Video Article

Production and Visualization of Bacterial Spheroplasts and Protoplasts to Characterize Antimicrobial Peptide Localization

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Abstract

The use of confocal microscopy as a method to assess peptide localization patterns within bacteria is commonly inhibited by the resolution limits of conventional light microscopes. As the resolution for a given microscope cannot be easily enhanced, we present protocols to transform the small rod-shaped gram-negative *Escherichia coli* (*E. coli*) and gram-positive *Bacillus megaterium* (*B. megaterium*) into larger, easily imaged spherical forms called spheroplasts or protoplasts. This transformation allows observers to rapidly and clearly determine whether peptides lodge themselves into the bacterial membrane (*i.e.*, membrane localizing) or cross the membrane to enter the cell (*i.e.*, translocating). With this approach, we also present a systematic method to characterize peptides as membrane localizing or translocating. While this method can be used for a variety of membrane-active peptides and bacterial strains, we demonstrate the utility of this protocol by observing the interaction of Buforin II P11A (BF2 P11A), an antimicrobial peptide (AMP), with *E. coli* spheroplasts and *B. megaterium* protoplasts.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57904/>

Introduction

Antimicrobial peptides (AMPs) have gained attention due to their potential use as alternatives to conventional antibiotics^{1,2,3,4,5}. AMPs kill bacteria by either translocating across the cell membrane and interacting with intracellular components such as nucleic acids or by permeabilizing the membrane causing leakage of cell contents⁶. In addition to their use as antibiotics, translocating AMPs may be adapted for drug delivery applications because they can non-disruptively cross the impermeable cell membrane^{7,8}. We, therefore, seek to understand fundamental AMP mechanisms of action to lay the foundation for their use in drug design.

Confocal microscopy offers a way to assess localization patterns of fluorescently labeled AMPs in bacterial cells providing insights into their mechanism of action^{9,10,11,12,13,14}. By labeling the membrane of the bacteria, one can determine if a fluorescently labeled peptide localizes to the membrane or the intracellular space of a bacterial cell. However, this technique is limited by the small size and rod shape of bacteria, which can make imaging challenging due to the resolution limits of conventional light microscopes and the variable orientation of the bacteria on the slide¹⁵.

The goal of the presented method is to enable enhanced visualization of the fluorescently labeled peptide localization patterns using confocal microscopy. Visualization is enhanced by turning the small, thin, rod-shaped gram-negative *Escherichia coli* (*E. coli*) and gram-positive *Bacillus megaterium* (*B. megaterium*) bacteria into enlarged, spherical forms referred to as spheroplasts (for gram-negative strains) and protoplasts (for gram-positive strains)^{16,17,18,19,20,21}. Spheroplasts and protoplasts are easier to image because of both their increased size and their symmetric shape, which makes the orientation of a bacterium on a slide irrelevant for its imaging. In addition, we present a systematic approach to quantitatively analyze confocal microscopy data in order to characterize AMPs as either membrane localizing or translocating. Applying these methods makes it easier to distinguish fluorescently labeled peptide localization patterns. The protocols presented here can be used to assess the localization of a variety of membrane-active agents other than AMPs, including cell-penetrating peptides.

One distinct advantage of this technique is that it provides insights into the mechanism of action of AMPs on a single cell level, which may reveal cell-to-cell heterogeneity¹⁵, as opposed to other fluorescence assays commonly used to identify the mechanisms of action of AMPs, which only provide bulk estimates^{9,22,23,24,25}. The use of spheroplasts and protoplasts in order to assess AMP cell entry is particularly useful²⁶ because they are more physiologically relevant¹⁵ than other models used for assessing cell entry, such as lipid vesicles²⁴.

Protocol

1. Solution Preparation

NOTE: Prepare solutions described in steps 1.1–1.9 and 1.8–1.11 in order to produce *E. coli* spheroplasts and *B. megaterium* protoplasts, respectively.

