Journal of Visualized Experiments

Visualization of Bacterial Resistance using Fluorescent Antibiotic Probes

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60743R2
Full Title:	Visualization of Bacterial Resistance using Fluorescent Antibiotic Probes
Section/Category:	JoVE Immunology and Infection
Keywords:	antibiotic, resistance, bacteria, efflux, fluorescent, microscopy
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	St Lucia, QLD, Australia 4072

TITLE:

Visualization of Bacterial Resistance using Fluorescent Antibiotic Probes

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KEYWORDS:

antibiotic, resistance, bacteria, efflux, fluorescent, microscopy

SUMMARY:

Fluorescently tagged antibiotics are powerful tools that can be used to study multiple aspects of antimicrobial resistance. This article describes the preparation of fluorescently tagged antibiotics and their application to studying antibiotic resistance in bacteria. Probes can be used to study mechanisms of bacterial resistance (e.g., efflux) by spectrophotometry, flow cytometry, and microscopy.

ABSTRACT:

Fluorescent antibiotics are multipurpose research tools that are readily used for the study of antimicrobial resistance, due to their significant advantage over other methods. To prepare these probes, azide derivatives of antibiotics are synthesized, then coupled with alkyne-fluorophores using azide-alkyne dipolar cycloaddition by click chemistry. Following purification, the antibiotic activity of the fluorescent antibiotic is tested by minimum inhibitory concentration assessment. In order to study bacterial accumulation, either spectrophotometry or flow cytometry may be used, allowing for much simpler analysis than methods relying on radioactive antibiotic derivatives. Furthermore, confocal microscopy can be used to examine localization within the bacteria, affording valuable information about mode of action and changes that occur in resistant species. The use of fluorescent antibiotic probes in the study of antimicrobial resistance is a powerful method with much potential for future expansion.

INTRODUCTION:

Antimicrobial resistance (AMR) is a rising crisis which poses a major threat to human health around the world. Resistance to most antibiotics has been reported, and infections caused by bacteria resistant to all clinically available drugs are emerging. In order to combat the rise of AMR, we need to increase our understanding of this multifaceted phenomenon and the

underlying mechanisms and interactions between antibiotics and bacteria. One aspect that has been historically poorly understood is the permeation of antibiotics into bacteria, along with the phenomena of accumulation and efflux. This knowledge is crucial in designing new drugs and understanding mechanisms of resistance. Hence, this plays a critical role in AMR research.

There are two main approaches that may be taken in order to measure antibiotic concentration: measuring the drug directly or tagging with a moiety designed to facilitate quantification. Although tagging the antibiotic improves detection, this can perturb the biological activity of the drug, such as antimicrobial activity and permeability. This is not a problem for untagged methods; however, detection can be challenging. In the past few years, technological advancements have led to a boom in research utilizing mass spectrometry (MS) to directly measure the antibiotic concentration in bacteria[1–7]. These studies have shown that it is possible to study intracellular accumulation in a variety of bacteria, with gram-negative bacteria the most widely studied. Quantification of molecule permeability has then been linked to activity and used to inform drug development[2–4], though caution must be taken when directly conflating accumulation and target activity[5]. Prior to MS development, the only antibiotics whose concentration could be directly measured were those possessing intrinsic fluorescence, such as tetracycline and the quinolones[8–11]. Although obviously limited in scope, accumulation and efflux were examined and quantified, illustrating the usefulness of fluorescence-based quantification.

Tagged antibiotics have been used for many decades to study distributions, modes of action, and resistance, with radioactive and fluorescent tags being common. Radio-tagged probes have the advantage of being almost identical to the parent compound, hence the biological activity is unlikely to be significantly different. Isotopes such as ^{3}H, ^{14}C, and ^{15}N have been frequently used due to the prominence of these elements in antibiotics, and a variety of antibiotic scaffolds have been examined[1,10,12,13]. While the detection of radio-probes is simple, there are a number of logistical concerns (e.g., safety, isotope half-life) that have limited the use of this approach. Another strategy is fluorescently-tagged antibiotics. These probes can be used to examine the distribution and modes of action and resistance of the parent drug, using simpler technology than MS and without the logistical problems of radiation[8]. The main drawback to this approach is that antibiotics are generally relatively small molecules, hence the introduction of a fluorescent moiety poses a significant chemical change. This alteration can impact physiochemical properties and antibacterial activity. Therefore, care must be taken to assess these factors in order to generate results representative of the parent antibiotic.

In this work, a method is described to synthesize, assess, and use fluorescent antibiotics, as in our previous publications[14–16]. Through previous work, a number of fluorescent antibiotics have been prepared and used for a variety of purposes (see Stone et al.[8]). In order to minimize the likelihood of impacting biological activity, very small fluorophores are used in this work: nitrobenzoxadiazole (NBD, green) and 7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl (DMACA, blue). Further, the assessment of antibacterial activity using the microbroth dilution minimum inhibition concentration (MIC) assay is described, so that the effect of modifications on activity can be measured. These fluorescently-tagged probes can be used in spectrophotometric assays, flow cytometry, and microscopy. The range of possible applications is where the advantage of

fluorescent antibiotics lies. Cellular accumulation can be quantified, categorized, and visualized, something not possible using MS alone. It is hoped that the knowledge gained through the use of fluorescent antibiotics will aid in our understanding of resistance, and the fight against AMR.

PROTOCOL:

1. Synthesis of alkyne-fluorophores

1.1. Synthesis of NBD-alkyne (7-nitro-*N*-(prop-2-yn-1-yl)benzo[*c*][1,2,5]oxadiazol-4-amine)

1.1.1. Dissolve 1,031 mg of 4-chloro-7-nitro-benzofuran (5.181 mmol) in 60 mL of tetrahydrofuran (THF). Add 1,857 mg of $CsCO_3$ (5.696 mmol), then 0.39 mL of propargylamine (6.1 mmol). Heat the reaction to 50 ˚C for 2 h, which will turn from brown to green, then cool to room temperature (RT).

1.1.2. Filter the reaction using a filtering aid (see **Table of Materials**), and wash with ethyl acetate (EA). Concentrate the filtrate under reduced pressure, then dissolve the residue in 150 mL of EA and move to a 500 mL separating funnel.

1.1.3. Wash the EA solution with 100 mL with water and brine respectively. Then combine the aqueous phases and wash 2x with 100 mL of EA.

1.1.4. Dry the combined organic phases over anhydrous magnesium sulfate, then filter and concentrate under reduced pressure.

1.1.5. Purify the crude product by flash chromatography on silica gel (20–30% EA in petroleum ether [PE]), checking purity by liquid chromatography mass spectrometry (LCMS, $[M+H]^+ = 219.1$) and/or nuclear magnetic resonance (NMR) spectroscopy, chemical shifts as follows:

^{1}H NMR (CD_3OD, 600 MHz) δ 8.54 (d, J = 8.7 Hz, 1H), 6.35 (d, J = 8.4 Hz, 1H), 4.31 (dd, J = 5.7 Hz, J = 2.5 Hz, 2H), 2.43 (t, J = 2.5 Hz, 1H); ^{13}C NMR (CD_3OD, 150 MHz) δ 144.3, 143.7, 142.2, 135.8, 125.7, 100.0, 76.5, 74.2, 33.4.

NOTE: When performing purification by silica gel chromatography, prepare the column using the less polar of the solvents listed. Crude compound may either be loaded as a concentrated solution or adsorbed onto the silica if solubility does not allow it. After the compound has been added to the top of the silica, run through 1–2 column volumes of the same solvent used for silica wetting. Then start with the solvent ratio listed, running through at least 1 column volume of each solvent, and making sure to not make large jumps in solvent composition. Collect fractions, and check purity/identity by LCMS or TLC (thin layer chromatography). Combine pure fractions and concentrate under reduced pressure by rotary evaporation.

1.2. Synthesis of DMACA-alkyne (2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)-*N*-(prop-2-yn-1-yl)acetamide).

1.2.1. Dissolve 5.02 g of 3-(dimethylamino)phenol (36.6 mmol) in 30 mL of ethanol, then add 6.7 mL of diethyl 1,3-acetonedicarboxylate (36 mmol). Add 10.5 g of $ZnCl_2$ (77.2 mmol), then reflux the red solution for 42 h. Add an additional 9.20 g of $ZnCl_2$ (67.6 mmol), then reflux for 8 h.

1.2.2. Cool the reaction and concentrate under reduced pressure. Disperse the resulting red solid in 200 mL of EA, filter, then transfer to a 500 mL separating funnel.

1.2.3. Wash the EA with 200 mL each of water and brine, then dry over anhydrous magnesium sulfate. Filter the dried organic phase and concentrate under reduced pressure.

1.2.4. Purify the red solid (ethyl 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetate) by flash chromatography on silica gel (0–100% EA in PE), checking purity by LCMS ($[M+H]^+$ = 275.1) and/or NMR, chemical shifts as follows:

^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.30 (d, J = 8.9 Hz, 1H), 6.52 (dd, J = 8.9 Hz, J = 2.6 Hz, 1H), 6.40 (d, J = 2.6 Hz, 1H), 5.87 (m, 1H), 2.96 (m, 8H), 2.25 (d, J = 1.2 Hz, 3H).

1.2.5. Dissolve 488 mg of ethyl 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetate (1.18 mmol) in 10 mL of THF, then add a solution of 157 mg of $LiOH{\cdot}H_2O$ (3.74 mmol) in 15 mL of water. Stir the reaction at RT for 3 h, then move to a separating funnel and dilute with an additional 50 mL of water.

