaged together to compile a model of the structure, providing no information about the dynamics of the machinery.

While some light microscopy techniques can be used to visualize pili, the most accessible method is immunofluorescence microscopy which uses bulky fluorescently conjugated antibodies that bind to extended pili and cause fiber aggregation in addition to impeding physical activity such as retraction[13–15]. Fluorescence microscopy of dynamic pilus structures has been achieved using succinimidyl-ester dyes that bind to primary amines on exposed bacterial proteins. However, this approach often results in high fluorescence signal of the cell body relative to the pili and requires the use of total internal reflection fluorescence (TIRF) microscopy to allow for pilus fiber visualization[16]. The method outlined here allows for direct visualization of pilus extension and retraction in the context of live cells in real-time by epifluorescence microscopy, providing a more comprehensive view of the role pili play in bacterial behaviors.

Selection of residues for cysteine substitution

Type IV pilins exhibit a “lollipop” structure consisting of a conserved alpha helical N-terminal transmembrane domain and a C-terminal globular head-like domain (Fig. 2a, b, c). The transmembrane domain is important for insertion in the inner membrane[17] and supports pilin-pilin interactions at the center of pilus fibers[18]. Parts of the globular domain are exposed to the extracellular environment, presenting an optimal target for making cysteine mutations that will be exposed to labeling by maleimide dyes. However, the globular head of many pilins also possesses conserved folds including a disulfide bond that is critical for maintaining protein structure and stable pilus formation. We found that mutating serine or threonine residues to cysteine was much more likely to result in labelable pili (Fig. 2d), likely because they share nucleophilic properties and are less likely to disrupt protein folding. The globular domain also exhibits variability between species, and structure prediction software based on homology is rarely sufficient for predicting exposed, non-deleterious sites for cysteine substitutions. To overcome this limitation, we use open source software NetSurfP[19] which calculates relative surface accessibility (RSA) of individual amino acid residues. While we have found that some outliers with extremely low or high RSA scores are unexpectedly labelable or not labelable respectively, we find that the majority of residues with RSA scores above 0.4 are often at least semi-labelable (Fig. 2d, Table 1). Interestingly, there is considerable variation in the number of residues selected that resulted in labelable pili in our test cases (*C. crescentus* PilA 2/7, *V. cholerae* PilA 2/9, *V. cholerae* MshA 14/24, *V. cholerae* TcpA 5/7). For this reason, we generally select ten residues for cysteine substitution with the highest RSA scores.

Labeling strategies

Labeling and visualization of dynamic nanomachine activity.

For a comprehensive methodology on labeling of flagella filaments and visualizing their dynamic activity see Turner and Berg 2018[20]. Here, we will focus on pili labeling. For assaying labeling efficiency of different cysteine-substituted pilus mutants, we use AlexaFluor 488 C_5 maleimide dye (AF488-mal), as other maleimide dyes do not always label pili of the same cysteine knock-in strain as efficiently (Table 1). Using AF488-mal, we successfully labeled cysteine-knock in mutants for the three subclasses of type IV pili[2]: the type IVa competence pilus and mannose-sensitive hemagglutinin (MSHA) pili found in *V. cholerae* (Fig. 3a), the type IVb toxin co-regulated pilus (TCP) found in *V. cholerae* (Fig. 3b), and the type IVc tight adherence (Tad) pili found in *Caulobacter crescentus* (Fig. 3c). Using time-lapse microscopy, cycles of extension and retraction can be observed for labeled pili, although due to differences in extension and retraction rates, the optimal time-lapse interval may vary (average extension rates of ~90 nm/s for type IVa *V. cholerae* competence pili[4] vs ~140 nm/s for type IVc tad pili[2] of *C. crescentus*).

One interesting observation for labeling type IV pili is that labeling of pilin subunits results in cell body fluorescence[2,4] (Fig. 3). Diffusion of fluorescently labeled cysteine-substituted pilins residing in the cytoplasmic membrane results in peripheral cell body fluorescence. In *C. crescentus*, AF488-mal is not permeable through the outer membrane, and thus cell body fluorescence is directly dependent on retraction of labeled pilins into the inner membrane[2]. However, in *V. cholerae*, maleimide dyes permeate the outer membrane and label pilins residing in the inner membrane independent of dynamic activity (unpublished data). Cell body fluorescence is a useful tool for determining whether a cysteine knock-in strain can be labeled (see protocol below), although it also underscores the importance of labeling a non-cysteine knock-in parent strain alongside any mutants as a negative control.

Disruption of dynamic nanomachine activity.

While physical perturbation of some pili is possible by genetic manipulation through deletion of the pili retraction ATPase genes or point mutations in minor pilins[21], these mutations often have pleiotropic consequences on global transcription profiles as in Neisseria[8]. In addition, deletion of the pili retraction ATPase genes can affect the physical activity of other pili, such as in *V. cholerae* where multiple pili share a retraction ATPase[4,22]. Furthermore, many type IV pili do not possess orthologs of a retraction ATPase, including many type IVb and all type IVc pili, excluding genetic perturbation from the experimental repertoire of tools for assessing the role of dynamics in these systems[2,23]. To overcome these issues, adding maleimide-conjugated bulky compounds disrupts dynamic pilus activity in real-time. Two methods for obstructing retraction have been established[2,4]. The first method involves a single incubation step with a bulky polyethylene glycol maleimide conjugate (PEG5000-mal), while the second involves a two-step process: incubation with a biotin-maleimide conjugate (biotin-mal) followed by a wash step and incubation with a streptavidin derivative. Surprisingly, we found that type IVa pili could only be obstructed by the two-step biotin-mal/streptavidin method (Fig. 4a), while the one step PEG5000-mal incubation robustly blocked dynamic activity in the type IVc pili (Fig 4b). Because type IVa pilins are

larger than type IVc pilins, they may form tighter connections with neighboring subunits, limiting accessibility of bulky conjugates to exposed cysteine residues. This may also explain discrepancies between labeling efficiencies of different positions with different maleimide dyes (Table 1), although this remains to be determined.

