

**One MedImmune Way**

**Gaithersburg, MD 20878**

**Editor, *Journal of Visualized Experiments***

**Re: Manuscript JoVE58390, revised version**

**Dear Editor:**

**Thank you for giving us the opportunity to revise our manuscript, “Automated Multiplex Immunofluorescence Panel for Immuno-Oncology Studies on Formalin- Fixed Carcinoma Tissue Specimens” (JoVE58390). This is a relatively complex paper, and we did our best to address all comments, suggestions, and concerns raised by the editor and reviewers.**

**We respectfully request you to reconsider our revised manuscript, which has been largely improved thanks to the valuable input from the reviewers. Please find below a point-by-point reply to the reviewers’ comments.**

**Thank you very much for your consideration.**

**Best regards,**

**Jaime Rodriguez-Canales, MD, FEBP**

**Senior Pathologist, Laboratory of Pathology**

**MedImmune**

**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.

**Answer:** The manuscript has been edited by a professional scientific editor.

2. Please remain neutral in tone when discussing commercial products. The accompanying video cannot become an advertisement.

**Answer:** The tone of the manuscript has been revised to comply with this request.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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: Phenochart™, InForm™, PerkinElmer, etc.  
Please reduce the number of instances of "Opal" and “Leica Bond RX” within your text.

**Answer:** All ™ and © symbols have been removed as requested.

4. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.

**Answer:** We have uploaded each individual image as a TIFF file.

5. Figure 2: Please include a scale bar to provide context to the magnification used. Define the scale in the appropriate figure Legend.

**Answer:** We have included a scale bar as requested.

6. Please print and sign the attached Author License Agreement - UK. Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account. Answer: Done.  
7. As some authors are affiliated with UK companies, can you please check whether open access is required by your funding agencies?

**Answer:** This is not required.

8. Keywords: Please provide at least 6 keywords or phrases.

**Answer:** We have provided six keywords.

9. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

**Answer:** The Abstract has been rewritten to address this comment.

10. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

**Answer:** We have rephrased the goal of the protocol in the Abstract and the Introduction.

11. Please define all abbreviations before use (DAPI etc.).

**Answer:** Abbreviations are now introduced at first mention and are used consistently throughout the text.

12. Please change “Tip” to “Note” throughout the protocol.

**Answer:** This has been done throughout the manuscript.

13. Lines 164-175: The Protocol should contain only action items that direct the reader to do something. Please move the materials and equipment information to the Materials Table.

**Answer:** The protocol is now written in the second person as imperative sentences, per the journal’s requirements.

14. 1.4/4.1: Please write the text in the imperative tense

**Answer:** The protocol is now written as imperative sentences.

15. 6.3, 6.4, 7.1, 7.2, 7.4, 7.8, 8.7-8.14, 11.6, 11.7, 12.2-12.4, 12.7, 13.2-13.4, 14.6, etc.: Please write the text in the imperative tense in complete sentences.

**Answer:** The protocol is now written as imperative sentences.

16. For computational steps, please provide software screenshots as supplementary files to match each step.

**Answer:** Screenshots of relevant steps have been included.

17. 9.4: What are the conditions?

Answer: we completed the protocol

18. 10.23-10.27: Please combine these steps into one numbered step.

**Answer:** This has been done as requested.

19. 11.3: Please avoid the use of any pronouns.

**Answer:** The text has been edited to omit the use of personal pronouns.

20. Line 367: Please use a superscripted numbered reference.

**Answer:** The references have been generated within EndNote in the journal’s bibliographic output style (with superscripted text citations).

21. Please include single-line spaces between all paragraphs, headings, steps, etc.

**Answer:** Single line spacing has been used throughout the manuscript.

22. 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.

**Answer:** Highlighting has been added as requested.

23. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**Answer:** Highlighting has been added as requested.

24. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Answer:** Highlighting has been added as requested.

25. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.

**Answer:** Information on critical steps and troubleshooting has been added and the Discussion section rewritten to this purpose.

26. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

**Answer:** References have been edited in EndNote, using JoVE’s bibliographic output style.

27. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

**Answer:** These symbols have been removed from the manuscript.

**Reviewers' comments:**  
  
  
  
**Reviewer #1:**  
Manuscript Summary:  
This manuscript describes a nice workflow of an automated multiplex staining protocol performed on Leica Bond RX system. This to study at PD-L1 and PD-1 expression on cytotoxic T cells, macrophages and tumor cells. Also the proliferation (Ki67) can be studied on tumor cells and T cells. This tremendously reduces the manual multiplex protocol from nearly one week (my experience 3 days) to overnight.  
  
Major Concerns:  
The title: "Optimization" does not fit the actual content of the manuscript. This concerns a protocol/workflow/demonstration of multiplex staining of tissue. I don't agree with the use of the word "Optimization". Therefore, it would be in place to refer to other articles that describe methods of optimizing TSA multiplex fluorescence staining such as the already referred Parra, et al (2017) Scientific Reports, but also Gorris, et al (2017) Journal of Immunology which has not been cited yet.  
The use of drop-controls can be beneficial, but is also quite complex to interpret. It is unfortunate that there is nothing about single stained TSA slides in the protocol.

-**Answer:** We agree with the reviewer and have modified the title accordingly, eliminating the word “optimization.” The Gorris et al reference has also been added.  
  
Minor Concerns:  
In supplemental material it is stated that less spectral bleed, amongst others, is observed between 520/540, 570/620. However, in my experience these are actually the ones that have the most spectral bleed and I actually don't have it at all between 540/570 and 650/690. So, it might also be the case that it really depends on how certain protocols are optimized. I would like to see this emphasized less strong, as it can be user-dependent. Also, no concerns about overexposed images are given that are also major problems to spectral bleed. Please include something about being careful for oversaturated pictures/exposure times.

-**Answer:** We thank the reviewer for this very interesting observation. With our protocols, we have noticed more spectral bleeding in 520/540, but it was interesting to see that other spectral interface can also bleed depending on the protocol and perhaps also the scanner (Vectra seems to be slightly different than Polaris). We have modified the text accordingly, de-emphasizing the ranges of spectral bleed. We have also added a phrase in the Discussion section about overexposure.   
  
Drop-controls are not explained until the supplementary material. I didn't know what they were. Please introduce better or refer to supplementary material.

-**Answer:** We have added a reference to the Supplementary Material, where drop controls are described. We are considering a follow-up study to explain in more detail the drop controls as well as other troubleshooting methods.   
  
Line 178: It should be stated that in this example tonsil is a good control, but for someone who might be eventually optimize a different panel, maybe another tissue type would be a better control

-**Answer:** The text has been changed accordingly.

Line 179: In this case you use lung, but this is also an example. Other tumors/tissues could possibly also be used.

**Answer:** The text has been changed accordingly.

Line 182: "necrosis". Sometimes one would not have any choice but to deal with necrotic areas tumor samples.

**Answer:** We have changed the text, trying to clarify that for technical optimization it may be better to avoid samples with extensive necrosis.

Line 200: "new" is in my system "add"   
Line 201: "for example"  
Line 208: I am not sure if OPAL peroxidase Block should go to hazardous waste, but if so, mention that you can select it to go to hazardous waste container.  
Line 220: explain how to load the base OPAL 7 Multiplex protocol into the Bond RX system.  
Line 240: state that it is optional to bake slides. Sometimes in labs, slides have already been baked or have dried long enough. This also reduces the protocol time.  
Line 270: For the 7ml titration containers the dead volume is nicely mentioned, however not for the 6ml Opal titration tubes, which is 300ml I believe. Please include.  
Line 277: + select a researcher  
Line 282: In my system it says "Test tissue"  
Line 284: "Single" "Routine" (2 separate boxes)  
Line 296: Rx should be RX (please check whole manuscript for this  
Line 299: Preferably select delayed start  
Line 350: Best to focus on the filter where the tumor for example is visualized which is Cy5 in this case. Maybe good to add a phenochart overview of the tumor as a figure.  
Line 367: no ref present

**Answer:** We thank the reviewer for this input and have revised the protocol according to these observations.

Line 456: I think it is unfortunate that singles TSA slides are taken along. This makes it much easier to identify regions where certain proteins are expressed and to find regions to adjust your exposure times on. Drop-controls are a good quality control, but much more difficult to interpret.

