Published in final edited form as:

Curr Protoc Chem Biol. 2013 December 1; 5(4): 239–250. doi:10.1002/9780470559277.ch130102.

Penicillin-Binding Protein Imaging Probes

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Abstract

Penicillin-binding proteins (PBPs) are membrane-associated proteins involved in the biosynthesis of peptidoglycan (PG), the main component of bacterial cell walls. These proteins were discovered and named for their affinity to bind the β -lactam antibiotic penicillin. The importance of the PBPs has long been appreciated; however, the apparent functional redundancy of the ~5–15 proteins that most bacteria possess makes determination of their individual roles difficult. Existing techniques to study PBPs are not ideal because they do not directly visualize protein activity and can suffer from artifacts. Therefore, development of new methods for studying the roles of distinct PBPs in cell wall synthesis was compulsory. Due to penicillin's covalent mode of inhibition, fluorophore-conjugated analogs can be utilized to visualize PBP activity. Herein, we describe a general protocol to label and detect subsets of active PBPs in live Gram-positive bacteria using fluorescent β -lactams.

Keywords

Penicillin-binding proteins; peptidoglycan; fluorescence imaging; activity-based probes; β -lactams

INTRODUCTION

Most bacterial cells are surrounded by a cell wall mainly composed of a structure called peptidoglycan (PG) that is unique to bacteria and essential for cell survival. Consequently, the biosynthesis of PG and the proteins required for its assembly, penicillin-binding proteins (PBPs), have been the targets for many antibacterial agents (Fisher et al., 2005). β -lactams, such as penicillin, are the most widely used class of antibiotics that inhibit bacterial cell wall construction by forming a covalent bond with a conserved serine residue in the transpeptidase domain of PBPs (Georgopapadakou and Liu, 1980). Because of this covalent mode of inhibition, fluorophore-conjugated β -lactams can be used to visualize PBP activity in living cells.

This article describes a general procedure to label PBPs in live Gram-positive bacteria using active site-directed small-molecule probes. With fluorescent β -lactam analogs, the localization of active PBPs can be observed by fluorescence microscopy. BOCILLIN-FL (Boc-FL), a commercially available fluorescent penicillin derivative, was designed to simultaneously detect all PBPs in a cell (Zhao et al., 1999)(Fig. 1A, Boc-FL). It has been successfully used for fluorescence microscopy and in-gel fluorescence detection of PBPs. Cephalosporins, another subclass of β -lactam antibiotics, are selective for high-molecular weight (HMW) PBPs. In particular, cephalosporin C specifically inhibits only certain HMW PBPs in *Bacillus subtilis* (Kleppe and Strominger, 1979). To selectively target a small

subgroup of PBPs in a cell, cephalosporin C-based probes were generated for *in vivo* PBP visualization (Fig. 1A, cephalosporin C-TAMRA (Ceph C-T)). As predicted, Ceph C-T exhibits selectivity towards a subset of PBPs in both *B. subtilis* PY79 and *S. pneumoniae* IU1945. Imaging of dual-labeled cells first saturated with Ceph C-T and then labeled with Boc-FL revealed that the subsets of PBPs are not co-localized, even within the same region of a cell. Contrary to previous hypotheses that PBPs are intermixed, the heterogeneous labeling pattern around the division site indicated that there are areas of non-uniform PBP activity (Kocaoglu et al., 2012). As shown, these new imaging tools have the potential to facilitate the construction of a better understanding of the functions of PBPs in PG biosynthesis.