Limitations and optimization

While this labeling method has been applied and successfully used to visualize surface-exposed nanomachines in several diverse bacterial systems, some limitations exist and optimization at the biological level is essential for each system labeled (Table 2). In the MSHA pili, some positions resulted in only weakly labeled pili for some dyes and while other cysteine substitutions resulted in bright labeling along the length of the pilus fibers for all three dyes tested, demonstrating the importance of testing multiple residues for cysteine substitution (Fig. 5a, b). While generally at least one out of ten cysteine-substituted positions result in at least weak fiber labeling with maleimide dyes (Table 1), some bacterial species are not easily genetically altered, limiting the number of cysteine knock-in mutants that can be rapidly tested. Pili are also often expressed maximally under different conditions in different species. For example, the type IVc tad pili of *C. crescentus* are produced in a cell-cycle dependent manner, while the type IVc tad pili of *Pseudomonas aeruginosa* are produced maximally in stationary cultures[24,25]. Determining optimal conditions for maximal pilus expression may not be trivial depending on the bacterial system.

Technical challenges can also pose a problem and require optimization for the best results (Table 2). As surface exposed nanomachines are often fragile, shearing during wash steps necessary for labeling can be an issue[20]. Furthermore, the labeling time directly impacts the fluorescence signal of the fibers (Fig. 5c), and the imaging conditions may result in more background fluorescence that obscures spatial resolution. For example, when no imaging pad is used, fibers often drift out of the plane of focus due to the lack of spatial confinement and result in blurry or smeared images from motion driven by Brownian forces. In addition, the media used to make pads can affect background fluorescence of the sample (Fig. 5d). An additional limitation common to all live imaging applications includes the potential effects of light exposure and the resulting phototoxicity to cells, which can affect their extension and retraction dynamics. We observed in some systems such as the *Vibrio cholerae* type IVa competence pili that pilus dynamic activity decreases over time upon light exposure, limiting imaging experiments to small time windows to prevent light interference in measurements of pilus-dependent phenotypes (Fig. 5e). However, the benefits of this technique far exceed its limitations. The use of the cysteine knock-in method for imaging surface-exposed nanomachines allows the visualization of these structures in live cells in real time and should prove valuable for the elucidation of their roles in the broad processes to which they contribute.

Reagents

- Growth medium appropriate for the growth of the bacteria under investigation such as Lysogeny broth (LB) medium sterilized by autoclaving (Fisher Scientific, cat. no. DF0446–17-3)

- Solid growth medium appropriate for the growth of specific species of bacteria, such as pre-poured LB agar plates (Sigma-Aldrich, cat. no. L5542)
- Instant Ocean (Aquarium Systems, cat. no. EAN3443981210100)
- Phosphate buffered saline (PBS) (ThermoFisher Scientific, cat.no. 10010023)
- Agarose (SeaKem LE agarose, Lonza, cat. no. 50004)
- Gelzan CM (Sigma-Aldrich, cat. no. G1910)
- High-quality anhydrous dimethyl sulfoxide (DMSO) (ThermoFisher Scientific, cat. no. D12345)
  - Caution – DMSO is inflammable, an irritant, and a permeator. Avoid contact with skin and eyes.
- Alexa Fluor 488 C_5 maleimide (AF488-mal) (ThermoFisher Scientific, cat. no. A10254)
  - Caution – maleimides are irritants. Avoid contact with skin and eyes. Harmful if swallowed.
- DyLight 405 maleimide (DyL405-mal) (ThermoFisher Scientific, cat. no. 46600)
  - Caution – maleimides are irritants. Avoid contact with skin and eyes. Harmful if swallowed.
- Alexa Fluor 594 C_5 maleimide (AF594-mal) (ThermoFisher Scientific, cat. no. A10256)
  - Caution – maleimides are irritants. Avoid contact with skin and eyes. Harmful if swallowed.
- EZ-Link Maleimide-PEG11-Biotin (biotin-mal) (ThermoFisher Scientific, cat. no. 21911)
  - Caution – maleimides are irritants. Avoid contact with skin and eyes. Harmful if swallowed.
- Methoxypolyethylene glycol maleimide, 5,000 (PEG5000-mal) (Sigma-Aldrich, cat. no. 63187)
  - Caution – maleimides are irritants. Avoid contact with skin and eyes. Harmful if swallowed.
- NeutrAvidin protein (ThermoFisher Scientific, cat. no. 31000)

Equipment

- Eppendorf snap-cap centrifuge tubes, 1.5 ml (ThermoFisher Scientific, cat. no. 3439), sterilized by autoclaving
- Tubes appropriate for the growth of the bacterial species under investigation such as Falcon™ Round-Bottom Polypropylene tubes (Fisher Scientific cat. no 352059)

- Incubator, appropriate for the optimum growth of the species of bacteria under investigation
- Microcentrifuge (ThermoFisher Scientific, cat. no. 75002451)
- Vortex mixer (Thermofisher Scientific, cat. no 88880017TS)
- A fluorescence microscope equipped with appropriate excitation/emission filters that cover the excitation/emission maxima of at least Alexa Fluor 488 C_5 maleimide, ThermoFisher Scientific, cat. no A10254s
- Glass slides (25 × 75 mm; VWR, cat. no. 48300–026)
- 22 × 22 cm glass coverslips (VWR, cat. no. 48366–227)
- Sterile cryogenic vials for bacterial freezer stocks (2 ml, polypropylene; VWR, cat. no. 66008–706)
- Sterile inoculating loops (1 μl, VWR, cat. no. 89126–870)
- Bunsen burner
- Micropipettors P2-P1000 (Fisher Scientific, cat. no. 14–388-100)
- Sterile pipette tips for P2 (Fisher Scientific, cat. no. 02–707-436), P20 and P200 (Fisher Scientific, cat. no. 02–707-409), and for P1000 (cat. no. 02–707-407)

Reagent Setup

1. 7 g/L Instant Ocean medium
    a. Dissolve 7 g of Instant Ocean into 1 L of water before autoclaving to sterilize. Store at room temperature.
2. DyLight 405 maleimide, Alexa Fluor 488 C_5 maleimide, Alexa Fluor 594 C_5 maleimide stocks
    a. Dissolve 1 mg of compound into 200 μL of anhydrous DMSO to make a 5 mg/mL stock and vortex to mix until compound is completely dissolved
        i. Critical step - stock solutions of maleimide conjugates should be stored at −20 °C and they are sensitive to both light and oxidation. The stocks can be kept at −20 °C for several months and should go through minimal cycles of freezing and thawing to prevent decomposition and loss of efficacy. It is therefore critical to aliquot into small fractions, for example 20 μL aliquots.
3. PEG5000-mal
    a. Dissolve 50 mg of PEG5000-mal into 1 mL of water to make a 100 mM stock solution and vortex vigorously to mix until compound is completely dissolved

i. Critical step - stock solutions of maleimide conjugates should be stored at −20 °C and they are sensitive to both light and oxidation. The stocks can be kept at −20 °C for several months and should go through minimal cycles of freezing and thawing to prevent decomposition and loss of efficacy. It is therefore critical to aliquot into small fractions, for example 50 μL aliquots.