**Answer:** We thank the reviewer for these very valuable suggestions. Unfortunately, it is difficult to expand the present protocol to include all the variables in detail. Therefore, we are considering a follow-up publication dedicated to the troubleshooting of multiplex IF.   
  
**Reviewer #2:**  
Manuscript Summary:  
In this technical study, the authors have worked with optimization of Automated Multiplex Immunofluorescence on a Leica Bond RX staining instrument and using a Vectra-Polaris slide scanner and Inform software for multispectral un-mixing (MSI). Optimization of all these three steps are essential to obtain a clean output - that is, complete biomarker visualization and separation. The study focuses on staining of cancer-related cell markers and biomarkers within immuno-oncology. The paper contains detailed methodological procedures and allows others to implement the same or similar antibody panel for automated multiplex staining (on the Leica instrument) and subsequent slide scanning (with the Vectra Polaris / Perkin Elmer instrument) and image analyses - in particular the spectral un-mixing process (using the associated MSI software). The authors state that the duration of the staining procedure is strongly reduced, from days to hours by automation. Automation is a major advantage for reproducibility needed in a clinical diagnostic context.  
The study is well conducted and well written. The drop control studies and the autofluorescence issues are highly relevant and essential control analyses and are included as supplementary material.  
  
Major Concerns:  
The Figure material is however poor. I suggest to extend Figures 1 and 2 to include (smaller) panels with the individual markers (including autofluorescence) in black and white - this allows to examine overlap between biomarkers/fluorescence signals (Suppl Fig 1 does not compensate for this).  
Annotations in the two Figures would also be helpful for readers not specialized in histology - please indicate where is the squamous epithelium, germinal centers and interfollicular areas.

**Answer:** We thank the reviewer for the input and comments. In our revised version, we have included the original TIFF files. We have also added an explanation of the histology in the figure legends.

Please address in the Results or Discussion how compatible the spectral unmixing can work equally well from patient sample to patient sample (the variation in autofluorescence disturbance between individuals/individual samples).

**Answer:** We have added new text in the Discussion section about spectral unmixing (critical steps section).   
  
**Reviewer #3:**  
Manuscript Summary:  
Considering that immunohistochemistry and immunofluorescence (IF) evaluation of combined markers in specimens formalin fixed paraffin embedded (FFPE) has become an important procedure for detection of predictive biomarkers for cancer immunotherapy, Dr Rodriguez-Canales and colleagues propose here a detailed protocol. They employed a multiplex IF staining of malignant specimens with a 6-plex antibody panel including PD-L1, PD1, CD68, CD8, Ki67, AE1/AE3 cytokeratins, and DAPI as a cell marker. They also described several controls and techniques suitable for the optimization and validation of the method.  
  
Major Concerns:  
The protocol makes a great contribution to the detection of predictive markers for immunotherapy. However, some questions about the method need to be better clarified.  
1. Detail on different antibodies and respective clones, standardization such as dilution, specificity and sensitivity are not reported. A table containing such information would be useful to readers.

**Answer:** Tables 3 and 4 now contain the information requested by the reviewer.

2. What are the advantages and disadvantages of the presented method in relation to other current methods?

**Answer:** The reviewer poses an interesting question. The main goal of our study was to present a protocol for multiplex IF, employing this particular method (Opal) rather than to compare it with other methods. We can point out some advantages, such as customization of the panels and QC steps by pathologist. However, some disadvantages include the relatively low number of markers (6 to 8) compared with 20+ by other methods such as CyTOF and IonPath.

3. What is the cost of the equipment and its maintenance for private laboratories and associated with Public Institutions?

