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.