4. Biotin-mal

   a. Dissolve 500 μg of biotin-mal into 100 μL anhydrous DMSO to make a 5 mg/mL stock and vortex vigorously to mix until compound is completely dissolved

      i. Critical step - stock solutions of maleimide conjugates should be stored at −20 °C and they are sensitive to both light and oxidation. The stocks can be kept at −20 °C for several months and should go through minimal cycles of freezing and thawing to prevent decomposition and loss of efficacy. It is therefore critical to aliquot into small fractions, for example 20 μL aliquots.

5. NeutrAvidin

   a. Dissolve 10 mg NeutrAvidin into 300 μL of water to make a 33 mg/ml stock and vortex vigorously to mix until compound is completely dissolved.

      i. Critical step - stock solutions of proteins should be stored at −80 °C and should go through minimal cycles of freezing and thawing to prevent denaturation and loss of efficacy. It is therefore critical to aliquot into small fractions, for example 10 μL aliquots.

Preparation before maleimide labeling

1. Grow bacterial cultures (4–24 hours, depending on the strain).

   a. From a −80 °C bacterial culture freezer stock or a single colony on a Petri agar plate, inoculate cells into sterilized liquid medium and grow them in optimal, species-specific conditions until they reach optimal growth for specific pilus expression.

      i. Critical step – different pili are maximally expressed under different conditions; it is important to know the conditions of maximal pilus expression for each species before labeling

2. Prepare desired stock solution(s) of maleimide conjugates as described in Reagent Setup (25 min)

i. Critical step – stock solutions of maleimide conjugates should be stored at −20 °C and they are sensitive to both light and oxidation. The stocks can be kept at −20 °C for several months and should go through minimal cycles of freezing and thawing to prevent decomposition and loss of efficacy.

3. Prepare agarose or Gelzan pad for imaging bacteria (25 min)

   i. Critical step – make no more than one hour prior to labeling as pads can dry out

      a. To make 1% agarose pad, dissolve 0.1 g of agarose into 10 ml of medium or buffer and microwave until boiling to melt agarose

         1. Note – 0.2% Gelzan pads can be made and used the same way by dissolving 0.02 g of Gelzan into 10 ml of PBS or 7 g/L Instant Ocean medium

         2. Caution – agarose solutions easily boil over and can cause burns if not handled properly

   ii. Spot ~80 μl of melted agarose solution onto a glass slide and cover with a 22 × 22 cm glass coverslip and apply pressure until agarose is thinly spread between the two glass surfaces without creating excessive bubbles or holes in the agarose

      1. Critical step – applying too much pressure will result in tearing of the pad and difficulty imaging, applying insufficient pressure may result in an uneven pad and difficulty imaging

   iii. Let pad solidify for at least 10 min at room temperature to dry appropriately

      1. Critical step – if pad does not dry sufficiently, bacteria will not be confined, and imaging will be difficult

   iv. Remove glass coverslip by sliding it gently of the pad just prior to use

      1. Critical step – do not leave exposed to air for more than 5 min as pads will dry out, which may cause difficulty imaging

Labeling with maleimide dyes for observation of dynamic pilus activity (7–35 min)

1. Pipette 100 μL of culture at appropriate growth phase into 1.5 ml Eppendorf centrifuge tube and centrifuge cells at appropriate speed and time for species of interest for 1 min (for example 5,000 × *g* for *C. crescentus*, 16,000 × *g* for *V. cholerae*) and resuspend in 100 μL of buffer lacking free thiols

   a. Critical step (only necessary for rich, complex media) – if there is excess free thiols in the culture medium, binding of the maleimide dye

to the cysteines in the pili will be competed by its binding to the free thiols in the medium

2. Pipette 0.5 μL of 5 mg/ml AF488-mal dye stock into tube containing cells for a final concentration of 25 μg/mL dye
3. Incubate at room temp for 5 – 30 min
    a. Critical step – some pili may need longer labeling times for best results, optimize by testing various labeling times before imaging
4. Centrifuge cells and discard supernatant
5. Add 100 μL of fresh buffer or medium and centrifuge cells again
    a. Critical step - failure to wash cells may result in high background fluorescence that will make imaging surface structures difficult
6. Discard supernatant and resuspend cells in appropriate volume for imaging
    a. Critical step – resuspension volume will vary depending on starting concentration of cells; ideally 1000 cells per field of view is optimal
7. Image using wide-field fluorescence microscopy as specified below

Labeling with maleimide compounds for disruption of dynamic pilus activity (7–70 min)

1. One-step blocking with PEG5000-mal
    a. Pipette 100 μL of culture at appropriate growth phase into 1.5 ml Eppendorf centrifuge tube and centrifuge cells at maximum speed for species of interest for 1 min (for example 5,000 × *g* for *C. crescentus*, 16,000 × *g* for *V. cholerae*) and resuspend in 100 μL of buffer lacking free thiols such as PBS or Instant Ocean
        i. Critical step (only necessary for rich, complex media such as LB medium) – if there are excess free thiols in the culture medium, binding of the maleimide dye to the cysteines in the pili will be competed by its binding to the free thiols in the medium
    b. Pipette 5 μL of 100 mM PEG5000-mal stock into tube containing cells for a final concentration of 500 μM PEG5000-mal. Quickly pipette 0.5 μL of 5 mg/ml AF488-mal dye stock into tube
        i. Critical step – PEG5000-mal must be added prior to AF488-mal dye because adding dye first will reduce the number of cysteines available for binding of the PEG5000-mal, possibly resulting in incomplete blocking
    c. Incubate for 5 – 30 min

i. Critical step – some pili may need longer labeling times for best results, optimize by testing various labeling times before imaging

ii. Critical step – some species may have more active pili if incubated at different temperatures. For best results, optimize be testing labeling at different temperatures such as room temperature or 37 °C.