1.2.6. Wash the reaction mixture 2x with 50 mL of diethyl ether (Et_2O), then wash the combined organic phase 2x with 25 mL of water. Take any yellow precipitate with the organic layer. Concentrate the organic phase under reduced pressure using a rotary evaporator.

1.2.7. Acidify the aqueous phase to pH = 2 with concentrated HCl, and cool to 4 ˚C overnight. Filter the acidified aqueous phase and add the yellow solid to the concentrated organic phase.

NOTE: The 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetic acid can be used without further purification, but this can be checked by LCMS ($[M+H]^+$ = 247.1) and/or NMR, chemical shifts as follows:

^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.41 (d, J = 9.0 Hz, 1H), 6.62 (dd, J = 9.2 Hz, J = 2.8 Hz, 1H), 6.52 (d, J = 2.8 Hz, 1H), 5.98 (d, J = 0.9 Hz, 1H), 3.05 (s, 6H), 2.35 (d, J = 0.9 Hz, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 162.2, 155.7, 152.9, 152.8, 125.3, 109.7, 109.3, 109.1, 108.8, 98.3, 40.2, 18.5.

1.2.8. Dissolve 466 mg of 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetic acid (1.89 mmol) in 7 mL of dry *N*,*N*-dimethylformamide (DMF) and place under an atmosphere of nitrogen.

1.2.9. Dissolve 0.33 mL of propargylamine (5.1 mmol) in 7 mL of dry DMF under nitrogen. Add 1.30 mL of di-isopropylethyl amine (DIPEA, 7.50 mmol) to the dye solution, then 535 mg of *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, 1.29

mmol). Stir the activated dye solution for 15 min at RT, then add the amine solution dropwise, and leave to stir overnight.

1.2.10. The next day, dilute the reaction with 35 mL of water then concentrate under reduced pressure.

1.2.11. Partition the resulting orange solid between EA and brine (100 mL each) in a 250 mL separating funnel. Separate the layers (run the two layers into different flasks), and wash the aqueous phase with 100 mL of EA.

1.2.12. Concentrate the combined organic phases under reduced pressure, then redissolve the orange solid in 3 mL of 1:1 acetonitrile (ACN)/water (v/v). Purify the crude product by injecting onto a reverse phase medium pressure liquid chromatography (MPLC) system equipped with a C18 cartridge column (solvent A: water, solvent B: ACN).

1.2.13. Check fractions for purity by LCMS ($[M+H]^+$ = 284.1, NMR chemical shifts given below), then combine and lyophilize appropriate fractions to give 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)-*N*-(prop-2-yn-1-yl)acetamide, NMR chemical shifts as follows:

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.65 (t, *J* = 5.4Hz, 1H), 7.52 (d, *J* = 9.0Hz, 1H), 6.72 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.55 (d, J = 2.6 Hz, 1H), 6.00 (s, 1H), 3.88–3.87 (m, 2H), 3.62 (s, 2H), 3.13 (t, *J* = 2.5 Hz, 1H), 3.01 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 167.7, 160.7, 155.4, 152.9, 151.0, 126.0, 109.4, 109.1, 108.1, 97.5, 80.9, 73.3, 39.7, 38.4, 28.2.

2. Synthesis of fluorescent antibiotics

2.1. Prepare an azide-derivative of an antibiotic as described previously[14–16].

NOTE: The procedure is specific to each antibiotic and requires careful examination of the structure activity relationship (SAR) of the parent molecule to ensure that the functionalized antibiotic retains activity comparable to the parent. (e.g., ciprofloxacin[16], linezolid[14], and trimethoprim[15]). See **Figure 1** for examples of published fluorescent antibiotics, and the general synthesis scheme.

2.2. Click reaction procedure A

NOTE: For most of the antibiotics, follow the procedure detailed here for copper catalyzed Huisgen [2+3] cycloaddition of azide (step 2.1) and fluorescent alkyne (prepared in step 1).

2.2.1. Place the azide-antibiotic in a round bottom flask and add *tert*-butanol (^{t}BuOH) and water (1:1 v/v, 25 mL each per mmol azide).

2.2.2. Add the fluorophore-alkyne prepared in step 1.1 (3 eq.) and heat the reaction to 50 ˚C. Then add copper sulfate (100 mM in water, 0.6 eq.) to the reaction, followed by ascorbic acid

(500 mM in water, 2.4 eq.).

2.2.3. Stir the reaction at 50 ˚C for 1 h, or until analysis by LCMS upon indication of the reaction completion (complete consumption of starting azide).

2.2.4. Cool the reaction and purify as appropriate for the antibiotic scaffold and proceed for purification either by step 2.3 or 2.4.

NOTE: Several different purification methods are possible, depending on the polarity and stability of the scaffold.

2.3. Click reaction procedure B

NOTE: Follow this procedure for peptide-based antibiotics, to provide stronger reaction conditions (unpublished work, Phetsang, 2019).

2.3.1. Place the peptide azide-antibiotic in a round bottom flask and add enough DMF (750 mL/mmol azide) to dissolve.

2.3.2. Add the fluorophore-alkyne prepared in step 1 (5 eq.) and heat the reaction to 50 ˚C for 1 h.

2.3.3. Add copper (I) iodide (20 eq.), then DIPEA (120 eq.), then acetic acid (240 eq.).

2.3.4. Stir the reaction at 50 ˚C for 1 h, or until the analysis by LCMS indicates reaction completion (i.e., complete consumption of starting azide). Cool the reaction and proceed for purification by method 1 (see step 2.3).

2.4. Purification method 1 (used for ciprofloxacin, trimethoprim, and linezolid)

2.4.1 Inject the cooled click reaction directly onto a MPLC C18 cartridge column.

2.4.2. Incorporate a long wash phase (roughly 10 min) at the beginning of the run (100% solvent A), then run a gradient up to 100% solvent B, followed by a return to solvent A.

NOTE: Solvent A can be chosen from water, 0.05–0.1% formic acid (FA) in water, 0.05–0.1% trifluoroacetic acid (TFA) in water, depending on solubility, stability, and the best resolution of peaks. Solvent B can be chosen from acetonitrile (ACN), 0.05–0.1% FA in ACN, 0.05–0.1% TFA in ACN, to match solvent A. If elution proves difficult, methanol may be used in place of ACN.

2.4.3. Collect and combine appropriate fractions, as indicated by LCMS and color (correct mass seen, singular peak), then lyophilize to give the (semi)pure fluorescent antibiotic.

2.4.4. Further purify the product if needed. Assess purity by NMR and/or LCMS and high-pressure

liquid chromatography (HPLC), using a column and method appropriate for the scaffold.

2.5. Purification method 2

NOTE: If solubility allows, prepurification may be carried out by the aqueous workup (used for macrolides, unpublished work, Stone 2019).

2.5.1. Dilute the cooled click reaction with water and Et_2O (1:1 v/v, approximately 10-fold dilution from initial reaction volume), transferring to an appropriately sized separating funnel.

2.5.2. Separate the layers and wash the aqueous phase twice with Et_2O.

2.5.3. Wash the combined organic phases 2x with water (equal volume with organic phase), then dry over Na_2SO_4.

2.5.4. Filter the dried organic phase and concentrate under reduced pressure.

2.5.5. Purify the crude product by MPLC and/or HPLC as described in step 2.4.4.

NOTE: See **Figure 2** for examples of incomplete, complete, and purified click reaction LCMS traces. Typical purified yields for the antibiotic-fluorophore click reactions range from 30–80%.

CAUTION: Most of the chemicals used in these syntheses possess specific safety hazards. Caution must be taken at all times, including the use of personal protective equipment. Et_2O, ^tBuOH, FA, acetic acid, PE, EA, THF, ACN, DMF, EtOH, DIPEA, propargylamine, and HCTU are all flammable; avoid contact with heat or spark sources. THF, propargylamine, DIPEA, ^tBuOH, FA, DMF, and PE are all toxic; avoid exposure. Propargylamine, $CsCO_3$, $ZnCl_2$, LiOH-H2O, DIPEA, CuI, FA, acetic acid, and HCl are all corrosive; avoid contact and be aware of surface contact. $ZnCl_2$, $CuSO_4$, CuI, and PE present environmental hazards; be mindful of disposal conditions. THF can form explosive peroxides; take care with storage conditions. Organic azides are explosive; take care especially with large scale production.

3. Evaluation of antimicrobial activity

NOTE: All work involving bacteria should be carried out under sterile conditions to avoid contamination of either the assay or the laboratory. All media should be autoclaved before use, and plasticware and equipment such as pipettes must be kept sterile. It is recommended that work be done in a biocontainment hood (type 2).

3.1. Streak glycerol stocks of bacterial strains appropriate for the antibiotic scaffold onto lysogeny broth agar (LB, prepared per manufacturer's instructions), and grow overnight at 37 °C.

NOTE: The choice of bacteria to test antibacterial activity must be made based on the antibiotic scaffold being used. A representative range of 5–10 bacteria should be chosen from the species

that are known to be susceptible to the antibiotic, with consideration given to the logistical capabilities of the lab. If possible, resistant bacteria should also be tested. The protocol given below will work on most bacteria, but check if special conditions are required (e.g., CO_2, special media) and make alterations as necessary. Bacteria successfully assayed using these conditions include *Staphylococci, Streptococci, Bacilli, E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Enterococcus faecium.*

3.2. Pick a single colony from the plate, and culture overnight in 5 mL of cation adjusted Mueller-Hinton Broth (CAMHB, prepared per manufacturer's instructions) at 37 ˚C.