**Answer:** This information is available directly from the manufacturer, but as an estimation, a Vectra-Polaris scanner can cost nearly $400,000. Autostainers such as the one described in our protocol are commonly used in pathology laboratories.

4. What is the inter and intraobserver variability? How will pathologists and other specialists be trained to use the equipment?

**Answer:** The reviewer raises an important question. We have previously published an article including variability (Sci Rep. 2017 Oct 17;7(1):13380). We and other laboratories are conducting extensive tests to answer this question.

5. How the authors demonstrate the efficacy of the method?

**Answer:** Overall, the Opal method has been supported by the literature (some of which is included in our references) and is in use in several centers, including MD Anderson Cancer Center, Yale University, and Johns Hopkins University. At MedImmune we are running several projects using the same method described in the paper that unfortunately cannot be disclosed at present.

6. An unbiased discussion of the protocol is necessary

**Answer:** We have revised the Discussion section to incorporate critical points in an unbiased fashion.

7. The efficacy of the protocol must be demonstrated

**Answer:** Please see our answer to point #5.

**Reviewer #4:**   
Manuscript Summary:  
The paper describes a practical approach for automated multiplex IHC protocol using Opal method and LeicaBondRX. It also introduces a new concept of drop control, which is similar to building a spectral library from monoplex slides but with the promise to be a better tool to assess bleed through and umbrella effect in a multiplex setting. There are few points that need to be addressed, although I think it is a good paper to be published after adjustments to be made.  
  
Major Concerns:  
- The paper mentions the use of lung carcinoma cases. I have not seen representative images of the lung cancer cases staining. This is important to have since optimization on a tonsil might be different on a cancer tissue with variable expression of markers.

**Answer:** We thank the reviewer for this observation. We have included Figures 2B, 3, 5, S1, and S2, with images of lung cancer staining.

- Some of the staining problems mentioned in the manuscript are not backed up with evidence (representative images of bleed through or umbrella effect) and how a change in reagent concentration led to a better result.

**Answer:** Figure 4 shows a real case of spectral bleeding from our laboratory. We plan to write a follow-up manuscript focused on troubleshooting; this manuscript shows a protocol that we are currently using for internal studies.

- I did not see a comparison between the use of monoplex (spectral library) stains to assess bleed through and the drop out control. The drop out seems to serve more the umbrella effect. This raises the question, whether the stains were optimized in monoplex to have a signal between 10-20 normalized counts would still lead to an umbrella effect in multiplex setting. If this is not the case, then this might eliminate the need for the drop out control.

**Answer:** We thank the reviewer for making this is very important point. The spectral library is not detailed in our protocol, as our goal is to publish the automated panel protocol that can be replicated by any laboratory investigator. We have decided to present the drop control method in the Supplementary Material, as it has proved to be helpful in troubleshooting or testing a new panel.

- Since there will be always some degree of bleed through or umbrella effect, do the authors foresee a way to quantify the 2 phenomenon and suggest a threshold beyond which, these optimizations are absolutely necessary.

**Answer:** We agree with the reviewer and we are working on a publication focused on image analysis and troubleshooting as a follow-up.

- In the explanation of umbrella effect, the authors believe the effect is due to blocking of antigen. Couldn't this be due to partial saturation of cytosine residues at the site of colocalization.

**Answer:** The reviewer makes a very interesting observation. Our original thinking was that blocking is due to changing the position in the sequence of the markers that were blocked, which sometimes obviated this effect. However, currently our first step is to dilute the TSA, which may also support the hypothesis of cytosine saturation. Additional experiments may be needed to fully answer this question.   
  
Minor Concerns:  
- The details of the protocol seem to be more of a supplemental material.

**Answer:** We thank the reviewer for this comment. Because JoVE is focused on the presentation of protocols for laboratory investigators, we have included important details in the main body of the manuscript.

- The supplemental material discussing the bleed through and umbrella phenomenon would be better suited as part of discussion–

**Answer:** We agree with the reviewer. Due to the length of the manuscript, we kept the Discussion section focused on the main protocol and moved part of the troubleshooting and drop controls to the Supplemental Material.