d. Centrifuge cells and discard supernatant

e. Add 100 μL of fresh buffer or medium and centrifuge cells again

i. Critical step - failure to wash cells may result in high background fluorescence that will make imaging surface structures difficult

f. Discard supernatant and resuspend cells in appropriate volume for imaging

i. Critical step – resuspension volume will vary depending on starting concentration of cells; ideally 1000 cells per field of view is optimal

g. Image using wide-field fluorescence microscopy as specified below

2. Two-step blocking with biotin-mal/streptavidin derivative

a. Pipette 100 μL of culture at appropriate growth phase into 1.5 ml Eppendorf centrifuge tube and centrifuge cells at maximum speed for species of interest for 1 min (for example 5,000 × *g* for *C. crescentus*, 16,000 × *g* for *V. cholerae*) and resuspend in 100 μL of buffer lacking free thiols

i. Critical step (only necessary for rich, complex media) – if there are excess free thiols in the culture medium, binding of the maleimide dye to the cysteines in the pili will be competed by its binding to the free thiols in the medium

b. Pipette 0.5 μL of 5 mg/mL biotin-mal stock into tube containing cells for a final concentration of 25 μg/mL. Quickly pipette 0.5 μL of 5 mg/ml AF488-mal dye stock into tube

i. Critical step – Biotin-mal must be added prior to AF488-mal dye because adding dye first will reduce the number of cysteines available for binding of the biotin-mal, possibly resulting in incomplete blocking

c. Incubate at room temp for 5 – 30 min

i. Critical step – some pili may need longer labeling times for best results, optimize by testing various labeling times before imaging

d. Centrifuge cells and discard supernatant

e. Pipette 100 μL of fresh buffer or medium and centrifuge cells again

- **i.** Critical step - failure to wash cells may result in high background fluorescence that will make imaging surface structures difficult
- **ii.** Critical step – failure to wash cells may result in NeutrAvidin binding to excess biotin-mal in the media resulting in incomplete blocking of dynamic pilus activity

f. Discard supernatant and resuspend cells in appropriate volume for imaging

- **i.** Critical step – resuspension volume will vary depending on starting concentration of cells; ideally 1000 cells per field of view is optimal

g. Pipette appropriate amount of NeutrAvidin stock to achieve 1.32 mg/mL final concentration in cells and incubate cells for additional 30 min at room temp

- **i.** Critical step – failure to add sufficient NeutrAvidin will result in incomplete blocking of dynamic pilus activity
- **ii.** Critical step – failure to incubate with NeutrAvidin for sufficient length of time will result in incomplete blocking of dynamic pilus activity
- **iii.** Note – 30 min incubation may not be necessary to achieve complete blocking of every system; optimize by testing different incubation times

h. Image using wide-field fluorescence microscopy as specified below

Microscopy setup and imaging

1. Wide-field fluorescence microscopy can be used to image pili labeled with maleimide dyes; for imaging cells labeled with AF488-mal, use a fluorescein isothiocyante (FITC) filter set; for DyL405-mal, use a 4′,6-diamidino-2-phenylindole (DAPI) filter set; for AF594-mal, use a mCherry filter set

- **i.** Critical step – imaging with a low numerical aperture (NA) objective (such as non-oil objectives) may result in fluorescence signal that is too low for visualizing pili. Example microscopy set up that works well for imaging pilus fibers includes Plan Apo 60X oil objective with an EM CCD camera
- **ii.** Troubleshooting – see Table 2 for trouble shooting issues with pilus imaging

2. Take 1 μl of labeled, washed cells, and spot onto pads prepared for imaging

i. Critical step – labeled pili will be difficult to see if cells are not confined under a pad, as the pad confines the cells and pili in the field of focus necessary for visualization

3. Cover with 22 × 22 cm glass coverslip and image

   a. For time-lapse imaging, first focus on the bacterial cells, and try 10 s intervals for imaging pili for at least one minute; this interval should allow for visualization of dynamic fibers if present

      i. Note – Different microscopes will have different light intensities and may require optimization of exposure times

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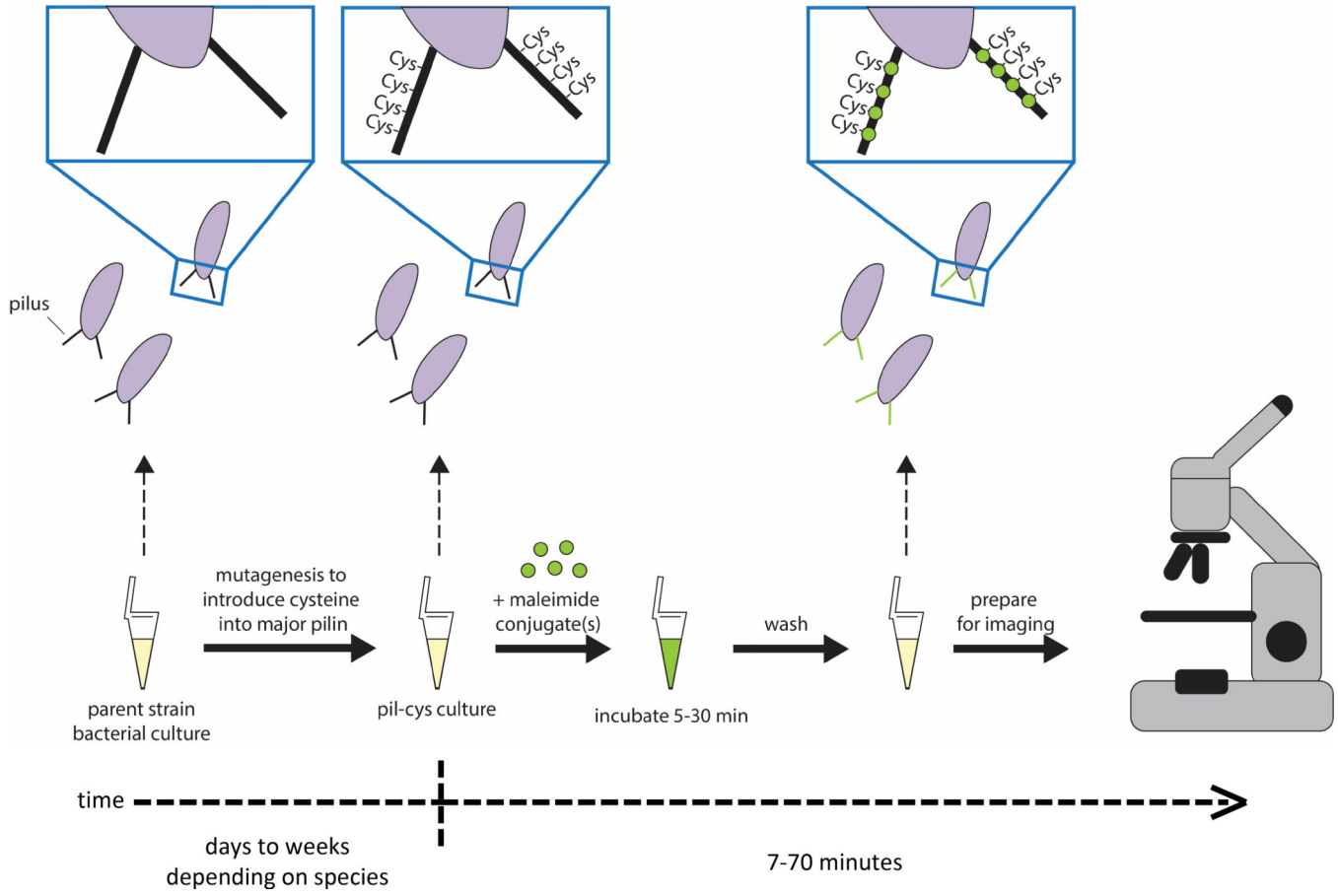