3.3. Dilute the overnight cultures ~40-fold in CAMHB and grow to a mid-log phase, optical density at 600 nm (OD_{600}) = 0.4–0.8, volume 5 mL).

3.4. Prepare stock solutions of each fluorescent antibiotic at 1.28 mg/mL in sterile water, and pipette 10 μL of antibiotic to the first column of a 96 well plate.

3.5. Add 90 μL of CAMHB to the first column and 50 μL to all other wells. Then, perform serial 2-fold dilution across the plate.

3.6. Thoroughly mix, then dilute the mid-log phase cultures to ~10^6 colony forming units (CFU)/mL and add 50 μL to all wells, to provide a final concentration of ~5 x 10^5 CFU/mL.

$$volume\ of\ culture\ (mL) = (media\ volume\ in\ mL)/(OD_{600} \times 1{,}000)$$

e.g., for an OD_{600} = 0.5 culture in a desired media volume of 12 mL, add (12/(0.5 x 1,000) = 0.024 mL of culture to 12 mL of media

3.7. Cover the plates with lids and incubate at 37 ˚C for 18–24 h without shaking.

3.8. Visually inspect the plates, with the MIC being the lowest concentration well with no visible growth.

NOTE: See **Table 1** for some examples of active and inactive fluorescent antibiotics.

4. Analysis of probe accumulation by spectrophotometry and flow cytometry

NOTE: These centrifugation times have been optimized for *E. coli*, so slight alterations may be needed for other species. Representative data for probe accumulation is reported for the NBD-labeled ciprofloxacin probe.

4.1. Streak glycerol stocks of the bacterial strains onto LB agar and grow overnight at 37 ˚C.

4.2. Pick a single colony from the plate and culture overnight in LB at 37 °C.

4.3. Dilute overnight cultures ~50-fold in media and grow to mid-log phase (OD_{600} = 0.4–0.8).

4.4. Centrifuge the cultures at 1,470 x *g* for 25 min and decant the media.

4.5. Resuspend the bacteria in 1 mL of phosphate-buffered saline (PBS), then centrifuge at 1,470 x *g* for 15 min.

4.6. Decant the media and resuspend the washed pellets in PBS to a final OD_{600} = 2.

4.7. If desired, add 10.1 μL of carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 mM in PBS) to 1 mL of bacteria (final concentration 100 μM) and incubate at 37 ˚C for 10 min.

NOTE: CCCP is an efflux pump inhibitor. Addition of CCCP will allow the examination of the impact of efflux.

4.8. Centrifuge the cultures at 18,000 x *g* for 4 min at 20 ˚C and decant the media.

4.9. Add 1 mL of fluorescent antibiotic solution (10–100 μM in PBS) to the pellet, and incubate at 37 ˚C for 30 min.

4.10. Centrifuge the cultures at 18,000 x *g* for 7 min at 4 ˚C and decant the media.

4.11. Resuspend the bacteria in 1 mL of cold PBS, and repeat step 4.9.

4.12. Repeat step 4.10 a total of 4x.

4.13. If desired, lyse bacteria by adding 180 μL of lysis buffer (20 mM Tris-HCl, pH 8.0, and 2 mM sodium EDTA) then 70 μL of lysozyme (72 mg/mL in H_2O).

4.14. Incubate at 37 ˚C for 30 min, then freeze-thaw 3x (−78 °C for 5 min, then 34 °C for 15 min).

4.15. Sonicate the samples for 20 min, then heat to 65 °C for 30 min.

4.16. Centrifuge the lysed samples (18,000 x *g*, 8 min), then filter through a 10 kDa filter membrane.

4.17. Wash the filter the 4x with 100 μL of water.

4.18. Transfer the lysate to a flat-bottom black 96 well plate and measure the fluorescence intensity on a plate reader with excitation and emission wavelengths appropriate for the fluorophore (i.e., DMACA: λ_{ex} = 400 nm, λ_{em} = 490 nm; NBD: λ_{ex} = 475 nm, λ_{em} = 545 nm).

NOTE: See **Figure 3** for examples of spectrophotometric analyses of bacteria using the fluorescent

NBD-labeled ciprofloxacin antibiotic.

4.19. For analysis by flow cytometry, the same growth and staining conditions may be used (steps 4.1–4.17), with changes solely in the final preparation.

4.19.1. Bring the total volume to 1 mL of PBS.

4.19.2. Read samples on a flow cytometer at a flow rate of approximately 60 µL/min, using logarithmic amplification for the data acquisition (F1 excitation = 488 nm; emission = 525/20 nm).

4.19.3. Record a total of 10,000 events, then analyze the data using appropriate software.

4.19.4. Plot the fluorescence intensity from F1 against the number of events counts, estimating the median fluorescence intensity from the histogram peaks after the bacteria was stained.

NOTE: See **Figure 4** for examples of flow cytometry analyses of bacteria using the NBD-labeled ciprofloxacin antibiotic.

5. Preparation for microscopic analysis

5.1. Grow subcultures to OD_{600} = 0.4, as for MIC assessment, then divide into 1 mL aliquots and centrifuge at 18,000 x *g* for 3–5 min.

5.2. Decant and discard media, then resuspend bacterial pellet in 500 µL of HBSS.

5.3. Centrifuge at 18,000 x *g* for 3 min, then decant and discard media.

5.4. Prepare solutions of antibiotic probes in HBSS at concentrations of 1–100 µM.

5.5. Resuspend the washed bacteria in 500 µL of the probe solution, and incubate at 37 °C for 30 min.

5.6. Repeat step 5.3. For a multiple labeling experiment, resuspend the pellet in 500 µL of an orthogonally colored nucleic acid dye. For green, use Syto9 (5 µM in HBSS); for blue, use Hoechst 33342 (20 µg/mL in HBSS). Incubate at RT for 15–30 min.

5.7. Repeat step 5.3, then resuspend in 500 µL of FM4-64FX (5 µg/mL in HBSS) and incubate at RT for 5 min.

5.8. Repeat step 5.3, then resuspend in 500 µL of HBSS, and repeat step 5.3 once more.

5.9. Repeat step 5.8, then finally suspend the washed, dyed bacteria in 15 µL of mounting medium (see **Table of Materials**).

5.10. Pipette mounting medium on a microscope slide and top with a high-performance cover slip, then seal edges with clear nail polish.

NOTE: See **Figure 5** for examples of confocal microscopy images taken with NBD-labeled ciprofloxacin and trimethoprim antibiotics, and **Figure 6** for DMACA-labeled oxazolidinone (linezolid) antibiotic.

REPRESENTATIVE RESULTS:

Figure 1 Illustrates the key click chemistry reaction (**A**) for the preparation of the fluorescent antibiotics, and with (**B**) examples of structures of our published fluorescent antibiotics based on ciprofloxacin (cipro), trimethoprim (TMP), and linezolid. These probes were all synthesized from the corresponding antibiotics via an azide intermediate. They were then coupled to the NBD and DMACA fluorophores, each functionalized with an alkyne.

Figure 2 shows example LCMS traces from a ciprofloxacin-N_3 and NBD-alkyne click reaction, where the azide eluted at 3.2 min and the product at 3.8 min. Comparing **1** and **2** shows how the progress of the click reaction could be followed by the disappearance of the azide peak (by UV or MS detector). Spectra **3** demonstrate the impact of purification, with erroneous peaks disappearing from the MS and UV traces. Both purity and reaction progress could be quantified by the integration of the product peak and any impurity peaks.

Figure 3 demonstrates typical results from the assessment of intracellular accumulation by fluorescence spectroscopy in the presence and absence of efflux. In this experiment, *E. coli* was treated with TMP-NBD with or without the addition of CCCP, which collapses the proton motive force (PMF). The intracellular fluorescence of the bacteria was significantly higher when pretreated with CCCP, indicating that efflux reduced the accumulation in these bacteria. This experiment was repeated using bacteria deficient in *tolC*, displaying the capacity of this assay to examine the impact of individual efflux pump components. In this case, although there was an increase in intracellular fluorescence compared to the wild type bacteria, CCCP accumulation still increased. These findings indicate that *tolC* takes part inTMP efflux but is not the sole PMF-drive pump involved.

Figure 4 shows the result of the same experiment as **Figure 2**, but with the accumulation measured by flow cytometry instead of spectroscopy. The same data trends were observed, demonstrating that either technique may be used to study the phenomenon of efflux mediated intracellular accumulation.

Figure 5 shows representative confocal microscopy images of gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*) labeled with TMP-NBD (**1**) and cipro-NBD (**2 + 3**) fluorescent probes, respectively. In both cases, the red membrane dye FM4-64FX was added in order to compare co-localization. For TMP-NBD, the blue nucleic acid dye Hoechst-33342 was also used. By overlaying these images, the localization of the antibiotic in the bacteria was visualized. Comparing panels **2** and **3** shows how the impact of efflux was examined, with the efflux inhibitor CCCP used in **2**,

resulting in intracellular accumulation. In panel **3**, no CCCP was added. Hence, efflux is active and no probe accumulation was seen.

Figure 6 shows representative confocal microscopy images of Gram-positive (*S. aureus*) bacteria labeled with DMACA-labeled oxazolidinone probe Lz-NBD. The red membrane dye FM4-64FX was added in order to compare co-localization, and the green nucleic acid dye Hoechst-33342 was also used. By overlaying these images, the localization of the antibiotic in the bacteria was visualized, showing internal localization distinct from the membrane and nucleic acid.

