26 April 2019

Nam Nguyen, Ph.D.
Manager of Review
JoVE

Dear Dr. Nguyen,

We would like to thank you and the reviewers for your comments and questions concerning our manuscript "An excitation-scanning hyperspectral imaging microscopy method to efficiently discriminate fluorescence signals." The comments received from the reviewers helped us clarify aspects of the manuscript, as well as revise the structure for a more consistent style. Please find below our responses to the comments and suggestions put forth by you and the reviewers. As indicated in the "revisions required" email, we have used the "track changes" feature to draw attention to our edits. Should you have any questions or additional formatting concerns regarding the manuscript, please contact me at your convenience.

Sincerely,

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RESPONSES TO THE COMMENTS OF THE EDITOR:

COMMENT: There are image artifacts in the upper left corner of every microscopy image. Please remove them.

Thank you for bringing this to our attention. We believe that something happened during the conversion from (.pdf) to (.eps). As we do not see the same artifacts in the original (.pdf)s, we have chosen to upload them in that format instead. Please let us know if this problem persists or if you would like the figures saved in an alternative file type.

COMMENT: Please revise the highlighting of the protocol to be 2.75 pages or less. This is a hard production limit to ensure that the videography can occur in a single day.

We have eliminated large portions of the highlighted section that refer to walkthrough of software and have instead indicated the parent function required to progress in the protocol. (e.g. "Unmix the spectral image data.")

RESPONSES TO THE COMMENTS OF REVIEWER 3:

COMMENT: In 4.5.1), the authors mentioned the mitochondrial label may have different optimal staining concentration/time depends on the color or brand. Could they specify the specific color/brand was used here?

We had brand names and colors in the original draft of the manuscript but were advised by JoVE that commercial names shouldn't appear in the body of the manuscript and should be confined to the table of materials appearing at the end. As such, all product names, companies, catalog numbers, and descriptions are included in the table of materials at the end of the manuscript to conform to JoVE criteria.

COMMENT: In 2.1.4.5.6), could the authors explain when should the users consider change the "Skip Fr." setting.

We have added the following note to the instruction of the "Skip Fr." Setting: "Note that it may be useful to selectively skip frames in the event that one or few excitation wavelengths are significantly more powerful than others or if individual excitation wavelengths prove to be especially phototoxic."

COMMENT: in 5.3), the last word should be "sample" not "same".

Thank you for pointing this out. It has been fixed.

COMMENT: In 5.11.2), Could the authors educate the users where to find the detection limit of the camera? The authors used an example of 65,535 in this manuscript, but we know different camera has different properties. One easy way is to open an image in ImageJ and then open the "image-->Adjust-->Brightness & Contrast" window. The maximum pixel that can be set is usually the detection limit.

This is a great point. We had previously failed to mention that a histogram containing information about the dynamic range is inherently displayed in the bottom portion of the main window of MicroManager. However, as this particular substep suggests the user check for overexposure using ImageJ, we have included information to check the detection limit in both programs. Specifically, we have added the note: "Note that the detection limit of the camera depends on the camera itself. The upper limit is displayed on the top right portion of the histogram in the main window of MicroManager. Alternatively, the upper

limit can be determined by opening an image in ImageJ and navigating to Image > Adjust > Brightness/Contrast. Click the "Set" button in the resulting pop-up window, enter an excessively large value (e.g. 999,999) in the blank next to "Maximum displayed value", and click the "OK" button to continue. The maximum value displayed in the new histogram should be the upper detection limit of the camera."

COMMENT: In 6.1.1), Could the authors describe in detail the steps to perform background subtraction in MATLAB or ImageJ.

Rather than describing how to script the necessary code or develop the appropriate linear algebra, we have chosen to include MATLAB code that will be available on our resources website and describe, in detail, how to use that code for background subtraction and image correction, much like we did in section 6.3 to describe the steps for spectral unmixing.

RESPONSES TO THE COMMENTS OF REVIEWER 4:

Thank you for your concise summary.