**Rebuttal letter with point-by-point response to Reviewers/ Editorial team**

**Editorial comments:**  
Changes to be made by the Author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly proofread the manuscript and we are sure that there are no spelling or grammar issues.

2. Please provide an email address for each author.

Please find the email address for each author as following:

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

The keywords for our manuscript are: Multiplexed immunofluorescence, Multispectral imaging, Quantitative analysis, Biomarkers**,** Lymphoma,Digital Pathology.

4. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Histoclear, Dako, Perkin Elmer, Mowiol, Vectra, Microsoft Excel, Graphpad Prism, Excel, Inform, etc.

We have deleted or modified all the commercial language.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Also see examples below:  
1.1-1.3: Please describe how these steps are actually performed. They do not have enough detail to replicate as currently written.

We have modified these parts, please refer to 1.1-1.2 in the modified manuscript. The IHC experiments and cell block preparation mentioned here are performed with standard protocols, and referenced accordingly.

2.1: Please specify the source of tissue samples. What is used to cut into sections? Are the sections placed on slides?

This has been modified in the text. We have used tonsil and lymphoma samples from excision biopsies. Of them, tonsil is our control tissue for protocol optimization. We used a microtome to cut tissue into sections and placed the sections on adhesive microscope glass slides.

2.9(now 2.4): What volume of Dako antibody diluent is used?

This is specified in the text now. The volume of antibody diluent depends on the size of the tissue, ranging from 50 µl (for biopsy samples) to 400 µl (for excision samples or tissue microarray samples).

2.11, 2.13, 2.15, 3.9, etc.: What is the incubation temperature? Please specify throughout the protocol.

All the antibodies mentioned in this manuscript are incubated at room temperature. We have specified this condition throughout the protocol.

Step 4, 6.1.3: Please describe any specific actions being performed here so that we can adequately film this step.

We have expanded on these steps.

5.1.1: What are the criteria for choosing the different filters?

We have included the criteria for filter selection in 5.1.1, and Table 1 was added to illustrate this.

5.1.2: Please specify how to optimize the assay conditions.

We have described how these steps are performed in more detail.

6. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We agree with this comment. We have combined the shorter protocol steps.

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

Single-line spaces between all paragraphs, headings, steps have been included.

8. After you have made all the recommended changes to your protocol (listed above), please re-evaluate your protocol length. There are a 10 page limit for the Protocol, and 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.

We have re-evaluated the length, the protocol is less than 10 pages and the highlighted parts are less than 2.75 pages.

9. 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.

We have ensured that the highlighted part of the step includes at least one action.

10. 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.

We have included all relevant details to perform the highlighted steps.

11. Figures 1, 2, 5: Please include a space between the numbers and their units of the scale bar (i.e., 100 µm).

We have modified all the scale bars in all the figures.

12. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.

We have included the acknowledgements section.

13. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

We have included a disclosures section.

14. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

We have provided the required information in the Table of Equipment and Materials.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
  
I reviewed the manuscript, "Multiplexed fluorescent immunohistochemical staining, imaging and analysis in histological samples of lymphoma".  
  
Below is the comments for each line indicated on JoVE58711R1.

We are grateful to the reviewer for their thorough review of our manuscript, and for all the suggestions for improvement.  
  
Line 102: Transfection to these cells may be a bit tricky. However, using lentiviral particle to knockout or knockdown is also available. This kind of alternate procedure should also be described here.

We thank the reviewer for this comment. We have modified 1.1-1.2 sections.  
  
Line122: The authors may refer Milestone's microwave. However, not all laboratory has same equipment. Therefore, the authors should describe at least the pressure (opened to air or the particular pressure).

We thank the reviewer for highlighting this point. We are using the microwave under normal pressure (opened to air).  
  
Line 125: Again, this sounds like a necessity of buying a material from a particular company. Therefore, this should show H2O2 concentration as an alternate reagent

We agree with this comment. We have included additional note: 3% hydrogen peroxide can be used as an alternate reagent (please refer to 2.4).

Line126: TBS may be different in each lab, therefore the contents should be described.

We thank the reviewer for raising this point. We have provided more details in 2.4.

Line127: What can be an alternate for DAKO diluent? It should be described.

We appreciate the review’s suggestion. Bovine serum albumin can be used as an alternate for DAKO antibody diluent. Please refer to 2.5.  
  
Line128: Each microwave has different power setting. What power of microwave was used in this manuscript? 1100W? 1200W? Which reagent was used?

We thank the reviewer for raising this point. We have added the power of microwave as 800 -1100 Watt.  
  
Lin2 127: and Line 128. If this order, blocking reagent could also be stripped by microwave. This is most probably because these lines are in wrong order.

We thank the reviewer for highlighting this part. The microwave stripping step was indeed in the wrong order, and has been revised.

Line135: This may be "concentrated" antibody from DAKO. It should be noted along with clone number.

We appreciate this comment. We have included the antibody clone number in the table of equipment and material.  
  
Line 139, 141, and many others: washing step is only once? How much volume of TBS was used? No Tween added? (and no need for adding tween?) And Tween should not be added to stripping buffer (safety issue).

We thank the reviewer for highlighting this. We usually perform the washing steps with TBST for three times. Antigen Retrieval buffer is used for stripping. TBST is used as a wash buffer only, and this has been clarified in the manuscript.