Table 1 shows MIC values for three series of fluorescent antibiotics, ciprofloxacin, trimethoprim (TMP), and linezolid (Lz), with data presented for the parent antibiotic, NBD and DMACA derivatives of each. Representative species for each antibiotic were chosen, including both gram-positive and gram-negative. For the ciprofloxacin series, both fluorescent probes lost antibiotic activity compared to the parent drug, but retained some activity against all species. Similarly, the linezolid probes lost some activity, but remained a moderate to weak antibiotic. The TMP probes lost almost all activity against wild type bacteria, but were active against efflux deficient *E. coli*, indicating that the loss of antibacterial activity was due to lack of accumulation.

FIGURE AND TABLE LEGENDS:

Figure 1: Synthesis and structures of antibiotic-derived probes. (**A**) The general reaction scheme for the synthesis of fluorescent antibiotic probes from azide-antibiotics and alkyne-fluorophores. (**B**) The structures of our published probes based on ciprofloxacin, trimethoprim, and linezolid.

Figure 2: Measurement of antibiotic-derived probe purity by LCMS. Analytical LCMS traces from (1) incomplete, (2) complete, and (3) HPLC purified ciprofloxacin-N_3 + NBD-alkyne click reactions demonstrating the disappearance of starting material upon reaction completion, and miscellaneous peaks on purification. A = UV-Vis trace (absorbance at 250 nm), B = MS trace (positive and negative mode).

Figure 3: Plate reader measurement of antibiotic-derived probe accumulation. Fluorescence spectroscopic measurement of cellular accumulation of TMP-NBD (50 μM) in wild type (1, ATCC 25922) and Δ*tolC* (2, ATCC 25922) *E. coli* incubated (**A**) with and (**B**) without addition of CCCP (100 μM). Statistical significance ($**p \leq 0.01$; $***p \leq 0.001$) is shown between the absence or presence of CCCP and between wild type and Δ*tolC E. coli*. Data reported are the mean ± SD for three experiments. This figure is adapted from our previous publication[15], and illustrates the use of spectroscopy to elucidate the role of efflux on intracellular accumulation.

Figure 4. **Flow cytometry measurement of antibiotic-derived probe accumulation.** Flow cytometry measurement of cellular accumulation using TMP-NBD in in wild type (1, ATCC 25922) and Δ*tolC* (2, ATCC 25922) *E. coli* incubated with and without addition of CCCP (100 μM). Median fluorescence activity is shown from 10,000 bacterial events, Statistical significance (***, $p \leq 0.001$; ****, $p \leq 0.0001$) is shown between the absence and presence of CCCP and between wild type and Δ*tolC E. coli*. Data reported are the mean ± SD for three experiments. This figure is adapted from our previous publication[15], and illustrates the use of flow cytometry to elucidate

the role of efflux on intracellular accumulation.

Figure 5. Confocal microscopy visualization of NBD-probe localization. Confocal microscopy images of 1) live *S. aureus* labeled with Hoechst-33342 (blue, nucleic acid), TMP-NBD (green), FM4-64FX (red, membrane), and overlaid; 2) live *E. coli* treated with CCCP (efflux inhibitor) labeled with cipro-NBD (green), FM4-64FX (red, membrane), and overlaid; 2) live *E. coli* labeled with cipro-NBD (green), FM4-64FX (red, membrane), and overlaid. This figure is adapted from our previous publications[15,16], and illustrates the use of microscopy to examine probe localization, including the impact of efflux.

Figure 6. Confocal microscopy visualization of DMACA-probe localization. Confocal microscopy images of live *S. aureus* labeled with oxazolidinone probe Lz-DMACA (blue), Sytox green (green, nucleic acid), and FM4-64FX (red, membrane).

Table 1: Antibiotic activities of fluorescent antibiotic probes based on ciprofloxacin, trimethoprim, and linezolid against appropriate clinically relevant bacterial strains, as measured by broth microdilution MIC assays. In most cases, the probes lost some activity compared to the parent drug, but retained some measurable antibiotic potency (sufficient to be useful in further studies).

DISCUSSION:

The creation of a successful fluorescent antibiotic probe must begin with careful planning and consideration of the SAR of the parent drug. If the SAR is not known or fully explored, several options may need to be tested to find a site which may be selectively modified without abolishing biological activity. Once a site/s have been identified, the installation of a linker moiety is often essential in order to provide steric spacing between the biological site of action and the inactive fluorophore. Care must be taken that the reaction used to attach the linker to the antibiotic leaves a bio-stable functional group, avoiding, for example, esters that are susceptible to cleavage by esterases in vivo. Depending on the pharmacodynamic and pharmacokinetic profile of the antibiotic, a simple alkyl linker may be used, or else a less lipophilic option such as a polyethylene glycol (PEG) linker should be considered. With the linker attached, the antibacterial activity should be assessed to ensure the MICs against relevant bacteria are similar to the parent compound.

In this work, we recommend the use of Huigsen azide-alkyne [3+2] dipolar cycloaddition (click chemistry, see **Figure 1**) to ligate fluorophore to antibiotic, for a number of reasons. Click reactions are highly selective, meaning that protection of reactive groups on the antibiotic is not necessary, and further, the reaction leaves a stable, biocompatible triazole moiety. The azide component is introduced to the antibiotic portion in our procedures, as this is generally more easily accomplished with a variety of structural types than introduction of an alkyne. The syntheses of two alkyne-derivatized fluorophores are described here, though others could be explored if desired. NBD and DMACA were chosen due to their small size, minimizing the possibility of interfering with cell penetration and target interaction. The click reaction itself is carried out using copper catalysis, where either Cu^{2+} ($CuSO_4$, with an ascorbic acid reducing

agent) or Cu^{+} (CuI) may be used as the starting reagent. Following purification (**Figure 2**), the MICs should then be tested as with the azide. Even with careful consideration of fluorophore choice and site of attachment, it is possible that poor antibiotic activity will be observed. This does not, however, mean that an inactive probe is without use. As shown with the TMP probes, compounds with poor antibacterial activity may still bind to the same target as the parent drug. This can enable studies on the mode of action and examination of phenomena leading to resistance, such as efflux.

As outlined in the protocols section, it is possible to analyze bacterial labeling by the fluorescent antibiotics using either a simple spectrophotometry assay (**Figure 3**) or flow cytometry (**Figure 4**). Both methods are capable of quantifying cellular accumulation, and by lysing cells and examining fluorescence localization in lysate, it is possible to assess intracellular accumulation. In this protocol, the use of lysozyme for cell lysis is described, as this is a rapid, universal technique. Other lysis conditions, such as overnight treatment with glycine-HCl[7], have also been successfully used. Using this technique, it is possible to study the impact of efflux on antibiotic cellular accumulation, which is a major mechanism of resistance. If efflux is indeed present in the bacteria, a lack of intracellular accumulation will be observed, though this can be rescued using an efflux inhibitor like CCCP.

Microscopy may also be carried out to visually inspect probe localization in different bacteria, garnering information on mode of action, and potentially also resistance (see **Figure 5** for representative examples). In order to see localization within bacteria, a high resolution confocal microscope is required, equipped with capabilities such as SIM (structured illumination microscopy), SR-SIM (superresolution-SIM), Airyscan, or STED (stimulated emission depletion). Furthermore, high-performance cover slips should be used, and post-imaging analysis carried out on an appropriate software (e.g., FIJI, Zen, or Imaris). Localization of probes is compared to dyes that stain specific architectures, such as Hoechst-33342 (blue, nucleic acid), Syto-9 (green, nucleic acid), and FM4-64FX (red, membrane). The choice of dyes should be made to match the fluorescent antibiotic, so that each color used has minimal spectral overlap. In order to obtain the best possible images, optimization may be required. For example, if bacteria are too crowded on the slide, take only part of the suspended pellet, then dilute with more mounting medium. In contrast, if bacteria are too sparse on the slide, simply start with more bacteria. In this protocol, the use of a thermo reversible gel that is compatible with live cells (e.g., Cygel) is recommended for live cell imaging, as it immobilizes bacteria (including motile bacteria), but other mounting media or agarose have also been successfully used.

Overall, despite challenges that may be faced in the preparation of a biologically active fluorescent antibiotic derivatives, the simplicity of their use and their versatility make these probes attractive tools for research in AMR. Future work using fluorescent antibiotics has the potential to provide insight into mechanisms of antibiotic resistance, improve our understanding of how current antibiotics operate, and aid the development of better drugs.

ACKNOWLEDGMENTS:
MRLS is supported by an Australian Postgraduate Award (APA) and an Institute for Molecular

Biosciences Research Advancement Award. Wanida Phetsang was supported by UQ International Scholarship (UQI) and IMB Postgraduate Award (IMBPA). MAC is a NHMRC principle research fellow (APP1059354) and also holds a fractional professorial research fellow appointment at the University of Queensland, with his remaining time as CEO of Inflazome Ltd, a company developing drugs to address clinical unmet needs in inflammatory disease. MATB is supported in part by Wellcome Trust Strategic Grant WT1104797/Z/14/Z and NHMRC Development grant APP1113719. Microscopy was performed at the Australian Cancer Research Foundation (ACRF)/Institute for Molecular Bioscience Cancer Biology Imaging Facility, which was established with the support of the ACRF.

DISCLOSURES:

The authors have nothing to declare.