STRATEGIC PLANNING

In Vivo PBP Labeling with Antibiotic-Based Probes

In Basic Protocol 1 and 2, *in vivo* labeling of PBPs with fluorescent β -lactam probes is demonstrated. After labeling of active PBPs with the probes, the cells were washed to remove unbound probe. Subsequently, live cells were imaged using fluorescence microscopy, which reveals PBP localization patterns. Alternatively, cells were lysed, and the membrane proteome was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to visualize the labeled proteins by in-gel fluorescence detection (Fig. 1B). Complementing the live-cell imaging, the gel-based studies provide information about which proteins are being covalently modified and with what relative intensity. The selectivity of antibiotic probes can be modulated by dose; therefore, it is recommended that a titration experiment is performed with the candidate probe on the organism of interest. In previous work, probe concentrations and incubation times were optimized for Boc-FL and Ceph C-T labeling of PBPs of *Bacillus subtilis* PY79 and *Streptococcus pneumoniae* IU1945. However, for other antibiotic-based probes and organisms, these conditions may need to be adjusted. Since these probes are based on antibiotics, using high probe concentrations or long incubation times is discouraged because it could kill the cells. Biotinylated versions of the antibiotic-based probes can also be used to detect functional PBPs. Using streptavidin-peroxidase, labeled proteins can be analyzed by immunoblotting or chemiluminescence detection (Dargis and Malouin, 1994). Alternatively, using streptavidin-functionalized agarose beads, PBPs can be enriched from whole proteomes and identified by mass spectrometry.

Additional Considerations and Controls

Extended exposure to light will decrease the intensity of the fluorescence signal. As a result, it is important to perform the labeling reactions with fluorophore-conjugated molecules in the dark and to minimize light exposure during sample preparation for gel-based analysis. Additionally, the derivatization of an antibiotic with a fluorophore could alter its original selectivity profile. Consequently, it is recommended that competition experiments with underivatized antibiotics are performed to confirm that the generated probes bind to the same PBP targets as the original scaffold. This experiment can be accomplished by pretreating the cells with the antibiotic followed by washing and then incubating the cells with the fluorophore-tagged probe. If both molecules target the same PBPs, fluorescent signal should not be observed.

BASIC PROTOCOL 1

In-gel detection of *in vivo*-labeled PBPs with β -lactam probes: The following is a standard protocol for *in vivo* labeling of PBPs in Gram-positive bacteria using β -lactam-based probes.

Materials: *B. subtilis* PY79 cells grown in Luria-Bertani (LB) broth at 37 °C with shaking at 150 rpm to OD₆₀₀ 0.4–0.5.

S. pneumoniae IU1945 cells grown statically in Becton-Dickinson brain heart infusion (BHI) broth at 37 °C in an atmosphere of 5% CO₂ to OD₆₂₀ 0.15–0.20.

2 mg/mL Ceph C-T probe stock in DMSO (store at –80 °C, protected from light, stable for months)

1 mg/mL Boc-FL probe stock in DMSO (Invitrogen cat. no. B-13233; store at –80 °C, protected from light, stable for months)

Phosphate-buffered saline (PBS, 1 ×, pH 7.4; see recipe)

1.5-mL microcentrifuge tubes

Eppendorf centrifuge 5424

10 mg/mL lysozyme (Sigma, cat. no. L4919), prepared in Tris buffer (see recipe)

37 °C incubator

CO₂ incubator shaker (set to 37 °C and 5% CO₂)

Branson Sonifier 250 with 1/8" tapered microtip

NanoDrop 1000 Spectrophotometer (Thermo Scientific)

4 × SDS-PAGE loading buffer (see recipe)

Analog heat block (VWR)

XCell *SureLock* Mini-Cell gel electrophoresis system (Invitrogen, cat. no. EI0001)

Disposable plastic gel cassettes, 1.5 mm (Invitrogen, cat. no. NC2015)

1.5 mm 15 well plastic combs (Invitrogen, cat. no. NC3515)

10% resolving and 4.5% stacking SDS-PAGE gel (see recipe)

Fluorescent molecular weight standards (see recipe)

Tris-glycine SDS running buffer, 10 × (Invitrogen, cat. no. LC2675-5)

Typhoon 9210 gel scanner (Amersham Biosciences)

ImageJ software (NIH) for gel data analysis

1. Dispense 1 mL of cell culture from each sample type into a 1.5-mL microcentrifuge tube, and centrifuge at 16,100 × g for 1–2 min at room temperature. Discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4, by gently pipetting up and down 2–3 times and then pellet the cells using the same centrifugation conditions, again discarding the supernatant.
2. Suspend the cell pellets in 50 µL of PBS containing 10 µg/mL of Ceph C-T or 5 µg/mL of Boc-FL. Incubate at room temperature for 30 min or 10 min, respectively.