1. Prepare 1 M Tris-Cl, pH 7.8 by dissolving 10.34 g Tris HCl and 4.17 g of Tris OH in 50 mL of dH₂O in a 125 mL flask. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane and store in a conical tube at room temperature.
2. Prepare solution A (20 mM MgCl₂, 0.7 M sucrose, 10 mM Tris-Cl, pH 7.8) by dissolving 0.10 g MgCl₂ (95.2 g/mol) and 11.98 g sucrose (342.3 g/mol) in 25 mL dH₂O in a 125 mL flask. Add 500 µL 1 M Tris-Cl (pH 7.8) and adjust the volume to 50 mL. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane and store in a conical tube at room temperature.
3. Prepare solution B (10 mM MgCl₂, 0.8 M sucrose, 10 mM Tris-Cl, pH 7.8) by dissolving 0.05 g MgCl₂ (95.2 g/mol) and 13.69 g sucrose (342.3 g/mol) in 25 mL dH₂O in a 125 mL flask. Add 500 µL 1 M Tris-Cl (pH 7.8) and adjust the volume to 50 mL. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane and store in a conical tube at room temperature.
4. Prepare 0.8 M sucrose by dissolving 13.69 g sucrose (342.3 g/mol) in 50 mL dH₂O in a 125 mL flask. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane and store in a conical tube at room temperature.
5. Prepare 5 mg/mL deoxyribonuclease I (DNase I) by dissolving 0.015 g DNase I in 3 mL of dH₂O in a 50 mL flask. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane. Aliquot solution into microfuge tubes and store at -20 °C.
6. Prepare 0.125 M ethylenediaminetetraacetic acid (EDTA), pH 8.0 by dissolving 0.698 g EDTA disodium dehydrate (372.2 g/mol) into 15 mL dH₂O in a 125 mL flask. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane and store in a conical tube at room temperature.
7. Prepare 600 µg/mL cephalixin by dissolving 0.03 g of cephalixin hydrate (365.404 g/mol) in 50 mL of dH₂O in a 125 mL flask. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane and store in a conical tube at 4 °C.
8. Prepare 5 mg/mL lysozyme by dissolving 0.015 g lysozyme in 3 mL of dH₂O in a 50 mL flask. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane. Aliquot solution into microfuge tubes and store at -20 °C.
9. Prepare 3% w/v Trypticase Soy Broth (TSB) by dissolving 30 g of TSB in 1 L of dH₂O in a 2 L flask. Aliquot the solution into flasks containing 25 mL or 100 mL TSB to be used in the preparation of *E. coli* spheroplasts or *B. megaterium* protoplasts, respectively. Autoclave flasks to sterilize the liquid medium. Sterile TSB can be stored at either room temperature or 4 °C.
10. Prepare solution C (1 M sucrose, 0.04 M maleate, 0.04 M MgCl₂, pH 6.5) by dissolving 34.23 g sucrose (342.3 g/mol), 0.46 g maleic acid (116.07 g/mol), and 0.38 g MgCl₂ (95.21 g/mol) in 100 mL of dH₂O in a 250 mL flask. Adjust pH to 6.5. Sterilize by filtering through a 25 mm syringe filter with a 0.2 µm membrane and store in a conical tube at room temperature.
11. Prepare 200 mL protoplast medium by mixing 100 mL 3% w/v TSB and 100 mL solution C in a 1 L glass bottle. Autoclave to sterilize the solution and store at room temperature.

2. Preparation of Overnight Culture

NOTE: Perform sections 2–4 using appropriate sterile techniques. If desired, a bacterial strain can contain a plasmid for antibiotic resistance to reduce the potential contamination. If using a strain with antibiotic resistance, add the necessary antibiotics in steps 2.1, 3.1–3.2, and 4.1–4.4.

1. Prepare an overnight culture by picking a single colony of bacteria using a sterile pipette tip and placing it into a 14 mL culture tube containing 2–3 mL of 3% w/v TSB. Incubate at 37 °C, while shaking for 16–21 h.