Figure 1.

Pili can be visualized by maleimide-reactive fluorescent dyes. A culture of a PilA-cysteine knock-in mutant (pil-cys) is grown to the proper growth phase and incubated with maleimide dye at room temperature. Excess, unbound maleimide dye is washed away, and the cells are imaged by epifluorescence microscopy. Pili with cysteine substitutions accessible to labeling will fluoresce. Green balls represent maleimide dye. Cys represents cysteine knock-ins in the pilins that comprise the pilus fiber.

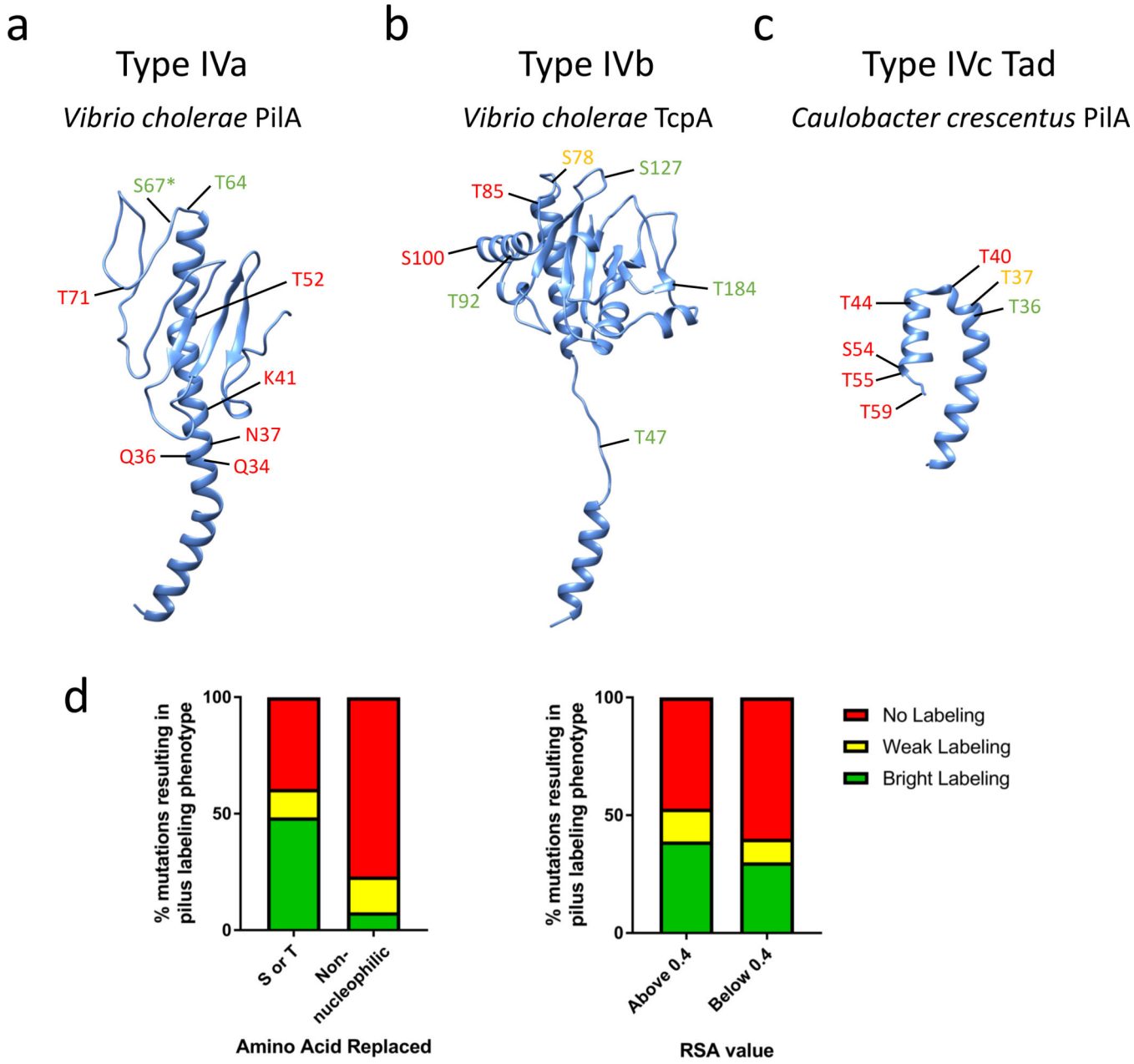

Figure 2.

Accessible residues are unique to each pilin gene. Structures were generated from individual, processed pilin sequences using Phyre2 software[26]. (a) Type IVa: type IV competence pilus of *V. cholerae*, (b) type IVb: TCP pilus of *V. cholerae*, and (c) type IVc: tad pilus of *C. crescentus*. Color of residues indicates whether cysteine replacement resulted in pili that could be labeled with AF488-mal dye: red – not labeled, yellow – very few filaments or weak labeling, green – labeled. Figures were generated using Chimera software[27]. Asterisk at S67 position is corrected annotation of the same position published previously as S81[4], renamed upon correction of misannotated translation start site. (d) Plots showing the % of mutations that resulted in bright, weak, or no pilus labeling based on the

amino acid mutated (left) or the relative surface accessibility (RSA) value (right). Data is based on values reported in Table 1.