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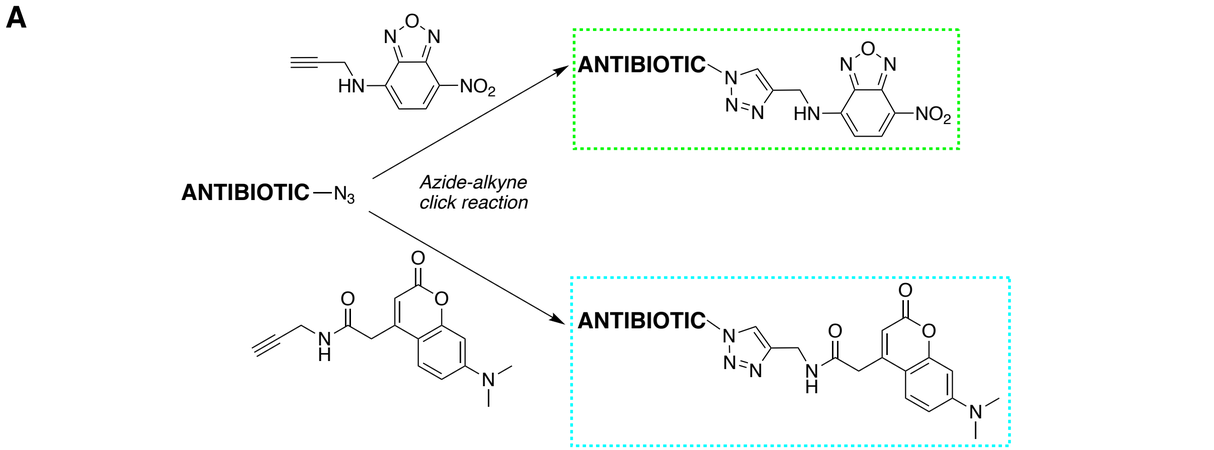
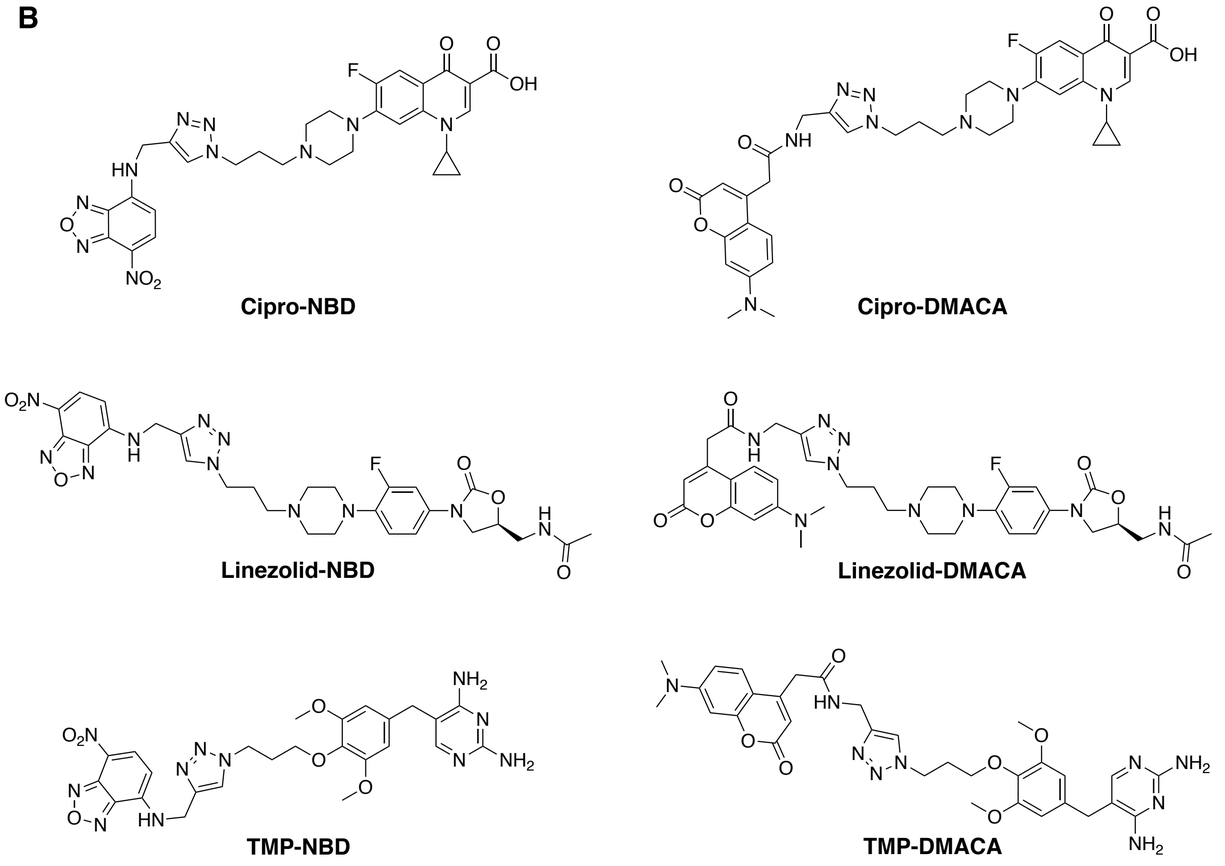
Figure 1