To make 10 µg/mL of Ceph C-T or 5 µg/mL of Boc-FL, add 0.5 µL of 2 mg/mL Ceph C-T or 1 mg/mL of Boc-FL stock solution to 99.5 µL PBS, respectively. The final concentration of DMSO is 0.5% in PBS. No difference in the results was observed with up to 5% final DMSO concentration.

3. Centrifuge the cell suspension using the same conditions and discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4, by gently pipetting up and down 2–3 times, centrifuge and discard the supernatant.
4. Suspend the cells in 100 µL PBS, and add 10 µL of 10 mg/mL lysozyme. Incubate the cells for 30 min at 37 °C.
5. Lyse the cells using a Branson Sonifier 250 with 1/8" tapered microtip (power setting 3, 30% duty cycle for 5 × 6 s intervals) on ice.
6. Isolate the membrane proteome by centrifugation at 21,000 × g for 15 min at 4 °C. Remove the supernatant. Resuspend the membrane in 100 µL PBS and homogenize the sample by sonication (power setting 1, 10% duty cycle for one second).
7. Determine the protein concentration by NanoDrop 1000 Spectrophotometer (Protein A₂₈₀ mode). Adjust the protein concentration to 2.5 mg/mL by diluting with PBS.
8. Dispense 51 µL of proteome sample into a clean 1.5-mL microcentrifuge tube, and add 17 µL of 4 × SDS-PAGE loading buffer. Using an analog heat block, boil the sample for 5 min at 90 °C to denature the proteins. Cool the sample to room temperature.
9. Prepare an SDS-polyacrylamide gel (see recipe).

The SDS-polyacrylamide gel is composed of a 10% resolving gel and a 4.5% stacking gel. The gel should be prepared ahead of time because polymerization of acrylamide takes a few hours.
10. Load 10 µL of sample onto the gel along with fluorescent molecular weight standards. Separate the protein bands by gel electrophoresis.

Dilute 10 × tris-glycine SDS running buffer to 1 × with Milli-Q purified water for gel electrophoresis. Perform the gel electrophoresis for 1 hr at 180 V, 400 mA, 60 W.
11. Rinse the gel with distilled water three times, and then scan the gel using a fluorescence gel scanner.

Use 580-nm bandpass filter for Ceph C-T and 526-nm short-pass filter for Boc-FL. The setting for the scan resolution should be 50 microns, and the voltage for photomultiplier tube (PMT) should be 525.
12. Analyze the gel image using ImageJ software (Figure 2). Subtract the background signal and adjust the brightness and contrast on the image in order to optimize the signal-to-noise ratio.

BASIC PROTOCOL 2

Fluorescence imaging of probe-labeled proteins: The following is a standard protocol for fluorescence microscopy imaging of PBPs that are labeled with β-lactam-based probes.

Materials: *B. subtilis* PY79 cells grown in Luria-Bertani (LB) broth at 37 °C with shaking at 150 rpm to OD₆₀₀ 0.4–0.5.

S. pneumoniae IU1945 cells grown statically in Becton-Dickinson brain heart infusion (BHI) broth at 37 °C in an atmosphere of 5% CO₂ to OD₆₂₀ 0.15–0.20.

2 mg/mL Ceph C-T probe stock in DMSO (store at –80 °C, protected from light, stable for months)

1 mg/mL Boc-FL probe stock in DMSO (store at –80 °C, protected from light, stable for months)

100 µg/mL 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) in Milli-Q water (Invitrogen, cat. no. D1306; store at –80 °C, protected from light, stable for months)

Phosphate buffered saline (PBS, 1 ×, pH 7.4; see recipe)

1.5-mL microcentrifuge tubes

Eppendorf centrifuge 5424

Glass microscope slides: 25 × 75 mm, 1.0 mm thick (VWR, cat. no. 48300-025)

Micro cover glass: 22 × 22 mm, 1.5 (VWR, cat. no. 48366-227)

Poly-L-lysine, 0.1% w/v in water (Sigma-Aldrich, cat. no. P8920)

OMX 3D-Structured Illumination Microscopy (SIM) super-resolution microscope (Applied Precision)

Immersion oil with a 1.512 refractive index

Imaging software: SoftWoRx (Applied Precision) and Imaris 3D (Bitplane) for OMX 3D-SIM.