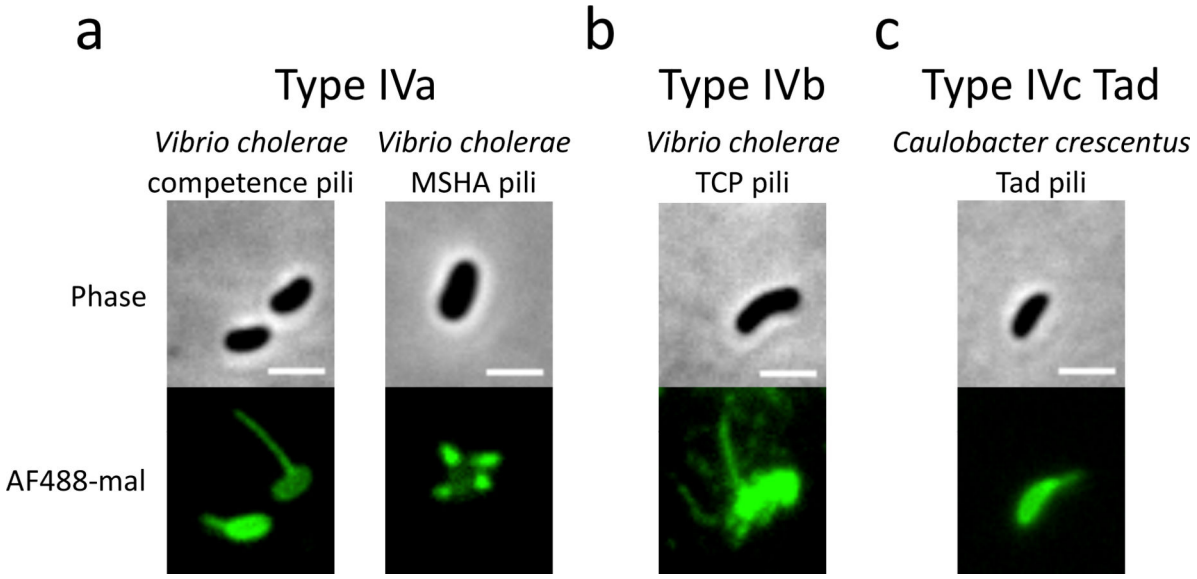

Figure 3.

Cysteine substitution and fluorescent maleimide labeling allows visualization of different classes of pili. (a) *V. cholerae* strain containing competence pilin PilAS67C (left) and *V. cholerae* strain containing MSHA pilin MshAS156C (right) labeled with AF488-mal. (b) *V. cholerae* strain containing TCP pilin TcpAT184C labeled with AF488-mal. (c) *C. crescentus* strain containing Tad pilin PilAT36C labeled with AF488-mal. Scale bar, 2 μm.

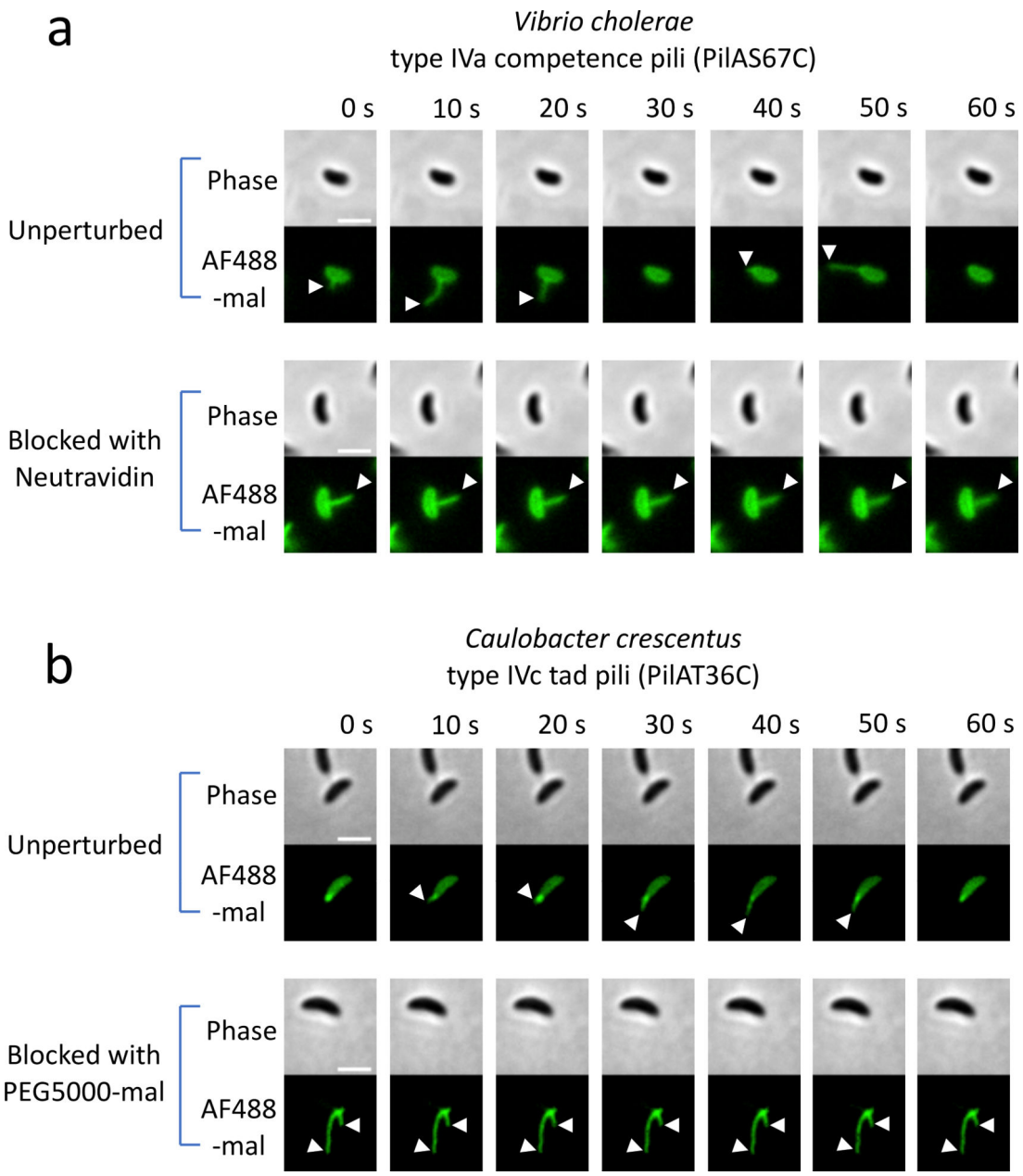

Figure 4.