3. Preparation of Gram-negative *E. coli* Spheroplasts

1. In a 250 mL flask, dilute the overnight culture 1:100 in 25 mL volume of 3% w/v TSB and incubate the bacterial solution at 37 °C, while shaking for approximately 2.5 h until the solution has reached an optical density of 0.5–0.8 at 600 nm. Measure the optical density using a spectrophotometer.
2. In a 250 mL flask, dilute this culture 1:10 in 30 mL of 3% w/v TSB and incubate at 37 °C, while shaking for 2.5 h in the presence of 60 µg/mL cephalixin (347.4 g/mol) to produce single cell filaments of about 50–150 µm in length, which are observable under 1,000X magnification using a light microscope (**Figure 1B**).
3. Harvest the filaments by centrifuging the bacterial solution at 1,500 x g, 4 °C for 4 min. Decant and discard the supernatant, reserving the pellet.
4. Wash the filaments by gently adding 1 mL of 0.8 M sucrose, being careful not to disturb the pellet. Incubate for 1 min, and then discard the supernatant without disturbing the pellet.
5. Add 150 µL of 1 M Tris-Cl (pH 7.8), 120 µL of 5 mg/mL lysozyme, 30 µL of 5 mg/mL DNase I, and 120 µL of 0.125 M EDTA in their respective order to the pellet and incubate the solution at room temperature for 10 min.
6. Add 1 mL of solution A gradually over 1 min to the solution prepared in 3.5 using a micropipette while gently swirling the solution by hand. Incubate the solution for 4 min at room temperature.
7. Put 7 mL of 4 °C solution B into two 15 mL conical tubes. Add equal amounts of the solution prepared in 3.6 to each of these two tubes. Centrifuge the solution at 1,500 x g, 4 °C for 4 min.
8. Using a serological pipette, carefully remove all but 1–2 mL of supernatant without disturbing the pellet. Resuspend the pellet by gently pipetting up and down using a P1000 micropipette. Visually check to see spheroplasts formation by observing the sample at 1,000X magnification using a light microscope (**Figure 1C**).
9. Store the spheroplasts at -20 °C for up to a week or until they have gone through 3 freeze-thaw cycles.

4. Preparation of Gram-positive *B. megaterium* Protoplasts

1. In a 250 mL flask, dilute the overnight culture 1:1,000 in 100 mL of 3% w/v TSB and incubate the bacterial solution at 37 °C, while shaking for approximately 4.5 h until the solution has reached an optical density of 0.9-1.0 at 600 nm. Measure the optical density using a spectrophotometer.
2. Pour the liquid culture into two 50 mL conical tubes and centrifuge at 2,000 x g, 4 °C for 10 min.
3. Using a serological pipette, discard the supernatant from both conical tubes. Resuspend each pellet in 2.5 mL protoplast medium and combine the resuspended solutions into a single conical tube. Pipette the combined resuspended solution in a 125 mL flask.
4. Add 1 mL of 5 mg/mL lysozyme and incubate for 1 h at 37 °C, while shaking.
5. Monitor the growth of protoplasts under 1,000x magnification using a light microscope, noting any irregularities, such as bacteria that appear as swollen rods instead of spheres (**Figure 2**). Using a serological pipette, transfer the solution to a 15 mL conical tube and centrifuge at 2,000 x g, 4 °C for 10 min.
6. Decant the supernatant and re-suspend the pellet in 5 mL protoplast media. Store protoplasts at -20 °C for up to a week or until they have gone through 3 freeze-thaw cycles.

5. Preparation of Peptide Solution and Membrane Dye for Imaging

1. **Peptide solution**
 1. In a microfuge tube wrapped in aluminum foil, dissolve 2 mg of FITC labeled BF2 P11A in 800 µL dH₂O. Use a peptide containing at least one tryptophan residue in order to conduct protein concentration measurements.
 2. Spectrophotometrically, measure the absorbance of the peptide solution at 280 nm in triplicate. Calculate the peptide concentration using the molar extinction coefficient for tryptophan (5,700/Mcm).
 3. Dilute the peptide concentration in dH₂O to a final concentration of 100–200 µM. Store in a microfuge tube at -20 °C wrapped in aluminum foil to protect from light.
2. **Membrane dye**
 1. In a microfuge tube, prepare a 10 mM stock of di-8-ANEPPS (592.9 g/mol) by dissolving 5 mg of di-8-ANEPPS in 843.3 µL of DMSO. Store at 4 °C wrapped in aluminum foil to protect from light.
 2. In a microfuge tube, prepare 1 mL of 0.03 mM di-8-ANEPPS by adding 3 µL of 10 mM di-8-ANEPPS to 997 µL DMSO. Store in a microfuge tube at 4 °C wrapped in aluminum foil to protect from light.