1. Dispense 1 mL of cell culture into a 1.5-mL microcentrifuge tube, and centrifuge at 16,100 × g for 1–2 min at room temperature. Discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4, by gently pipetting up and down 2–3 times and then pellet the cells using the same centrifugation conditions, again discarding the supernatant.
2. Suspend the cell pellets in 50 µL of PBS containing 10 µg/mL of Ceph C-T or 5 µg/mL of Boc-FL. Incubate the cells at room temperature for 30 min or 10 min, respectively.
3. Centrifuge the cell suspension using the same conditions and discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4 by gently pipetting up and down 2–3 times, centrifuge and discard the supernatant.
4. Suspend the cell pellets in 50 µL of PBS. Add 1 µL of 100 µg/mL DAPI, and incubate the cells for 3–4 min at room temperature.
5. Centrifuge the cell suspension using the same conditions and discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4 by gently pipetting up and down 2–3 times, centrifuge and discard the supernatant.
6. Suspend the cell pellets in 20–50 µL of PBS.
7. Spot 5 µL of cell suspension onto a clean glass micro slide. Cover it with a micro cover glass treated with poly-L-lysine.

Soak the micro cover glass in the poly-L-lysine solution for a few minutes before use and then dry it with a delicate task cleaning wipe.

8. Image the cells using super-resolution microscopy (OMX 3D-SIM) or any other fluorescence microscope.

Image acquisition should be performed using the following: a 405-nm laser for DAPI with 100 ms exposure at 100% laser strength; a 488-nm laser for Boc-FL with 100 ms exposure at 10% strength; and a 561-nm laser for Ceph C-T with 100 ms exposure at 10% strength.

9. Reconstruct the raw data to obtain three-dimensional (3D) image using SoftWoRx and analyze them using Imaris 3D software for the images taken with OMX 3D-SIM (Figure 3).

SUPPORT PROTOCOL 1

Dual labeling with Ceph C-T and Boc-FL: The following is a standard protocol for dual labeling of PBPs in Gram-positive bacteria using Ceph C-T and Boc-FL probes.

1. Dispense 1 mL of cell culture into a 1.5 mL microcentrifuge tube, and centrifuge at $16,100 \times g$ for 1 min at room temperature. Discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4 by gently pipetting up and down 2–3 times and then pellet the cells using the same centrifugation conditions, again discarding the supernatant.
2. Suspend the cell pellets in 50 μ L of PBS containing 10 μ g/mL of Ceph C-T, and incubate for 30 min at room temperature.
3. Centrifuge the cell suspension using the same conditions and discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4 by gently pipetting up and down 2–3 times, centrifuge and discard the supernatant.
4. Suspend the cell pellets in 50 μ L of PBS containing 5 μ g/mL of Boc-FL, and incubate for 10 min at room temperature.
5. Centrifuge the cell suspension using the same conditions and discard the supernatant. Wash the cell pellets once with 1 mL PBS, pH 7.4 by gently pipetting up and down 2–3 times, centrifuge and discard the supernatant.
6. Proceed with Basic Protocol 1, steps 4 through 12, for gel-based assay or Basic Protocol 2, steps 4 through 9, for fluorescence microscopy (Figure 4).

REAGENTS AND SOLUTIONS

Use Milli-Q purified water in all recipes.

Fluorescent molecular weight standards

Precision Plus Protein™ Unstained Standards (Bio-Rad, cat. no. 161-0363) in a Slide-A-Lyzer Dialysis Cassette, 3.5 K MWCO, 0.5 mL (Pierce, cat. no. 66333).

Put the cassette in a beaker filled with $1 \times$ PBS (500 mL) and dialyze it overnight at 4°C.

Repeat the dialysis with fresh $1 \times$ PBS.

Dissolve the fluorophore (TAMRA-SE or Fluorescein-SE) in DMSO to make 50 μ M probe solution.

Remove the protein standard from the dialysis cassette, and put it in a clean 1.5-mL microcentrifuge tube.

Add 50 μ M fluorophore to the dialyzed protein standard and incubate overnight at 4 °C (in the dark).