Click here to access/download;Figure;Figure 1.ai





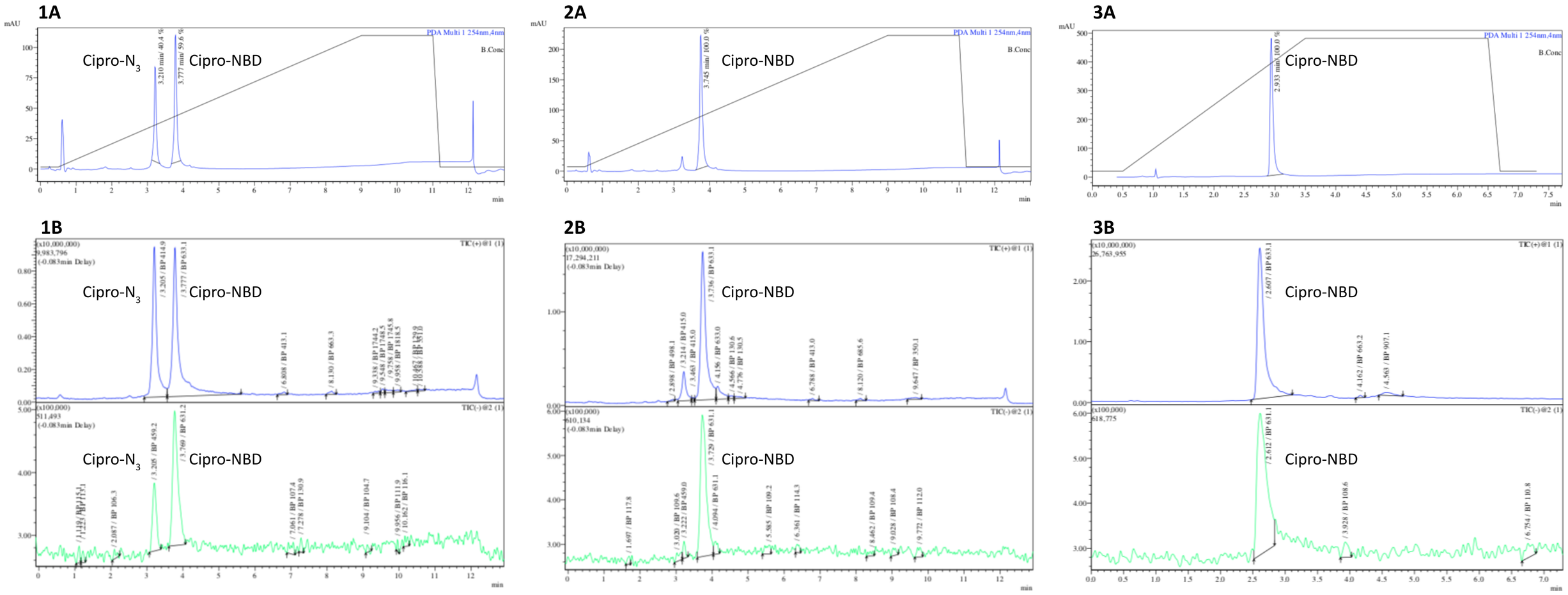


Figure 3

Click here to access/download;Figure;Figure 3.ai

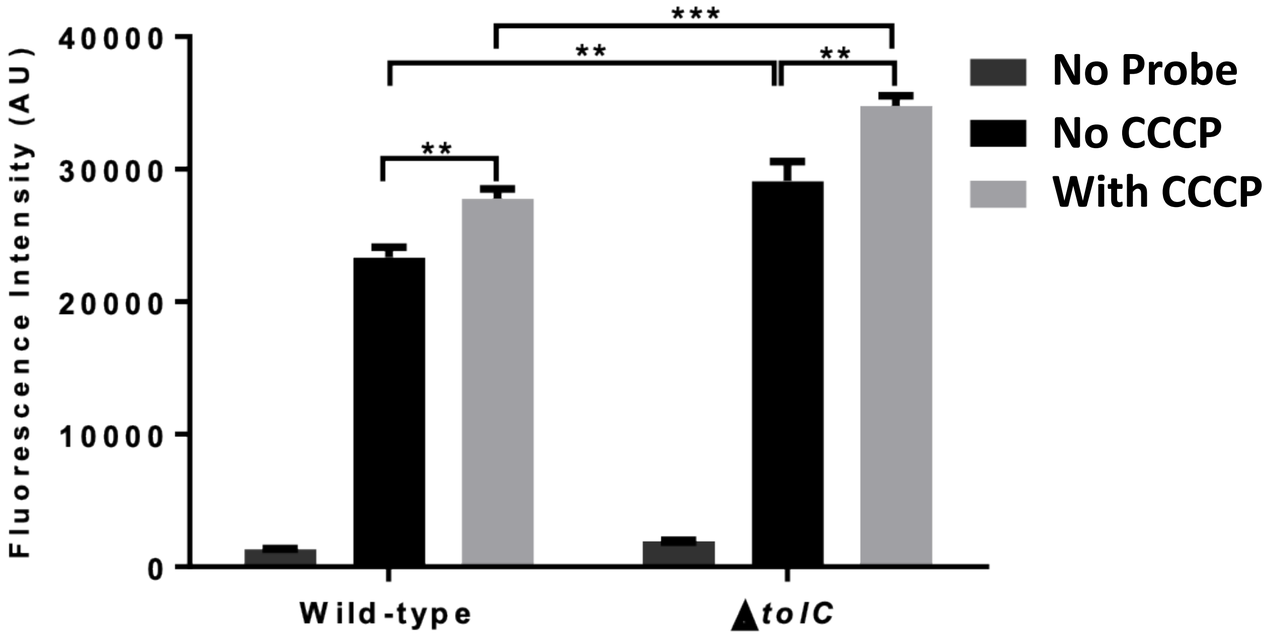


Figure 4

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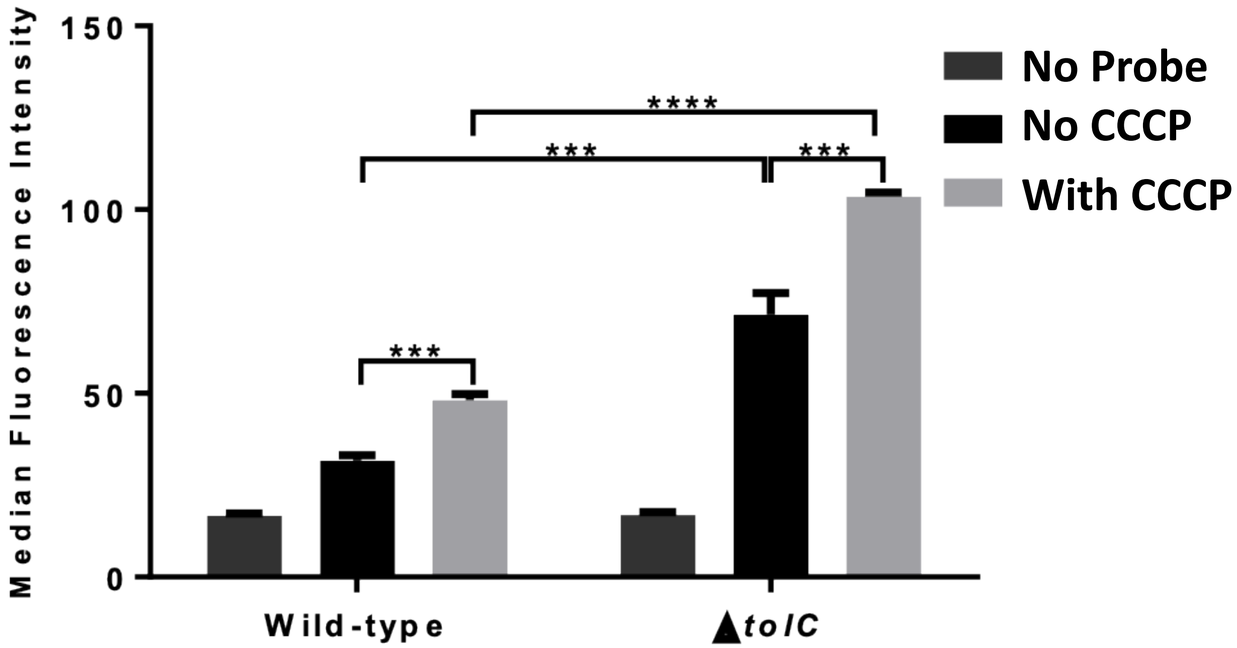

Figure 5

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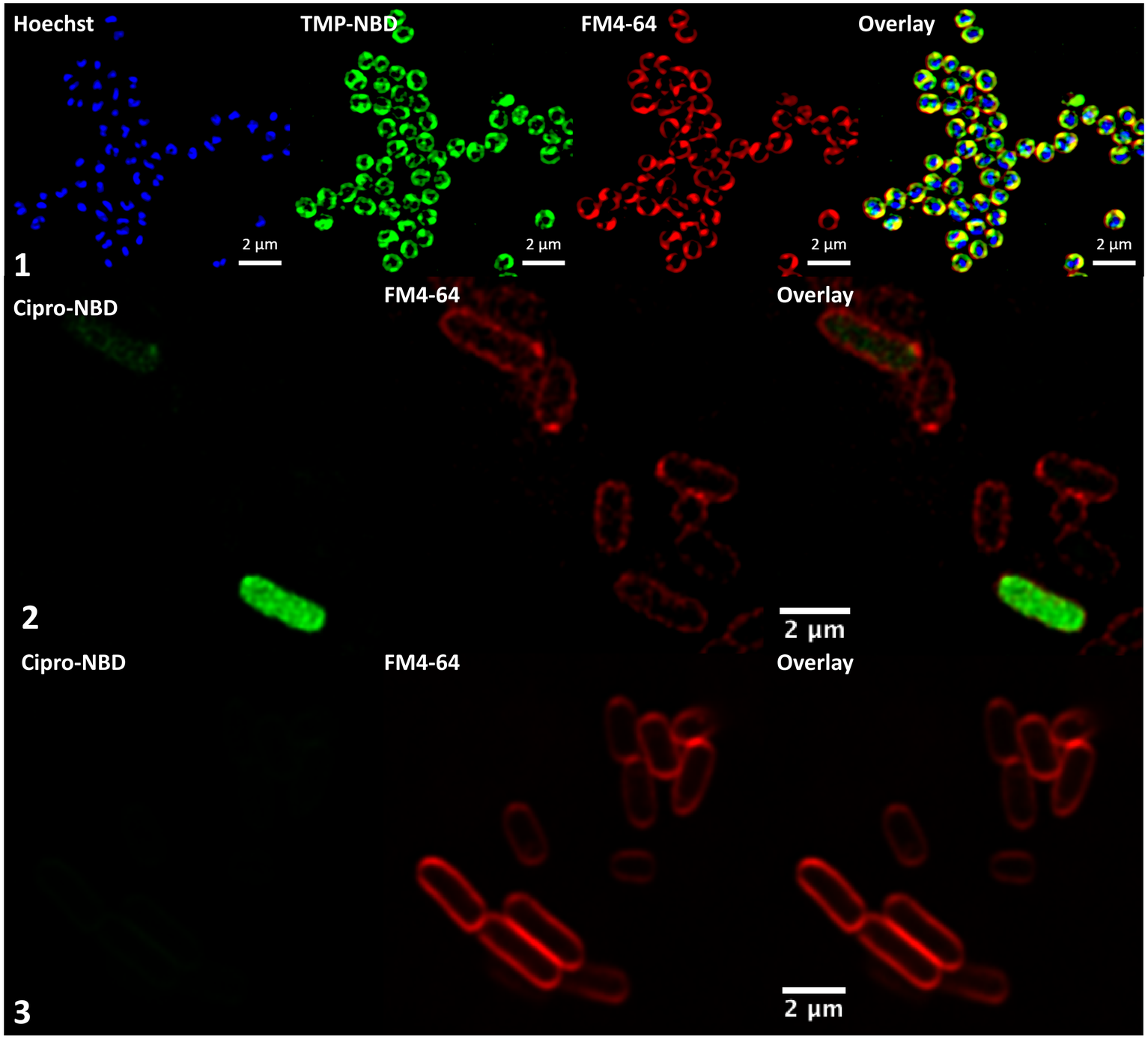
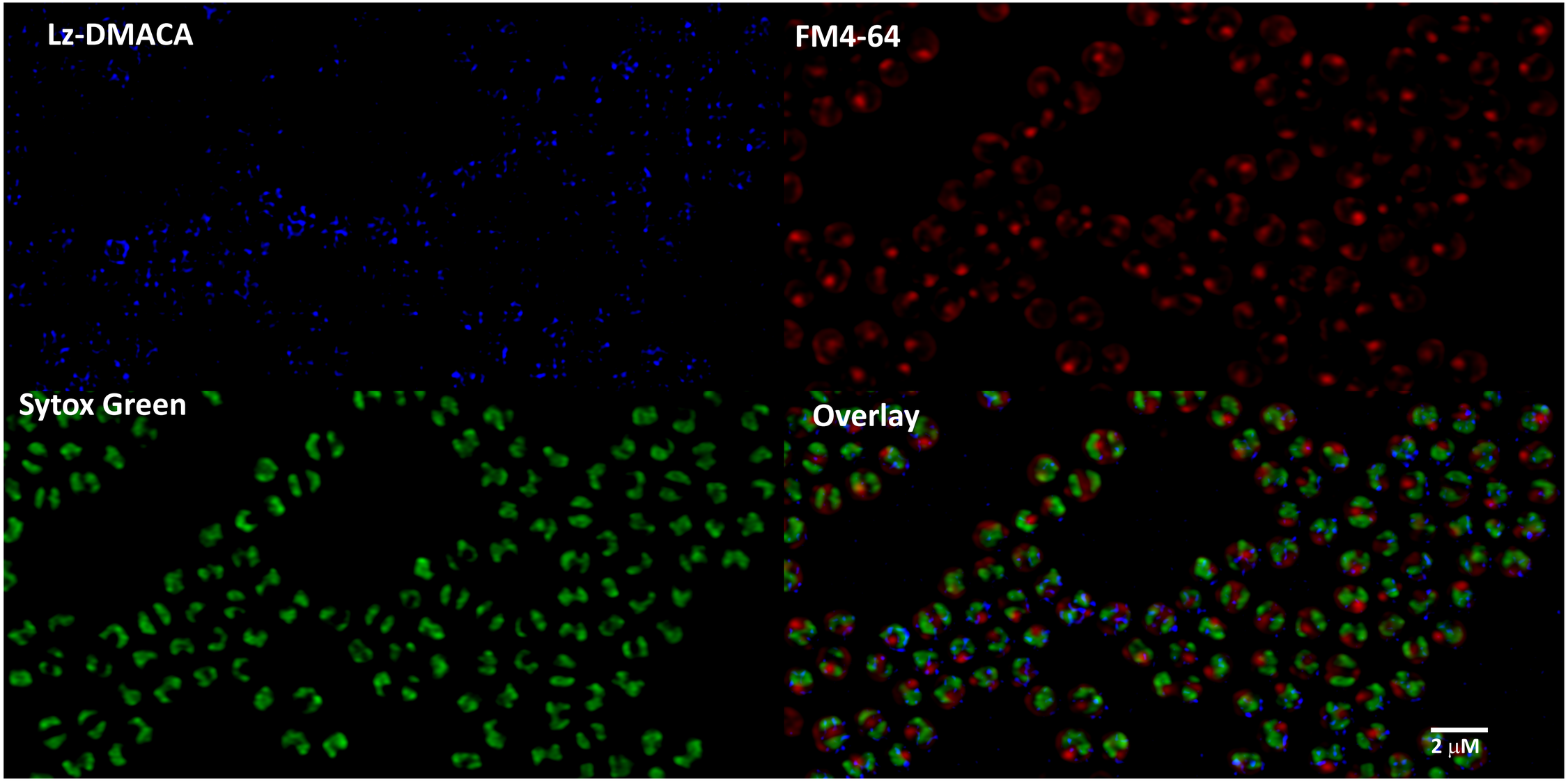