6. Visualization of *E. coli* Spheroplasts and *B. megaterium* Protoplasts Using Confocal Microscopy

1. Pipette 5 µL of spheroplasts or protoplasts onto a poly-L-lysine coated glass slide. Add 2 µL of FITC labeled AMP (100-200 µM) to the slide and incubate for 3 min protected from light.
2. Add 1 µL of the membrane dye di-8-ANEPPS (0.03 mM) to the slide and incubate for 3 min protected from light. Cover with a glass coverslip and seal with the nail polish.
3. Turn on the confocal microscope and argon laser. Adjust the argon laser to 20% power output and 20% transmission.
4. Set emission wavelength ranges of 499–532 nm and 670–745 nm for the FITC-labeled peptide emission channel and di-8-ANEPPS-labeled membrane dye emission channel, respectively.
5. Image protoplasts or spheroplasts (**Figure 1** and **Figure 2**) with a 63X objective. Use imaging software to obtain 8-bit, 512 x 512 composite z-stack images composed of 0.5 µm slices of the entirety of the spheroplast or protoplast.
NOTE: Take careful consideration to reduce the emission bleed-through while imaging spheroplasts and protoplasts. See discussion for details.

7. Characterization of AMP Localization

1. Open the composite z-stack image in imaging software. Locate the center-most slice of the spheroplast or protoplast and place one circular Region of Interest (ROI) (0.3 µm in diameter) on the membrane (ROI 1), one at the center of the spheroplast or protoplast (ROI 2), and one away from spheroplast or protoplast to measure the background fluorescence (ROI 3) (**Figure 5**). Avoid the inclusion of saturated pixels. The fluorescence intensity in each ROI can be calculated by imaging software.
NOTE: In instances when peptide does not localize to the entirety of the membrane, draw ROI 1 on an area of the membrane where the peptide is localized.

2. Use the following equation to determine the ratio of intracellular peptide fluorescence intensity to membrane peptide fluorescence intensity:

$$\frac{\text{Intracellular Fluorescence}}{\text{Membrane Fluorescence}} = \frac{\text{ROI 2} - \text{ROI 3}}{\text{ROI 1} - \text{ROI 3}}$$

NOTE: The fluorescence intensity in ROI 3 is subtracted from fluorescence intensities in ROI 2 and ROI 1 in order to account for background fluorescence.

Representative Results

By enlarging bacteria and making them spherical, we can easily distinguish whether peptides localize to the bacterial membrane or readily translocate across the bacterial membrane. The resolution limits of conventional light microscopes make it challenging to distinguish whether peptide signals arise from the membrane or intracellular space in normal bacteria because signals localized to the membrane will appear to overlap with the intracellular space (**Figure 3A**). In contrast, the enlarged size of spheroplasts (2–5 μm) and protoplasts (2–3 μm) compared to normal bacteria, which are typically only 1 μm in diameter, results in clear resolution between the membrane marker and the intracellular space making it easier to distinguish peptide localization (**Figure 3B**).

Here, spheroplasts and protoplasts are used to show the localization pattern of the N-terminal FITC labeled AMP BF2 P11A. We observe FITC labeled BF2 P11A to both localize to the membrane and translocate across the cell membrane of *E. coli* spheroplasts and *B. megaterium* protoplasts (**Figure 4**). While wild type BF2 has been observed to translocate across *E. coli* and lipid vesicle membranes, the P11A mutation is known to reduce this translocation^{13,27,28}. We utilized a non-antibiotic resistant *B. megaterium* and ampicillin resistant Top 10 *E. coli* (pET45B) in data presented. The ampicillin resistant *E. coli* was grown in the presence of ampicillin (349.4 g/mol) at a final concentration in solution of 25 $\mu\text{g/mL}$. For imaging, following the systematic approach outlined in section 7, ROIs were drawn on the membrane, intracellular space, and the background (**Figure 5B**). The ratio of intracellular peptide fluorescence intensity to peptide fluorescence intensity on the membrane was calculated for each spheroplast or protoplast interacting with BF2 P11A using the equation described in section 7.2. **Figure 5A** shows the distribution of this ratio for all spheroplasts and protoplast scored. The spheroplasts and protoplasts scored largely fell into two groups, with the majority of spheroplasts or protoplasts having a ratio of less than 0.3 or greater than 1.

Given the distinct groups that the ratios fall into, we defined peptide localization as translocating when the ratio calculated in section 7.2 was greater than or equal to 1. Conversely, peptide localization was defined as membrane localizing when the ratio described in section 7.2 was less than 1. Following this method of scoring, we observed a similar localization pattern for BF2 P11A in *E. coli* spheroplasts and *B. megaterium* protoplasts with BF2 P11A localizing to the membrane in 71% and 70% of cases for *E. coli* spheroplasts and *B. megaterium* protoplasts, respectively (**Table 1**).