Pilus retraction can be blocked using different maleimide conjugates. (a) Time-lapse images of *V. cholerae* strain containing competence pilin PilAS67C that has been labeled with a 1:1 ratio of AF488-mal:biotin-PEG11-maleimide followed by blocking with neutravidin (b) Time-lapse images of *C. crescentus* strain containing Tad pilin PilAT36C labeled with AF488-mal and +/− PEG5000-mal to block retraction. Scale bar, 2 μm. White arrows indicate tips of extended pili.

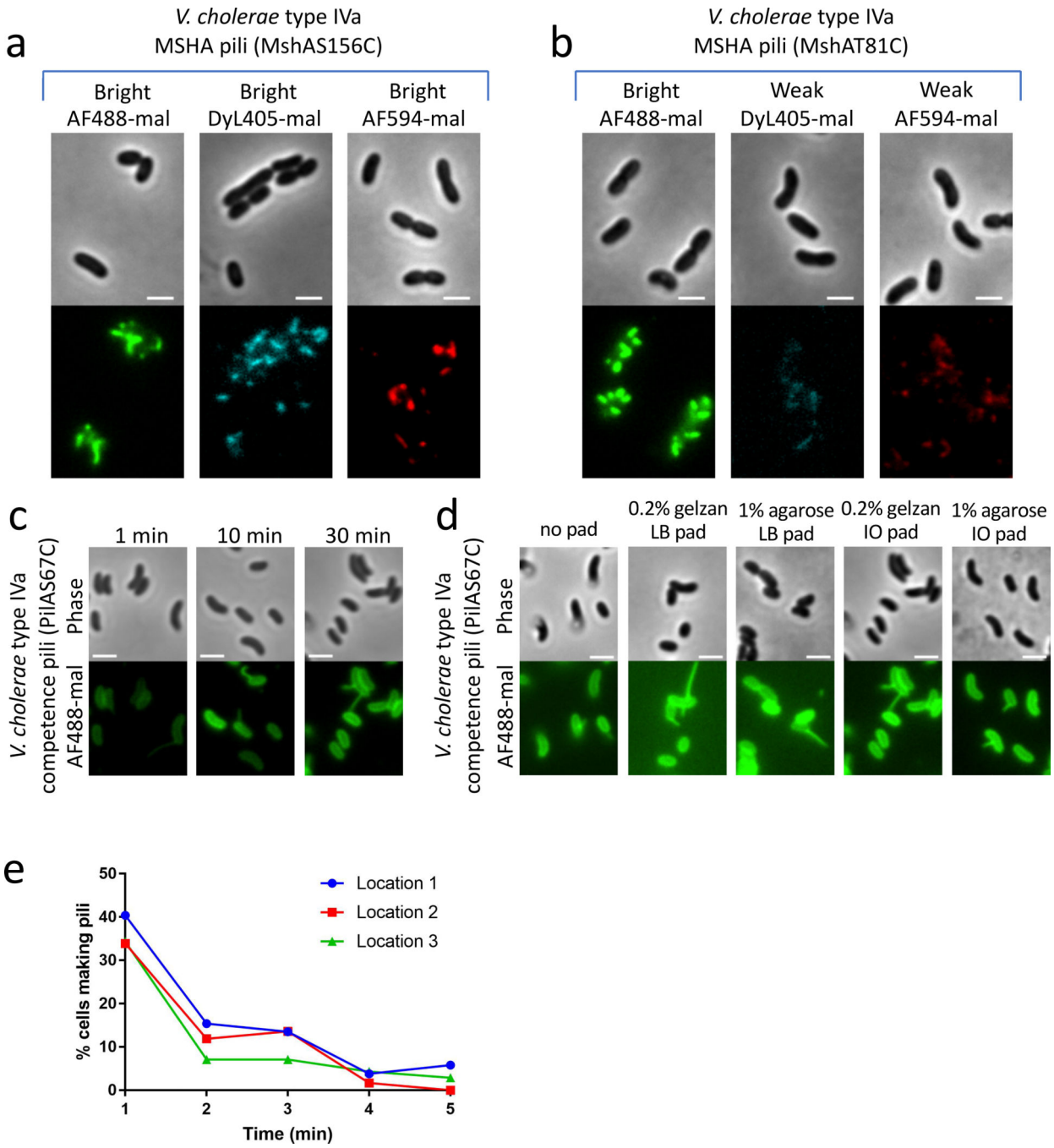

Figure 5.

Observation of pili is affected by multiple variables. (a) Bright labeling with all three maleimide dyes tested for *V. cholerae* strain containing MshAS156C. (b) Bright labeling with AF488-mal and weak labeling with Dylight405-mal and AF594-mal dyes for *V. cholerae* strain containing MshAT81C. (c) Labeling of *V. cholerae* strain containing PilAS67C with AF488-mal dye for 1 min, 10 min, or 30 min before washing. (d) Labeling of *V. cholerae* strain containing PilAS67C with AF488-mal dye for 30 min before washing and imaging under different types of pads. Pili are out of focus when no pad is present, and

both LB and gelzan separately and together have higher background fluorescence than a pad made with instant ocean (IO) buffer and agarose. (e) A *V. cholerae* strain containing PilAS67C was labeled with AF488-mal and then subjected to time-lapse fluorescence microscopy (10 s intervals). Data indicate the percent of cells that make a pilus binned by minute (i.e. within the 1st, 2nd, 3rd, 4th, or 5th minute of the time-lapse). Because the three separate fields of view were imaged on the same pad sequentially (i.e. the time-lapse for location 2 was started after completing the 5 min time-lapse at location 1) and all locations exhibited the same decrease in pilus activity, we attributed this reduction in activity to phototoxicity and not an artifact of pad drying, etc. Number of total cells quantified for locations 1, 2, and 3 are n = 52, 59, and 70 respectively.

Table 1.

Labeling efficiency and functionality of cysteine knock in mutants with different maleimide dyes.