Equilibrate a PD-10 desalting column (GE Healthcare, cat. no. 17-0851-01) with 25 mL of 50 mM Tris-Cl, pH 7.4.

Transfer fluorophore-tagged protein standard to the PD-10 column. Using 50 mM Tris-Cl, pH 7.4 buffer as the eluent, collect 12 $\times$ 500 μ L fractions.

Load 5 μ L from each fraction onto 10% SDS-PAGE gel to evaluate which fractions contain the protein standards.

Combine three eluted fractions that have the highest band intensities on the gel and add one volume of 2 $\times$ SDS-PAGE loading buffer (1 μ L marker + 65 μ L Milli-Q water + 66 μ L 2 $\times$ SDS-PAGE loading buffer) (see recipe).

Lysozyme stock solution

Dissolve lysozyme to a final concentration of 10 mg/mL in 10 mM Tris-Cl, pH 8.0.

Store up to 1 year at -20 °C.

Phosphate-buffered saline, pH 7.4 (1 $\times$)

137 mM NaCl

2.7 mM KCl

10.1 mM Na₂HPO₄

1.8 mM KH₂PO₄

Dissolve in 1 L water, and adjust to pH 7.4 with HCl or NaOH.

Filter sterilize the buffer using a 0.22 μ m filter (Millipore, cat. no. GVWP04700)

Store up to 1 year at room temperature.

SDS-PAGE gel (10% resolving gel with 4.5% stacking gel)

10% resolving gel

21 mL water

10.5 mL of 1.5 M Tris-HCl buffer, pH 8.8 (Bio-Rad, cat. no.161-0798)

10.5 mL bis-acrylamide 29:1 (40% solution; Fisher Scientific, cat. no. BP14081)

140 μ L of 10% ammonium persulfate (APS)

15 μ L tetramethylethylenediamine (TEMED; Bio-Rad, cat. no. 161-0800)

Add the reagents in the exact order listed. Pour the mixture into the gel cassette right away and seal the gel by adding 250 μ L ethanol. Let the gel polymerize for 1 hr. After the

polymerization is complete, discard the methanol and rinse the top part of the gel with Milli-Q purified water.

4.5% stacking gel

6.375 mL water

2.5 mL of 0.5 M Tris-HCl buffer, pH 6.8 (Bio-Rad, cat. no. 161-0799)

1.125 mL bis-acrylamide 29:1 (40% solution; Fisher, cat. no. BP14081)

30 μ L of 10% APS

10 μ L TEMED

Add the reagents in the exact order listed. Pour the mixture on top of the polymerized resolving gel in the gel cassette, insert the 15-well comb, and let the gel polymerize for 3 hr. The height of the stacking gel should be higher than the height of the comb's well.

Store up to 2 weeks in electrophoresis buffer (with comb still inserted) at 4 °C.

This recipe prepares 4 mini gels.

SDS-PAGE gel loading buffer (2 $\times$) for fluorescent molecular weight standards

125 M Tris-base

20% glycerol

Dissolve in 15 mL water, and adjust to pH 6.75 with HCl or NaOH.

4% sodium dodecyl sulfate (SDS)

5% β -mercaptoethanol

0.2% bromophenol blue

1 protease inhibitor tablet (Roche Complete EDTA free protease inhibitor tablets, cat. no. 04693159001), pre-dissolve in 1 mL water

Bring up the volume to 50 mL with water. Buffer may be aliquoted into smaller quantities.

Store up to 2 years at -20 °C.

SDS-PAGE loading buffer (4 $\times$)

200 mM Tris-Cl, pH 6.8

400 mM dithiothreitol (DTT)

8% (w/v) SDS

0.2% (w/v) bromophenol blue

40% glycerol

Bring up the volume to 25 mL with water. Buffer may be aliquoted into smaller quantities.

Store up to 2 years at -20°C .