Figure 1: Representative images of *E. coli*. (A) *E. coli* bacteria (B) *E. coli* snake and (C) *E. coli* spheroplast. Images were taken at 100X magnification. [Please click here to view a larger version of this figure.](#)

Figure 2: Representative images of *B. megaterium*. (A) *B. megaterium* bacteria and (B) *B. megaterium* protoplast. Images were taken at 100X magnification. [Please click here to view a larger version of this figure.](#)

Figure 3: Representative images of *B. megaterium*. (A) *B. megaterium* bacterial cell and (B) *B. megaterium* protoplast labeled with the membrane marker di-8-ANEPPS. Images from the middle z-stack are shown at 100X (A) and 63X (B) magnification. [Please click here to view a larger version of this figure.](#)

Figure 4: Representative *E. coli* spheroplasts and *B. megaterium* protoplasts interacting with FITC labeled BF2 P11A. (A) *E. coli* spheroplast showing BF2 P11A localizing to the membrane. (B) *E. coli* spheroplast showing BF2 P11A translocating across the membrane. (C) *B. megaterium* protoplast showing BF2 P11A localizing to the membrane. (D) *B. megaterium* protoplast showing BF2 P11A translocating across the membrane. *E. coli* spheroplasts and *B. megaterium* protoplasts are labeled with the membrane marker di-8-ANEPPS (red) and FITC labeled BF2 P11A (green). Images from the middle z-stack are shown at 63X magnification. [Please click here to view a larger version of this figure.](#)

Figure 5: Analysis of peptide localization patterns. (A) Distribution of ratio of intracellular fluorescence intensity (ROI 2) to membrane fluorescence intensity (ROI 1) after background subtraction ((ROI 2-ROI 3)/(ROI 1-ROI 3)) for *E. coli* spheroplasts and *B. megaterium* protoplasts labeled with BF2 P11A (B) *E. coli* spheroplast interacting with FITC labeled BF2 P11A. Circular Regions of Interest (ROI) 0.3 μ m in diameter drawn on the membrane (ROI 1) intracellular space (ROI 2) and background (ROI 3). The fluorescence intensity of peptide was quantified in each ROI and the background fluorescence was accounted for by subtracting the fluorescence intensity in ROI 3 from the fluorescence intensity in ROI 1 and ROI 2. [Please click here to view a larger version of this figure.](#)

Bacterial strain	No. spheroplast or protoplast	% Membrane localizing	% Translocating
<i>E. coli</i>	84	71	29
<i>B. megaterium</i>	70	70	30

Table 1: The localization pattern of BF2 P11A interacting with *E. coli* spheroplasts and *B. megaterium* protoplasts.

Discussion

The protocols presented here make it feasible for researchers to more rapidly obtain larger sample sizes of bacterial images because the enlarged, spherical bacteria are much easier to locate, orient, and image. This enhanced ability to collect data is valuable in several respects. First, it enables a more systematic quantitative analysis of peptide localization patterns. While qualitative trends can be demonstrated from smaller sets of images, only a large sample set of high-quality images reveal more nuanced trends in localization patterns, such as the percentage of cells where a peptide translocates versus localizes to the membrane. Also, the ability to better resolve the internal fluorescence from the membrane localization makes it easier to perform time course studies to assess how quickly peptides interact with bacterial cells as well as how localization patterns change over time. The improved resolution also allows researchers to consider trends in peptide localization that are not possible with smaller bacteria. For example, in some of our observed spheroplasts and protoplasts, peptides appear to localize to specific sections of the membrane, *i.e.*, the peptide signals do not form a complete, cohesive ring around the bacteria and instead show some punctate labeling. In other cases, peptides may not be distributed entirely evenly inside the bacterial membrane, *i.e.*, the peptide signals do not completely fill the inner space of the bacteria. While we have not pursued these trends in our current analysis, considering such localization trends becomes feasible when imaging spheroplasts and protoplasts instead of standard bacterial cells. These advantages would also apply to determining the localization of peptides other than AMPs, such as cell-penetrating peptides. Spheroplasts and protoplasts produced with these approaches could also be utilized for non-imaging experiments that benefit from increased cellular size, such as patch-clamp electrophysiology measurements^{18,19}.