Figure 6

Click here to access/download;Figure;Figure 6.ai



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3-(dimethylamino)phenol	Alfa-Aesar	B23067	
4-chloro-7-nitro-benzofuran	Sigma-Aldrich	163260-5G	
Amicon Ultra-0.5 centrifugal filter unit with Ultracel- 10 membrane	Merck	UFC501096	
Atlantis Prep T3 OBD (100 A, 5 uM, 10x250 mm)	Waters	186008205	
Atlantis T3 column (100 A, 5 uM, 2.1 × 50 mm)	Waters	186003734	
Bruker Avance 600 MHz spectrometer	Bruker		
Buchi Reveleris C18 12g Cartridge	Buchi	BUC145152103	
CCCP	Sigma-Aldrich	C2759	
Celite 545	Sigma-Aldrich	22140-5KG-F	
Cygel	ABCAM	Ab109204	
Elyra PS,1 SIM/STORM confocal microscope	Zeiss		
FM4-64FX, fixable analog of FM™ 4-64 membrane stain	Life Technologies Australia Pt	F34653	
Gallios flow cytometer	Beckman Coulter		
Gamma 2-16 LSCplus lyophilise	CHRIST		
Gilson HPLC 2020	Gilson		
Hanks' Balanced Salt solution, Modified, with sodium bicarbonat	Sigma-Aldrich	H6648-500ML	
Hettich Zentrifugen Rotofix 32	Hettich		
High performance #1.5 cover slips (18 x 18 mm)	Schott/Zeiss	474030-9000-000	
Hoechst 33342, Trihydrochloride, Trihydrate - Fluo	Life Technologies	H21492	
LB	AMRESCO	J106	
Leica STED 3X Super Resolution Microscope with White Light Laser excitation	Leica		
Lysozyme from chicken egg white lyophilized powder	Sigma-Aldrich	L6876	
Mueller Hinton II Broth Cation adjusted	Becton Dickinson	212322	

Propargylamine	Sigma-Aldrich	P50900-5G
Reveleris GRACE MPLC	Buchi	
Shimadzu LCMS-2020	Shimadzu	
Sigma 1-15 Microcentrifuge	Sigma-Aldrich	
Silica gel 60 (0.040-0.063 mm) for column chromatography (230-400 mesh ASTM)	Merck	1093859025
SYTO 9 Green Fluorescent Nucleic Acid Stain	Life Technologies Australia Pt	S34854
TECAN Infinite M1000 PRO	TECAN	

JoVE60743 "Fluorescent Antibiotic Probes for Visualization of Bacterial Resistance"

Rebuttal to Editorial and Reviewer Comments

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please use American English throughout.

This has been done

2. Please ensure that the Summary is within 50-word limit and clearly describe the protocol and its applications in complete sentences.

This has been done

3. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

This has been done

4. Please ensure that the Introduction contains all of the following with citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

This has been done

5. Please include more citations in the introduction section to cover the wider body of literature.

This has been done

6. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Celite, Cygel, etc.

This has been done, with the exception of the discussion on different mounting media and their advantages, which we feel is important

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. We cannot have non numbered step or subheadings. Please do not use bullets.

Non-numbered bullets have been removed

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

The non-bulleted text has been changed to a note

9. Please define all abbreviations during the first-time use.

This has been done

10. Please include volume and concentrations for buffers and reagents used throughout the protocol. Please also include centrifugation speed in x g wherever applicable.

This has been done

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

The non-imperative text has been changed to a note

12. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

This has been done

13. Please ensure you answer the “how” question, i.e., how is the step performed?

This has been done

14. 1.20: How is this done?

More detail has been added

15. 1.29: How do you separate the layers?

More detail has been added

16. 3: Any special strain used? How do you check the mid log phase?

More detail has been added, and the mid-log phase is covered in step 3.3

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing)

that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

This has been done

18. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

This has been done

19. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

This has been done

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

This has been done

21. Please include a better resolution figure.

This has been done

22. As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

This has been done

23. Please include a minimum of 10 citations in the reference section.

This has been done

24. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table alphabetically.

This has been done

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The protocol is interesting for a wider public.

Major Concerns:
I think the authors are well aware that labeling change the permeability and eventually target affinity.

No specific actions were taken in response to Reviewer 1

Reviewer #2:

Manuscript Summary:
Stone et al. describe the procedure of synthesizing and attaching an alkyne-fluorophore, (NBD or DMACA) to an azide-derivative of an antibiotic (linezolid, ciprofloxacin, trimethoprim) and assessing their uptake by bacteria by spectrophotometry, flow-cytometry and microscopy. The procedure is based on previous publications (6-8).

In general the procedure is described clearly-arranged. Overall I was missing the inclusion of controls, namely the free fluorescent dye, in all experiments described. To evaluate any accumulation of fluorophore-antibiotic probe this has to be compared to the accumulation of the free fluorophore (or alkyne-fluorophore) to exclude signals from non-specific binding to the bacterial cell wall. I strongly recommend to include such advise in the description of the method and also in the representative results that are shown.
Suggestion added to paper, but unfortunately it is not within the scope of these revisions to redo experiments, as these are taken from published work. Confocal microscopy has shown no evidence of non-specific binding by free fluorescent dye.

Furthermore I recommend to include a description why these two fluorophores were chosen (stability, low bleaching, low size, high quantum yield, small size, previously tested for non-binding to the bacterial species of interest?). It was well described that it is important to find the right site for attachment of the fluorophore, but there was no description about the choice of fluorophore. Also the impact of introducing a big modification like a fluorophore should be described more properly and the problems that can result (no uptake, no target activity, ..).
Introduction has been re-written and now addresses these concerns.

The introduction is lacking a hint to the latest and numerous publications about uptake measurements by LCMS and is not estimating such methods with their real feasibility. In my opinion the advantage of fluorescent measurements is overestimated in the introduction and discussion, although for sure it is a nice complementary method to aid in the research in the antibiotics field. For antibiotic drug development it is not feasible to apply such method due to the numerous compounds that need to be assessed.
Introduction has been re-written and now addresses these concerns.

Another point is that two fluorophores, NBD and DMACA, are presented for the labeling of antibiotics, but results are only shown for one. It would be nice to see results (LCMS, fluorescence spectroscopy, flow cytometry) from both of them. Otherwise the information should be included why results from only one of them is shown or if there is an advantage of one above the other (compare effectiveness and differences).
We agree that this information would be useful, but the JOVE author instructions ask for a "concise, written description of a representative outcome" rather than a traditional research article where the additional data might be expected. Furthermore, 'the article should be focused on the protocol and not the representative results." The article is focused on the procedure, not the results.