COMMENTARY

Background Information

The PG layer is the main structural component of the bacterial cell wall that gives it shape and prevents lysis from intracellular osmotic pressure. PG is composed of alternating β -(1, 4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid glycan chains that are cross-linked by pentapeptides (Vollmer et al., 2008). Polymerization and cross-linking between glycan chains are catalyzed by PBPs (Sauvage et al., 2008). PBPs are serine acyltransferases that react irreversibly with β -lactam antibiotics by making stable acyl-enzyme intermediates (Macheboeuf et al., 2006). Thus, PBPs can be tagged with radiolabeled β -lactams and subsequently separated by SDS-PAGE to be analyzed by fluorography (Spratt and Pardee, 1975). This is a long-standing method for PBP detection; however, it has several inherent limitations. First, radiolabeled β -lactams are hazardous. Therefore, their use and disposal is not ideal for routine or large-scale analyses. Second, it is a time consuming method as it may take several days to obtain sufficient signal with this procedure. Finally, fluorography cannot be used for *in vivo* visualization of PBPs to gain information about their localization in live cells.

Over the past decade, fluorescent labeling has become a very useful tool for studying gene products in cellular environments (Lippincott-Schwartz and Patterson, 2003). Fluorescent proteins such as green fluorescent protein (GFP) have been used to study protein expression and localization in living systems (Scheffers et al., 2004). However, this technique also has limitations as it requires genetic manipulation for each protein of interest, and the relatively large size of fluorescent proteins can potentially affect localization, expression and/or function of the fused proteins (Hao et al., 2011). The disadvantages of radiolabeled penicillin use and fluorescent protein fusions of target proteins can be avoided by using fluorescent β -lactam analogs. Fluorophore-conjugated penicillin derivatives have become available to detect bacterial PBPs and have been successfully used to visualize active proteins *in vivo* (Galleni et al., 1993). For example, Boc-FL was introduced as a fluorescent penicillin analog to label PBPs in an activity-dependent fashion in living cells (Zhao et al., 1999). It has been used for fluorescence microscopy and SDS-PAGE gel-based analyses. Since Boc-FL is a penicillin analog, it labels all of the PBPs present in bacteria. Alternatively, cephalosporin-based probes enable the examination of only a subset of PBPs, as it is well known that high molecular weight (HMW) PBPs have elevated affinity for this class of compounds (Kleppe and Strominger, 1979). Furthermore, fluorophore-conjugated cephalosporin can be used together with fluorescent penicillin (Boc-FL) for dual labeling experiments to visualize two subsets of PBPs in the cell. Biotinylated versions of these probes can also be used for protein enrichment using streptavidin-functionalized matrices to perform protein identification by mass spectrometry (Kocaoglu et al., 2012).

Critical Parameters and Troubleshooting

Choice of fluorophore—Testing of more than one fluorophore for each probe design is recommended to confirm that the candidate fluorophore does not cause non-specific protein binding. Fluorophores with visible color of green and red result in brighter signals when compared to blue.

In-gel detection of the probe labeled proteins—For well-resolved protein bands, gel electrophoresis should be performed for 1 hr at 180 V, 400 mA, 60 W. Fluorescent proteins can be visualized with a fluorescence gel imager, such as the Typhoon 9210 gel scanner (Amersham Biosciences), with a 580-nm bandpass filter for Ceph C-T and 526-nm short-

pass filter for Boc-FL. The setting for the scan resolution should be 50 microns, and the voltage for photomultiplier tube (PMT) should be 525. Analysis of the gel images should be performed using ImageJ or similar image analysis software. The brightness and contrast on the image should be adjusted in order to optimize the signal-to-noise ratio. Additionally, the background signal should be subtracted. For dual-labeled samples, the gel should be scanned for TAMRA first and then, without changing the position of the gel, should be rescanned for Boc-FL. Using ImageJ, the brightness and contrast for each gel image should be adjusted. Then these two images can be overlaid to obtain a gel showing both Ceph C-T and Boc-FL labeled PBPs. In general, any changes made to a gel using imaging software must be made to the entire gel such that all protein bands can be compared relative to one another.

