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Cover Letter with Detailed Point-by-Point Responses

Dr. Ronald Myers
Science Editor
Journal of Visualized Experiments

Dear Dr. Myers,

We would like to thank you for the review of our manuscript entitled "***In vivo* immunofluorescence localization (IVIL) for assessment of therapeutic and diagnostic antibody and antibody-conjugate biodistribution in cancer research**" by Jennifer Wischhusen, PhD and Katheryne E. Wilson, PhD for consideration of video production and publication in the *Journal of Visualized Experiments (JoVE)*. The authors would like to thank the reviewers for their thoughtful comments and for their time to help improve our manuscript. Their comments have helped improve the overall clarity and significance of the paper. We addressed all comments and questions that were raised by the reviewers and modified the manuscript accordingly.

To summarize the comments and questions of the three referees, the manuscript was described to **"[tackle] a technical question that has practical and scientific value**", "**could become a helpful reference**", and is "**well-written, clear, and provides an easy-to-follow protocol**". The referees question some technical aspects of our work, ask for additional information, and propose some ideas to be added to the introduction and discussion. We have added technical details, addressed formatting issues, and discussed the mentioned references.

Please find here a detailed point-by-point response (in **green**) to the comments (in **black**):

Editorial comments:

Changes to be made by the Author(s):

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.

We have thoroughly rechecked and proofread the final version of the manuscript.

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

We made sure to define abbreviations on first-time use.

3. 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." However, notes should be concise and used sparingly.

We revised the manuscript accordingly.

4. The protocol should contain only action items that direct the reader to do something.

We revised the protocol section to only enumerate action items and, when necessary, moved additional information into notes.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

We have modified the protocol to attempt to address the “how” question.

6. Step 2: please explain how is this done?

The authors feel section 2 of the **Protocol** is clear. If there is something more specific, or a specific sub step, that needs to be addressed, please let us know and we will correct it.

7. 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 and a maximum of 4 sentences per step

We have revised the **Protocol** to be more discrete steps without large blocks of instructions in the text.

8. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.). Please format step 5 to show actual button clicks, knob turns etc. performed in your experiment.

The authors have clarified the confocal imaging and software **Protocol** with more detailed and discrete steps.

9. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The authors have highlighted 2.75 pages of the **Protocol** in grey in the manuscript meant for video production (Steps 1-4).

10. 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].”

Figure 1. is original data originating from a study that has been previously published.

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The authors feel the Figure 2 caption clearly states this citation and permission, but please let us know if something further needs to be done.

11. Please alphabetically sort the materials table.

The materials table has been sorted alphabetically.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript by Wischhusen and Wilson describes a method for visualizing tumor binding and localization of in vivo administered antibody or antibody-conjugates by ex vivo fluorescence immunohistochemistry. Therefore, the antibody of interest is injected into tumor bearing mice, allowed to circulate for 4 days, then tumors are dissected, and cryosections are subjected to staining with a fluorescence-labeled secondary antibody to visualize the localization of the antibody within the tumor.

Minor Concerns:

1. The title implies an in vivo method of assessing antibody biodistribution, which usually described organ level distribution of antibodies. The method would be better described as ex vivo fluorescence immunohistochemistry to validate delivery and histological localization of in vivo administered antibodies in tumor compartments/tumor microenvironment.

We thank the reviewers for this comment. We chose the term "*in vivo* immunolocalization (IVIL)" to highlight the method's capacity to reveal the *in vivo* distribution/localization of antibody-based contrast agents or therapeutics as opposed to standard *ex vivo* immunofluorescence staining which reveals antigen presentation independent of *in vivo* accessibility. Furthermore, we have introduced this technique in a previous paper using the term IVIL. Thus, we chose the present title.

2. The Short Abstract claims in vivo labeling, this should say in vivo tumor targeting and ex vivo immunostaining.

Thank you for the comment. We have revised the short abstract accordingly.