A laser scanning confocal microscope was utilized in the development of our imaging protocol, but it is critical to optimize parameters for each specific imaging system. This includes selecting a set of parameters that maximize the detection of peptide and membrane dye fluorescence, while minimizing bleed-through from the membrane dye into the peptide's emission channel, and from the FITC-labeled peptide into the membrane's emission channel. Bleed-through from the peptide into the membrane's emission channel was avoided by selecting a wavelength range for the membrane's emission channel that did not include any portion of FITC's emission spectrum. Bleed-through from the membrane dye, di-8-ANEPPS, into the peptide's emission channel was more difficult to avoid because di-8-ANEPPS' emission spectrum overlaps with the majority of FITC's emission spectrum. Specific to this protocol, bleed-through from the membrane dye into the peptide's emission channel can result in the false characterization of AMPs as membrane localized. One can determine if the bleed-through of this nature is occurring by imaging spheroplasts or protoplasts that are exclusively labeled with membrane dye and checking to see if the fluorescence is visible in the peptide's emission channel. Various imaging parameters, including the wavelength range used to define the peptide's emission channel and the gain and offset values, can be systematically tested to determine a set of parameters that minimizes bleed-through. Once a set of parameters is established, it is critical to evaluate whether imaging yields strong detection of fluorescence signal when spheroplasts or protoplasts labeled with both peptide and membrane dye are utilized. Through this approach, we successfully established imaging parameters that minimized the effect of the bleed-through while maximizing the fluorescence detection of both FITC-labeled AMP and the membrane dye di-8-ANEPPS. Specifically, we found that the emission bleed-through from the membrane dye into the peptide channel was minimized using emission wavelength ranges of 499–532 nm (peptide) and 670–745 nm (membrane), and when the gain was adjusted to be ≤ 800 volts (V) (peptide) and ≤ 900 V (membrane) and the offset was adjusted to $\leq -10.0\%$ (peptide, membrane).

Although we focused on *E. coli* and *B. megaterium*, the presented protocols could be adapted to produce spheroplasts or protoplasts from many different bacterial strains. For example, we have been able to successfully produce *Bacillus subtilis* protoplasts that were 1–2 μm in diameter using the protocol outlined in section 4, and adaptations of the protocol could be made to further optimize size. The ability to observe peptide localization patterns in both gram-negative and gram-positive bacteria is particularly useful to the study of AMPs because differences in the cell wall structure between these two classes of bacteria may affect how AMPs interact with cellular membranes. However, many imaging studies of AMPs to date have solely focused on model *E. coli* strains and, thus, may not reflect the behavior of peptides with gram-positive bacterial strains.

Spheroplasts and protoplasts differ from normal bacteria, most notably in their lack of an outer cell wall, and consequently may not be good models for all experimental questions. For example, the outer membrane of gram-negative bacteria has been shown to act as a molecular sieve for larger molecules²⁹. Larger peptides, therefore, may be able to translocate in spheroplasts when they would not do so in normal bacteria. Additionally, detailed studies of the interaction of AMP with the bacteria cell wall, outer membrane, and cytoplasmic membrane cannot be performed using spheroplasts and protoplasts. For example, recent work from the Weisshaar Lab has utilized imaging to note the more precise positions of the peptide in the CM15 and melittin mechanisms of action^{30,31}. However, our approach, as described here, does not require the implementation of microfluidics into microscopy instrumentation in order to achieve the required resolution³¹. Although previous work has shown that spheroplasts are viable^{21,32}, they nonetheless likely have some metabolic and physiological differences from "normal" bacteria that could potentially alter membrane interactions in some cases. Additionally, because we have not assessed the viability of spheroplast populations, one cannot distinguish between a peptide that is readily able to translocate across the cell membrane of a living cell versus a peptide that can only translocate across a dead or dying cell. Despite these differences, we have seen localization patterns in *E. coli* spheroplasts for many AMPs that are consistent with known mechanisms of action¹⁵, and our results for BF2 P11A with *B. megaterium* protoplasts presented here seem consistent with other observations for that peptide^{13,27,28}. The membrane compositions of spheroplasts and protoplasts are also certainly more physiologically relevant than the typical mixtures used in other model systems, such as lipid vesicles. In summary, given the enhanced resolution and ease of imaging afforded by spheroplasts and protoplasts, we believe they are useful models for visualizing membrane active agents, such as AMPs, in a range of bacterial strains.

Disclosures

No conflicts of interest are declared.

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