OMX 3D-SIM microscopy and image analysis—For super-resolution microscopy, an OMX 3D-SIM super resolution microscope (Applied Precision) equipped with the E Cascade II 512 EM-CCD camera (Photometrics, Tuscon AZ) and Olympus UNIPLANAPO 100 ×, 1.4 NA objective should be used. Immersion oil with a 1.512 refractive index should be applied to the slides. Image acquisition should be performed using the following: a 405-nm laser for DAPI with 100 ms exposure at 100% laser strength; a 488-nm laser for Boc-FL with 100 ms exposure at 10% strength; and a 561-nm laser for Ceph C-T with 100 ms exposure at 10% strength. Image acquisition and reconstruction can be directed with DV-OMX software and SoftWoRx (Applied Precision), respectively. Afterward, image analysis can be performed using Imaris 3D imaging software (Bitplane). The brightness and contrast for each color channel should be adjusted individually before overlaying the images.

Anticipated Results

Basic Protocol 1 and Support Protocol 1 will generate gel images in which PBPs are labeled with fluorophore-conjugated β -lactam probes. Representative gels can be found in Figure 2, 4A and 4B for both *B. subtilis* PY79 and *S. pneumoniae* IU1945. Anticipated results for Basic Protocol 2 and Support Protocol 1 include generation of 3D images of Gram-positive bacterial cells labeled with fluorescent β -lactams (see Figure 3, 4C and 4D). If live-cell labeling is performed in non-synchronized populations, the localization patterns are expected to vary depending upon the divisional status of the cell.

Time Considerations

With all reagents prepared, the labeling procedure described above can be completed in 1 hr. Subsequent fluorescence microscopy can be done in less than 1 hr, and gel-based analyses can be finished in 2–3 hr.

Acknowledgments

This work was supported by NIH R00GM82983 (E.E.C.), NIH DP2OD008592 (E.E.C.), a Pew Biomedical Scholar Award (E.E.C.), and Indiana University-Bloomington, Department of Chemistry.

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Figure 1. Fluorescence labeling of PBPs with β -lactam probes. **(A)** Structures of Boc-FL and Ceph C-T. **(B)** PBPs are selectively labeled *in vivo* with the probes. Cells are incubated with dye and then washed. They are subsequently visualized by fluorescence microscopy or lysed for SDS-PAGE analysis of membrane proteins, followed by in-gel fluorescence detection.

Figure 2. In-gel fluorescence detection of *B. subtilis* PY79 and *S. pneumoniae* IU1945 with fluorescent β -lactams. **(A)** *B. subtilis* PY79 and **(B)** *S. pneumoniae* IU1945 PBPs are labeled with Boc-FL (green) and Ceph C-T (red) *in vivo*. The tagged proteins are separated using SDS-PAGE to visualize the PBP profiles of these two bacteria. Ceph C-T probe is more selective than Boc-FL, labeling only a small subset of PBPs in these organisms.

Figure 3.

Localization of Boc-FL and Ceph C-T labeled PBPs in living organisms, both *B. subtilis* PY79 and *S. pneumoniae* IU1945 as visualized with 3D-SIM super resolution fluorescence microscopy. (A–B) *B. subtilis* PY79 and (C–D) *S. pneumoniae* IU1945 cells were first incubated with Boc-FL (green) or Ceph C-T (red) and then stained with DAPI to visualize DNA (blue). Left panels: overlay images of DAPI stain and β -lactam dye to show whole cells; right panels: cross-section of septa in a rotated view with only β -lactam staining shown. PBPs that are labeled with the two β -lactam probes are not co-localized in areas of

active PG synthesis (i.e., division septa in both and equator in *S. pneumoniae*). Scale bar, 1 μm .

Figure 4. Imaging of *B. subtilis* PY79 and *S. pneumoniae* IU1945 PBPs after *in vivo* dual labeling with Ceph C-T (red) and Boc-FL (green) as visualized with gel-based and 3D-SIM super resolution fluorescence microscopy methods. Gel-based analysis of (A) *B. subtilis* PY79 and (B) *S. pneumoniae* IU1945 PBPs labeled first with Ceph C-T (30 min) followed by treatment with Boc-FL (10 min). 3D-SIM images of (C) *B. subtilis* PY79 and (D) *S. pneumoniae* IU1945 PBPs after dual labeling with Ceph C-T (red) and Boc-FL (green) *in vivo*. Left panels: overlay images of all three colors to show whole cells; right panels: cross-section of septa in a rotated view. Scale bar, 1 μ m.