Long Abstract:

3. It is unclear why this method would be applied to antibody based contrast agents for molecular imaging, as these antibodies are themselves fluorescence labeled (ICG)

The reviewer is correct that the method describes ICG labeling of the primary antibodies. As ICG is a near infrared fluorescent dye, very few microscopy systems are set up with the proper excitation lasers and filter sets to detect such a far red signal. Furthermore, the additional step of secondary labeling allows amplification of the signal from a single primary antibody as additional fluorophores can be added onto a secondary antibody without affecting binding (2-8 per antibody) to allow for increased confocal imaging sensitivity. For these reasons, in the original method it was decided to do additional secondary antibody labeling.

However, the reviewer is correct in suggesting that it is confusing in this case, and therefore this step has been removed from the **Protocol**, also addressing Reviewer #3's concern #5. However, the description of applying the IVIL method to a previously labeled antibody also extends the application to additional antibody based constructs making it more broadly applicable. Therefore the indication in the introduction has been retained.

Introduction:

4. The introduction into antibody engineering and cancer could be shortened. Instead introduce methods used to assess antibody biodistribution in preclinical models and their strength and weaknesses. For detailed tumor localization noninvasive methods (PET, fluorescence) are not sensitive enough and don't provide high enough resolution. Additionally, the signaling moiety (label) might alter biodistribution of the antibody.

The authors agree. We have shortened the involved paragraphs (**Introduction**, paragraphs 1 and 2), and complemented the description of preclinical methods with antibody distribution analysis by molecular imaging (**Introduction**, paragraph 4) and with the reference on immunofluorescence histology proposed by Reviewer #3 (Comment 1).

Important steps missing:

5. As this method is intended to study the antibody distribution within the tumor or in context of tumor structures (vasculature) the authors should explain if they assess the whole tumor slice, tumor margins, invasive margins, tumor histology, tumor penetration depth, distance from blood vessels etc.

Thank you for the useful comment. Indeed, this is a very important consideration and we are happy to add this information to our protocol. For this method, we randomly selected fields of view throughout the whole tumor slice excluding the tumor margins, which are often slightly damaged by the sectioning process on the cryostat. We added this information to the **Protocol** section, at step 5.5.

Reviewer #2:

Manuscript Summary:
The article "In Vivo immunofluorescence localization (IVIL) for assessment of therapeutic and diagnostic antibody-conjugate biodistribution in cancer research" describes an immunofluorescence method to visualize injected antibodies coupled or not the contrast agent ICG, within resected tumor tissues. The article tackles a technical question that has practical and scientific value to assess the biodistribution and processing of antibodies in vivo and facilitate correlative microscopy with immunofluorescence imaging. This article could become a helpful reference after further optimization of the method.

Thank you for your comments addressing the significance of the method.

Major Concerns:

The article presents immunofluorescence images which include controls. This demonstrates a solid technical approach to the topic. However, the protocol needs to be further troubleshooted, and new experiments need to be added to fully validate the protocol.

1. In figure 2, the isotype control is not clean enough to eliminate an issue such as crossreactivity between the rat and rabbit secondaries. The staining procedure may need to include an improved blocking step (fish gelatin? And remove the goat serum?) and secondaries' dilutions need to be optimized. Cross-referencing shows that the dilution of secondaries recommended by Lifesciences is 1/500. It may be a better option to use a streptavidin conjugated secondary with one of the used secondaries rather than the combination of the anti-rat and anti-rabbit secondary antibodies.

Thank you for the comments and questions with regard to the second example of our representative results. We fear our manuscript was not clear enough to prevent confusion and have adjusted the **Representative Results and Figure Legends** to aid in clarification.

The protocol for the second representative result, detection of netrin-1 protein in the tumor endothelium, used different antibody configurations: 100 µg of primary humanized NET1-H-mAb (Netris Pharma) or 100 µg of human IgG isotype control antibody (NBP1-97043, Novus Biologicals) were intravenously injected 24 h prior to tumor collection. Freely circulating antibody was removed by cardiac perfusion with PBS and tumor tissue was isolated, flash frozen, and sectioned at 15 µm thickness on a cryostat. Endothelial cells were labeled with primary rat anti-mouse CD31 antibody (550274, BD Biosciences) followed by secondary Alexa 488-coupled goat anti-rat IgG (A11006, Life Technologies) at 1:500 dilution. To reveal the primary antibody targeting netrin-1, secondary Alexa Fluor 594-coupled goat anti-human IgG (A11013, Life Technologies) at 1:500 dilution was used. To avoid unspecific interaction of the goat secondary antibodies, tissue samples were blocked with goat serum (and BSA), as goat is the host species of the secondary antibodies.