In sum I recommend to consider publication after major revisions as described above, also

after solving the following major and minor concerns:

line 29
In the summary fluorescent antibiotics are described to have a significant advantage over other methods for studying antimicrobial resistance. In my opinion their drawbacks are underestimated in a description like this. Completely neglected are the problems of non-specific binding of the fluorophore to the bacterial cell wall, the possible change/loss of activity, altered uptake behaviour of such fluorescent antibiotics.
The introduction section has been re-written to incorporate these comments.

line 36
I would consider rephrasing and to include the information for the non-expert why MICs are assessed.
This has been done.

line 58
With regard to the use of LCMS for quantifying antibiotic concentrations more citations should be included, giving a better overview about the current development and possibilities of this method. For example:
Prochnow et al., 2018 (DOI: 10.1021/acs.analchem.8b03586)
Bhat et al. 2013 (DOI: 10.1016/j.mimet.2013.05.010)
Cai et al. 2009 (DOI: 10.1016/j.ab.2008.10.041)
Phetsang et al. 2016 (DOI: 10.1021/acsinfecdis.6b00080) --> Publication of own group!
Davis et al. 2014 (DOI: 10.1021/cb5003015)
Iyer et al. 2018 (DOI: 10.1021/acsinfecdis.8b00083)
Richter et al. 2017 (DOI: 10.1038/nature22308)
These have been included.

line 59
...,remains technologically challenging' is to my opinion not completely true considering the amount of recent publications about uptake quantification by LCMS. See previous point and references.
The introduction section has been re-written to incorporate these comments.

line 60
There is a recent publication dealing with subcellular fractionation followed by compound quantification that provides insight into subcellular localization by LCMS: Prochnow et al., 2018 (DOI: 10.1021/acs.analchem.8b03586).
This has been incorporated into the new introduction.

line 60
,Fluorescent antibiotic derivatives are ideally suited to overcoming these limitations and offering more scope for use in AMR... ,
I disagree that fluorescently labelled antibiotics can overcome limitations that there may be for MS. The study of intracellular antibiotic concentration by MS is currently a vivid field and many successful cases have been reported. See citations mentioned before.

The introduction section has been re-written to incorporate these comments.

line 69
The presented probes are not retaining similar antimicrobial activity! Compare to table 1.
We have revised the introduction to remove this claim: the focus of this paper, and this set of probes, is that their reduced activity can be attributed to changes in uptake/efflux.

line 280
Maybe an introductory sentence about sterile working with bacterial cells for the non expert could be helpful.
This has been done.

line 282
I recommend specifying more precisely which bacterial strains are supposed to grow on LB agar because not all of them will do so.
A note has been added expanding on the choice of media.

line 284
Specify which strains grow in CAMHB. The Gram-negative E.coli and Gram-positive S.aureus need different growth media for optimal growth according to my knowledge.
A note has been added expanding on the choice of media.

line 286
Dilute overnight cultures in what?
This has been added.

line 294
Mention to be careful that bacterial cells may settle down if procedure takes too long. This may cause problems with equal seeding.
Detail to ensure mixing has been added.

line 297
Cover with?
This has been added.

line 306
See comment to line 282.
A note has been added expanding on the choice of media.

line 308
See comment to line 284.
A note has been added expanding on the choice of media.

line 312
Depending on the bacterial strain, centrifuge conditions may vary. Also the type of rotor should be mentioned: fixed angle or swinging bucket?

A note about different speeds has been added, and details of the centrifuge added.

line 324
How is it followed what effect the antibiotic has during these 30 min - are cells already dying? PI staining could be implemented as a dead stain control.
This timepoint was selected to provide enough time to see accumulation, while avoiding excessive cell death base on expected time-kill values.

line 329 and 331
Why are you using water here and not PBS?
Water was used in the published procedure, but PBS would be more appropriate. Corrected.

line 389
... repeat step 4.3 --> 5.3 (check numbering)
This has been fixed.

line 428
Why is the median fluorescence intensity used here? Is a broad distribution of values observed from the experiment? If so it could also be helpful to give the adviso to measure more events.
Suggestion included in procedure 4.16.

Looking at figure 3 makes me wonder about the low median fluorescence values. I think it would be good to include the hint to keep an eye on the scatter plots and to carefully compare the results from fluorophore-antibiotic with those from the free fluorophore.
Suggestion included in procedure 4.17.

line 438
The broth microdilution assay conditions were they really exactly the same for E.coli and S.aureus?
Detail has been added about conditions in a note.

line 482
‚Microscopy may also be carried out to visually inspect probe localization in different bacteria'
Maybe it could be helpful here to include the advice to add dyes for colocalization studies in order to assign probe localizaton properly.
This has been done.

line 509
All references should be written with the same style. Some are author et al., some are listing all author names.
This appears to be a problem with the JoVE End-Note style.

Figure 4:
The images from the CIP-DMACA staining seem to be out of focus. As FM4-64FX was also included in 1.) and 2). the resulting images should look similar.

This has been fixed.

In general the fluorescent signal only shows that the fluorophore-antibiotic has the same localization as FM4-64FX. The latter one is a dye for membrane labelling. As the controls for the signal of free fluorophore are missing, it is difficult to judge about the real value of this staining signals and if they show more than a binding of the fluorophore-antibiotic to the bacterial cell wall.
See earlier comment, these experiments have been performed, but for the sake of brevity, the data is not shown here.

Another question is why for confocal microscopy S.aureus was used but all experiments before were conducted with E.coli? For comparability it would be better to show all experiment results from the same species.
E. coli microscopy data has been added.

Reviewer #3:
In this manuscript, Stone et al. report protocols for the preparation of fluorescently labeled antibiotic derivatives using Click chemistry and subsequently detail how these molecules can be used to study how antibiotics interact with bacterial cells.

Major Comments
The "Representative Results" section that is commonly found in JoVE articles is absent from this manuscript. This section is necessary to describe the experiments that are detailed in Figures 2-4. E.g., what's the difference between the wild type and the tolC mutant E. coli and why do they behave differently? How do readers interpret the information on the graphs?
This has been added.

The authors should either expand detail for Step 6 on Confocal Live Microscopy or relegate this content to the discussion as an additional application of their fluorescent antibiotics.
Moved to discussion as requested.

Minor Comments
Reaction schemes detailing each step of the chemical syntheses may be useful for the reader.
This has been added.

There is inconsistent usage of significant figures throughout the manuscript.
This has been corrected

The purification described in steps 1.8, 1.16, and 1.31 should be expanded, such as described at lines 235 and 254.
A note has been added to expand on this method.

What are typical percent yields for each synthesis?
A note has been added about this.

How many bacterial strains have these fluorescent antibiotics been tested on?
A note has been added about this.

Line Comments
27-29: It is not fair to describe fluorescent antibiotics as having a "significant advantage" without direct comparison to other methods.
The introduction section has been re-written to incorporate these comments.

36 & 41: Remove the abbreviations for MIC and AMR as they are not used elsewhere in the abstract
This has been done.

47: Add an "s" to "antibiotic"
This has been done.

49: I would err against using the term "behavior" to describe an effect that an antibiotic exerts
This has been reworded.

52: How do radiolabeled antibiotics and mass spectrometry inform the emerging antibiotic crisis? These approaches provide information on mechanisms of action and resistance, but they do not provide detail at the level of epidemiology.
This has been reworded.

68-70: Discussion of the limitations should be moved to the Discussion Section
This has been done.

71, 105: Can the authors include a description as to why these fluorophores were chosen?
This has been done.

86: What is the pore size of Celite?
This has been added.

219: Add specific references to the alternative Steps that readers can follow
This has been added.

282, 306, 370: What specific bacteria are being used? Don't abbreviate lysogeny broth without writing it fully first. What's the recipe for LB? Also remove the period after "…LB agar".
A note has been added about the types of bacteria used. LB corrections have been made.

284: What is CAMHB and what is the recipe? What volume is the culture?
The full name has been added, with detail as to preparation. Volume added.

286: What volume is the culture?
Volume added.

294: What is the correspondence between OD600 and CFU/mL for your bacterial strains?
This has been added.

297: Are the plates incubated statically or with shaking?
This has been added.

317: Use an equals symbol "OD600 = 2" instead of "OD600 of 2"
This has been done.

329: Why are the bacteria resuspended in water instead of an isotonic solution?
Water was used in the published procedure, but PBS would be more appropriate. Corrected.

406: What is cygel?
A more general term has been used here instead.

422: Remove the hyphen in "wild-type" and italicize tolC. If this is the first instance of E. coli then it should be written out fully.
These have been done.

430: Italicize tolC
This has been done.

433-436: What do each of the stains listed bind to?
This has been added.

445: "tested" instead of "trialed"
This has been changed.

452-454: Is there a "rule of thumb" here for how similar the MIC of the fluorescent antibiotic should be to the parent molecule?
There is no accepted rule of thumb – for our purposes, we believe to fully model the parent antibiotic, the MIC should be within 2-3 dilutions. However, as long as the probe retains some measurable activity, we believe it can still usefully mimic the parent, particularly if alterations in permeability or efflux are found to affect its whole cell activity.

477-478: Is there a reference here?
This has been added.

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

Institute for Molecular Bioscience
Dr Mark Blaskovich

Jaydev Upponi, Ph.D.
Science Editor | Immunology and Infection
Editorial Department
JoVE
1 Alewife Center | Suite 200 | Cambridge | MA 02140 | USA

14 Oct, 2019

re: JoVE60743 "Fluorescent Antibiotic Probes for Visualization of Bacterial Resistance"

Dear Dr Upponi,

Please find attached our re-revised submission describing the synthesis, characterization and microbiological evaluation of fluorescent probes derived from antibiotics. Hopefully we have successfully addressed the remaining editorial concerns. We have added an additional Figure 6 that includes an example of the use of a DMACA fluorophore, as requested. The highlighted area is approx. 2.4 pages, and the protocol section just over 7 pages.

Yours sincerely,

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Figure 4B, 5B, and the 4 left-hand (S. aureus) pictures from Figure 7A

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The material will be used in a methodology article in JoVE (https://www.jove.com/) describing the techniques employing fluorescent probes to image bacterial and measure antibiotic uptake, including a video of the method.

Best regards,

Dr Mark Blaskovich
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Author(s):	M. Rhia L. Stone, Wanida Phetsang, Matthew A. Cooper, Mark A. T. Blaskovich

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