Line143: If it does not damage the slide, it is fine. However, for the safety issue, it might not be safe to handle boiled solution. Something about safety should be noted here.

We thank the reviewer for this suggestion. We recommend users to use forceps and heatproof gloves to remove slides into a separate jar of distilled water. Please refer to 2.8.  
  
Line162: antibody incubation time can be variable. It should be mentioned somewhere.

We appreciate the reviewer for raising this point. We have incorporated this into step 2.

Line167: Perkin Elmer's product is named as "Opal"+ number (Opal520 reagent). From this line, it is indicated as "Opal reagent"+ number. As far as this is the product from Perkin Elmer, the product name should be precise.

We agree with this comment. We have modified the reagent name in the table of equipment and material list.   
  
Line233: What are the setting and parameters for this analysis? Especially, since checking cell segmentation does not need pathologist to check, what parameters were used in this study? (especially, typical intensity of DAPI, splitting sensitivity, minimum nuclear size are important.  
And authors should describe if only Vectra can do this or there are some other alternative way to visualized these multiplex IHC samples (stained with Opal dyes). Furthermore, it is also important to describe if image taken by Vectra can be opened by software other than inForm.

We appreciate the reviewer’s comments and have incorporated the changes into the text. We always have trained pathologists to help check cell segmentation. The pathologist needs to review individual images, and decide if cell segmentation is adequate, or if additional tissue segmentation is required to select regions enriched for tumour cells/ stroma/ necrosis. If additional segmentation is required, then select appropriate control regions and check that the image analysis software is able to correctly identify such regions. We have also included a line on other machines and image anlalysis software that can scan and analyze multiplex IHC slides, such as Aperio FL as an alternative for Vectra, HALO from Indica Labs for image analysis1.

The appropriate changes have been made in the text

Figure1 is fine, but Figure 2 should show lymphoma and peripheral tissues.

We appreciate the reviewer for highlighting this. We stained the DLBCL cases using tissue microarray (TMA) sections. The TMA cores are all taken from tumour tissue with the highest tumour content and peripheral issues are not included in our TMA cores. Hence, we are not able to illustrate peripheral tissues for figure 2. However, we have added Figure 6 to demonstrate the staining in a normal tonsil control tissue sample.  
  
**Reviewer #2:**  
  
Manuscript Summary:  
Hong et al, described the multiplex IF/IHC method that recent years quite widely use in the translational research and tried to address a technical difficulty by looking at lymphoma.  
They already tried to provide their protocol on staining and analytic pipeline which is honest, open and generous.

We are grateful to the reviewer for their thorough review of our manuscript, and for all the suggestions for improvement.  
  
Major Concerns:  
  
In general, as a technical paper/protocol paper, i have several concerns about this paper:  
  
1) the image provided not super high resolution, and in fact is quite blurry (maybe due to the copy that I am reviewing)

We apologize for the quality of the first submission. We have provided all the figures in eps format with higher resolution for the revised manuscript.

2) I would suggest the author to provide the same images with higher magnification too.

We appreciate the point being made. In this case, 20x images are routinely used for Vectra imaging, as the quantitative data is similar with 20x and 40x images, but the speed is much faster with 20x for large data sets. We have retained 20x images as they are a more accurate representation of the images that are typically used for quantitation.

3) the protocol is unclear about the difference between multiplex and single, is there any different condition of the same antibody that the authors adopted for single or multiplex? how was the optimization/validation done? on single, or on multiplex; on IHC or on IF? If there is optimization on IHC and IF done, please kindly provide the correspondent image too.

We thank the reviewer for highlighting this. Some of the images are at lower magnification to illustrate the spatial relationship of tumour cells with surrounding microenvironment (i.e. peripheral tissue as requested by reviewer 1). However, we have taken reviewer 2’s suggestion and replaced some images with higher magnification, eg. Figure 5B. In general, the optimization is largely performed at the monoplex IF step, after which the conditions are replicated in the multiplex. Please refer to Figure 6 for multiplex IF optimization images.

4) can the authors show some internal control when they analyse the data? e.g. a cell that not suppose to be expressing one particular marker, will be showing 0 or 0% in their analysis.

We appreciate the reviewer for raising this point. For most lymphoma antigens, we use tonsil slides as our control tissue, where there is an admixture of cells, some of which serve as negative controls. We have added Figure 6 B to show germinal center CD20 positive cells are also positive for BCL6 and Ki67, while being negative for BCL2.  
  
Minor Concerns:  
  
1) I am not sure whether it is limited by the journal, the citation in this article is very limited. many places that needed citation are lacking or insufficient.

We thank the reviewer for this comment. We have added more citations within the manuscript.

2) the citation is obsolete. some of the reference about this technique is almost 10yrs ago which make this paper not so timely. I suggest the authors cite at least some papers on the field from 2017-2018.

We apologize for this oversight. We have added more recent citations in this regard.

**Reference:**

1 Parra, E. R. Novel Platforms of Multiplexed Immunofluorescence for Study of Paraffin Tumor Tissues. *Journal of Cancer Treatment and Diagnosis.* **2** (1), 43-53, doi:10.29245/2578-2967/2018/1.1122, (2018).