As far as dilution of the secondaries, Life Technologies recommends that each application of the secondary undergo appropriate optimization (reference: https://www.thermofisher.com/antibody/product/Goat-anti-Human-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11013), and for the results in Figure 2., we found that 1:500 was appropriate. We've addressed the comment in **Representative Results**.

The results in Figure 2 demonstrate that the goat anti-human secondary antibody does not bind to rat anti-CD31 antibody as the distribution profiles between netrin-1 and CD31 differ largely. Isotype control signal and CD31 overlap as the isotype control antibody is best retained in the endothelium which is the site of antibody arrival.

2. It would be helpful to add a DAPI staining to facilitate the reading of the tissue morphology and labeling.
   Despite the possibility of crossreactivity, the differences between the signals of CD31 and Netrin after injection are sufficient to support the idea that the approach can be validated after further optimization of the protocol.
   The article would benefit from additional examples to further validate the method. It would be worth considering examples of a therapeutic antibody (PD1/PDL-1?, Her2?) as this topics is well introduced. The image could also include a cancer cell marker (such as CD31) and T cell labeling.

Thank you for the very helpful comment. We fully agree with the reviewer that additional stains are useful to decipher cell morphology. In the **Protocol**, we suggest nuclear (*e.g.* DAPI), vascular (*e.g.* CD31), or cytoplasmic markers (*e.g.* actin) in step 4.9.

We further agree with the reviewer that the proposed method is a good tool to study the distribution of therapeutic antibodies such as PD-1/PD-L-1 or HER2. We added this information to our discussion.

3. Another interesting experiment would be to include an additional staining of the tissue section after resection, using the injected antibody but detected with a differently labeled secondary antibody (or use the unconjugated and the conjugated versions of the injected antibody to facilitate the staining). This would allow to visualize antigen-antibody complexes that were formed by the injected antibody as well as the remaining free antigens which can be detected after resection of the tumor. That would give indication about the penetration of the tumor by the injected antibody or other possible processes. interests would be those associated to immune cells (T cells especially).

Thank you for this interesting comment. A comparison that would give similar information concerning penetration of the injected antibody compared to the target expression, would be to liken images collected between *in vivo* injected antibody labeling and a standard, *ex vivo* applied antibody labeling. This is very useful information for the development of a novel therapeutic or diagnostic approach relying in the accessibility of the antigen by the antibody. For the B7-H3 target (Figure 1.), native immunofluorescence staining was also performed on invasive carcinoma from the MMTV-PyMT mouse without injected primary antibody. We added the comparison images and information to the **Representative Results and Figure Legend** for Figure 1. The distribution patterns of the antibody *in vivo* and the marker on the *ex vivo* tissue are very different. *In vivo*, the antibody strongly binds the vasculature (the first point of contact) and only heterogeneously

extravasated into the tumor tissue, a common problem for antibody-based therapeutics. In standard *ex vivo* immunofluorescence, the B7-H3 target is clearly located on all epithelial cell membranes as well as the vasculature.

Minor Concerns:

4. In Figure 1, there is an issue with aggregates of secondary antibodies for the isotype control in the cancer tissue. Maybe spinning down the secondaries and taking the supernatant for staining would allow to solve this issue. In term of imaging, showing images of full tissue sections in addition to the high resolution ones would strengthen the illustration.

Thank you for highlighting this potential confusion. The secondary solution used to highlight the primary antibodies was the same solution for both the isotype control agent and specific agent (and now *ex vivo* standard IF staining) to allow for comparison of staining. This is noted in the **Protocol** section before step 4.1. The differences in patterns highlights the different *in vivo* biodistribution patterns observed for the different antibodies, representing nicely the working principle of the presented method to distinguish between targeted and background labeling.