Pilus class	Species and pilus name	Position mutated	Relative surface accessibility (RSA) score	Labeling efficiency with DyL405-mal	Labeling efficiency with AF488-mal	Labeling efficiency with AF594-mal	Functionality
Type Iva	*V. cholerae* MSHA	None					ND
		P31	0.174	None	None	None	ND
		N35	0.519	None	Weak	None	ND
		G38	0.535	None	None	None	ND
		D39	0.449	None	None	None	ND
		G47	0.425	None	None	None	ND
		G50	0.422	None	None	None	ND
		K66	0.507	None	None	None	ND
		T70	0.422	None	Bright	Weak	ND
		T74	0.416	None	Bright	Weak	ND
		T77	0.427	Weak	Bright	Bright	ND
		T81	0.287	Weak	Bright	Weak	ND
		T84	0.443	None	None	None	ND
		T97	0.284	None	None	None	ND
		S102	0.369	Bright	Bright	Bright	ND
		T112	0.342	None	None	None	ND
		S116	0.557	None	Weak	None	ND
		S117	0.415	Weak	Bright	Weak	ND
		S124	0.450	None	None	None	ND
		S125	0.489	Weak	Bright	Bright	ND
		T126	0.534	Bright	Bright	Bright	ND
		G143	0.546	None	Weak	None	ND
		T155	0.461	Bright	Bright	Bright	ND
		S156	0.592	Bright	Bright	Bright	ND
		T171	0.518	Weak	Bright	Bright	ND
Type Iva	*V. cholerae* competence pilus[†]	None					+++
		Q34	0.253	None	None	None	-
		Q36	0.311	None	None	None	-
		N37	0.583	None	None	None	-
		K41	0.517	None	None	None	-
		T52	0.425	None	None	None	+
		T64	0.476	Weak	Bright	Weak	++
		S67[*]	0.453	Bright	Bright	Bright	+++

Pilus class	Species and pilus name	Position mutated	Relative surface accessibility (RSA) score	Labeling efficiency with DyL405-mal	Labeling efficiency with AF488-mal	Labeling efficiency with AF594-mal	Functionality
		T71	0.35	None	None	None	+
Type IVb	*V. cholerae* TCP[‡]	None					+++
		T47	0.093	Weak	Bright	Weak	++
		S78	0.616	Weak	Weak	Weak	-
		T85	0.436	None	None	None	-
		T92	0.441	Bright	Bright	Bright	+
		S100	0.507	None	None	None	-
		S127	0.604	Bright	Bright	Bright	-
		T184	0.578	Bright	Bright	Bright	+
Type IVc	*C. crescentus* Tad[◊]	None					++
		T36	0.407	Bright	Bright	Bright	++
		T37	0.377	Weak	Weak	Weak	+
		T40	0.58	None	None	None	-
		T44	0.578	None	None	None	-
		S54	0.61	None	None	None	-
		T55	0.643	None	None	None	-
		T59	0.868	None	None	None	-

ND – not determined

[*] Previously published as S81; correctly annotated position is S67

[†] Functionality assessed by transformation frequency assays

[‡] Functionality assessed by aggregation assays

[◊] Functionality assessed by phage sensitivity assays using pilus-dependent phage ϕCbK

Table 2.

Troubleshooting Table

Step	Problem	Possible Reason	Solution
11	No cell body fluorescence or labeled fibers	Pilin cysteine residue is not exposed and is therefore not labeled	Try longer labeling cells for longer time before imaging; if still no cell body fluorescence then likely not a labelable position
		Maleimide dye solution has deteriorated	Make new stock of maleimide dye or obtain fresh aliquot from freezer
		Excess free thiols in labeling buffer competed with pilin cysteines for labeling	Resuspend cells in thiol-free buffer prior to labeling
		Growth conditions are not optimized for pilus expression/ activity	Perform optimization to assess the growth phase and conditions at which pilus-dependent activity is maximal
11	There is cell body fluorescence but no labeled fibers	Not enough signal from fibers	Increase exposure time or light intensity settings on microscope
			Increase labeling time prior to imaging
		Other surface-exposed cysteines may be present and labeled by dye	Test parent strain lacking cysteine mutation for cell body fluorescence to compare to mutants
		Inappropriate time interval for time-lapse microscopy	Vary interval time, trying both shorter intervals of 1–3 s and longer intervals of 30 s
		Cell body signal occludes pili signal	Use TIRF microscopy
		If still no fibers, cysteine position may result in labelable pilins but may prevent pilin-pilin interactions resulting in all pilins remaining in membrane	Make an outer membrane secretin mutant to determine if maleimide dyes can pass through the outer membrane to label pilins in the absence of fiber formation Note that this may not always work, but is true in at least some systems. For example, deletion of the gene encoding the outer membrane pilus secretin CpaC in *C. crescentus* results in no cell body fluorescence while deletion of the gene encoding the outer membrane secretin PilQ in *V. cholerae* still allows for cell body fluorescence
11	Labeled fibers but very weak labeling efficiency	Not enough signal	Increase exposure time or light intensity settings on the microscope
			Increase labeling time prior to imaging
			Reduce background fluorescence by using a different growth medium/buffer to make pads
		Multiple pilins make up pilus fiber, resulting in a lower ratio of labeled pilins in the fiber	Increase labeling time
			Check genome for other pilins and make mutation to disrupt expression of other pilins if present
			Make a cysteine knock-in mutation in other pilins and combine mutations in same genetic background
12	Labeled fibers but no dynamic activity	Inappropriate time interval for time-lapse microscopy	Vary imaging time interval, trying both shorter intervals of 1–3 s and longer intervals of 30 s
		Pili are not dynamic	Test multiple cysteine knock-in position mutants for similar phenotype
			Check for cell body fluorescence: if no cell body fluorescence pili are not dynamic as fluorescently labeled pilins disseminate into the inner membrane after retraction; if cell body

Step	Problem	Possible Reason	Solution
			fluorescence, pili may be dynamic, but you will have to test if the dye permeates the outer membrane by making an outer membrane secretin mutant and looking for cell body fluorescence *Note – if outer membrane secretin mutant exhibits cell body fluorescence, no conclusions can be drawn about dynamic activity; if mutant exhibits no cell body fluorescence, but the original cysteine knock-in strain does, retraction is most likely occurring
		Phototoxicity is preventing visualization of dynamic activity	Decrease exposure time or light intensity settings on microscope
			Increase imaging interval time
		Growth conditions are not optimal for pilus activity	Grow cells under different conditions and in different media and test for increased activity; grow and image cells over a range of different temperatures
12	Blocked cells still exhibit dynamic activity of pilus fibers	Blocking reagent has deteriorated	Prepare fresh blocking reagents and attempt protocol again
		Protocol tested does not work with cysteine knock-in strain	Test alternative blocking protocol provided
		Not all pili are blocked for retraction	Incubate cells longer with blocking reagent
			Increase concentration of blocking reagent
			Add blocking reagent without dye and incubate for a few minutes prior to adding dye
			If none of the above solutions work, test other cysteine knock-in positions for dynamic activity and blocking capability