As we were interested in the cellular distribution of the B7-H3 and netrin-1 proteins, we focused our studies on higher magnification cellular analysis. Images of full tissue sections require extensive image tiling which limits the number of sections that can be imaged at the same time, and can cause photobleaching of the samples preventing quantitative analysis of fluorescence signal and are therefore not included with this study.

Reviewer #3:
Manuscript Summary:
In this work, the authors have demonstrated a protocol whereby they inject either an anti-B7-H3 antibody or an anti-netrin-1 antibody raised in rabbit into tumor-bearing mice. These primary antibodies are localized ex vivo using fluorescently tagged secondary antibodies after sacrifice, tumor excision, and sectioning. This technique is named In Vivo Immunofluorescence Localization (IVIL). They show representative results for the two antibodies and discuss limitations and potential applications of this technique. Overall, the article is well-written, clear, and provides an easy-to-follow protocol.

Thank you for your kind words concerning our manuscript.

Major Concerns:
1. The use of fluorescent secondary antibodies to stain ex vivo tissues for the localization of therapeutic mAbs or antibody-drug conjugates is a well-established technique. Although the authors do discuss some similar work using fluorescently-labelled primary antibodies or ex vivo analysis via flow cytometry, and the use of a well-established technique does not preclude publication in JoVE, it would be good to cite some works that use a near-identical protocol to the one outlined in this manuscript (ie. Cancer Res. 2018 78(3):758-768).

Thank you for bringing to the author's attention the references and uses of a method similar to the IVIL method. We have included citations to and added a description of the work in the **Introduction**. Our manuscript provides the opportunity to depict further applications of this type of method and to make it more broadly visible to the molecular imaging community and beyond in addition to the immunotherapy community.

2. The authors use the technique to localize antibodies raised in rabbits in a mouse model. However, therapeutic antibodies or antibody-drug conjugates are often species matched (i.e. human antibodies would be used for therapeutic applications in humans). Would this IVIL strategy be useful in this situation where native antibodies introduce significant background staining? Please discuss as a potential limitation.

Thank you for highlighting this interesting detail. We agree that the use of native antibodies that are species-matched and require the use of secondary antibodies that are directed against the model organism (*e.g.* fluorescent anti-mouse Ab in mice) could cause significant background noise and interfere with target detection. We added this limitation to the **Discussion**. Though, the problem could be addressed by using fluorescently labeled primary antibodies even though this in turn might slightly change the biodistribution profile. Additional controls would be required to validate the individual labeling approach.

Minor Concerns:

3. Radiolabeling is perhaps the most common method of determining antibody biodistribution. It would be good to include a discussion of how the IVIL method compares to radiolabeling (advantages and disadvantages).

Thank you for this very good remark that was also listed by Reviewer #1 (comment 4). We discussed the utility of non-invasive molecular imaging techniques such as PET and SPECT in the **Introduction.**

4. Could this method be used to study off-target effects in other tissues (ie. liver) - if yes consider adding optional processing of other tissues as a note to the protocol. The current protocol only indicates removal and sectioning of the tumor tissue.

Thank you for this interesting point. We fully agree that alternative tissues can be collected at the same time as the tumor tissue is recovered. We revised the **Protocol** at step 3.3 accordingly.

5. Consider removing the ICG-labelling of the anti-B7-H3 antibody from the protocol (step 2.2). It is interesting that the authors used this protocol to localize a labelled antibody; however, this step will not be useful for most readers and is confusing because the ICG is not used again during the course of the protocol outlined in this manuscript.

We thank the reviewer for this excellent comment. We agree that the ICG conjugation primarily added confusion to the manuscript (see response to Reviewer #1, comment #3) and is generally not required to perform the IVIL protocol. We have removed this step in the outline and added a comment in the **Discussion** to highlight that performing IVIL on a labeled primary antibody is achievable.

We are grateful to the reviewers for their comments, which we believe have clarified some very important aspects of our work and strengthened the manuscript. If any additional information or clarifications are needed, please don't hesitate to let me know.

We thank you in advance for your consideration of the revised manuscript.

Sincerely,

Katheryne (Katie) E. Wilson, Ph.D.