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Corresponding Author:	Dinesh Jaishankar Northwestern University Chicago, Illinois UNITED STATES
Corresponding Author's Institution:	Northwestern University
Corresponding Author E-Mail:	dinesh.jaishankar@northwestern.edu
Order of Authors:	Dinesh Jaishankar
	Cormac Cosgrove
	Ryan J. Deaton
	I. Caroline Le Poole
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TITLE:

A Rapid Method for Multispectral Fluorescence Imaging of Frozen Tissue Sections

AUTHORS AND AFFILIATIONS:

Dinesh Jaishankar[1], Cormac Cosgrove[1], Ryan J. Deaton[2], I. Caroline Le Poole[1,3]

[1]Robert Lurie Comprehensive Cancer Center and Department of Dermatology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

[2]Department of Pathology, University of Illinois at Chicago, Chicago, IL, USA

[3]Department of Microbiology and Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

Corresponding Author:

I. Caroline Le Poole (caroline.lepoole@northwestern.edu)

Email Addresses of Co-authors:

Dinesh Jaishankar (dinesh.jaishankar@northwestern.edu)

Cormac Cosgrove (cormac.cosgrove@northwestern.edu)

Ryan J. Deaton (rdeaton@uic.edu)

KEYWORDS:

multispectral imaging, frozen tissues, cancer, quantitative pathology, multiplexing, immune profiling

SUMMARY:

We describe a rapid staining method to perform multispectral imaging on frozen tissues.

ABSTRACT:

Multispectral fluorescence imaging on formalin-fixed paraffin-embedded (FFPE) tissues enables the detection of multiple markers in a single tissue sample that can provide information about antigen coexpression and spatial distribution of the markers. However, a lack of suitable antibodies for formalin-fixed tissues may restrict the nature of markers that can be detected. In addition, the staining method is time-consuming. Here we describe a rapid method to perform multispectral fluorescence imaging on frozen tissues. The method includes the fluorophore combinations used, detailed steps for the staining of mouse and human frozen tissues, and the scanning, acquisition, and analysis procedures. For staining analysis, a commercially available semiautomated multispectral fluorescence imaging system is used. Through this method, up to six different markers were stained and detected in a single frozen tissue section. The machine learning analysis software can phenotype cells that can be used for quantitative analysis. The method described here for frozen tissues is useful for the detection of markers that cannot be detected in FFPE tissues or for which antibodies are not available for FFPE tissues.

INTRODUCTION:

Recent advances in microscopic imaging techniques have significantly improved our knowledge and understanding of biological processes and disease states. In situ detection of proteins in tissues via chromogenic immunohistochemistry (IHC) is routinely performed in pathology. However, detection of multiple markers using chromogenic IHC staining is challenging[1] and newer methods to use multiplex immunofluorescence (mIF) staining approaches, wherein multiple biological markers are labeled on a single tissue sample, are being developed. The detection of multiple biological markers is useful, because information related to tissue architecture, spatial distribution of cells, and antigen co-expression are all captured in a single tissue sample[2]. The use of multispectral fluorescence imaging technology has made detection of multiple biological markers possible. In this technology, using specific optics the fluorescence spectra of each individual fluorophore can be separated or "unmixed", enabling the detection of multiple markers without any spectral crosstalk[3]. Multispectral fluorescence imaging is becoming a critical approach in cell biology, preclinical drug development, clinical pathology, and tumor immune-profiling[4-6]. Importantly, the special distribution of immune cells (specifically CD8 T cells) can serve as a prognostic factor for patients with existing tumors[7].

Various approaches to multiplex fluorescence staining have been developed and can be performed either simultaneously or sequentially. In the simultaneous staining method, all the antibodies are added together as a cocktail in a single step to label the tissue. UltraPlex technology uses a cocktail of hapten-conjugated primary antibodies followed by a cocktail of fluorophore-conjugated anti-hapten secondary antibodies. InSituPlex technology[8] uses a cocktail of unique DNA-conjugated primary antibodies that are simultaneously added to the tissue followed by an amplification step and finally fluorophore-conjugated probes that are complementary to each unique DNA sequence on the primary antibody. Both of these technologies enable the detection of four markers plus 4’,6-diamino-2-phenylindole (DAPI) for nuclear staining. Two other approaches for simultaneous multiplex staining are based on secondary ion mass spectrometry[9]. The Hyperion Imaging System uses imaging mass cytometry[10] to detect up to 37 markers. This technology uses a cocktail of metal-conjugated antibodies to stain the tissues, and specific areas of the tissues are ablated by a laser and transferred to a mass cytometer where the metal ions are detected. Another similar technology is the IONPath, which uses multiplexed ion beam imaging technology[11]. This technology uses a modified mass spectrometry instrument and an oxygen ion source instead of laser to ablate the metal-conjugated antibodies. While all these simultaneous multiplex staining approaches enable the detection of multiple markers, the costs involved for conjugating DNA, haptens, or metals to antibodies, the loss of tissue due to ablation, and the extensive image processing for unmixing cannot be underestimated. Moreover, kits and staining protocols are currently available only for FFPE tissues and developing custom panels entails additional time and expenditure.

The sequential multiplex staining method, in contrast, includes labeling the tissue with an antibody to one marker, stripping to remove the antibody, followed by sequential repeats of this process to label multiple markers[12]. The tyramide signal amplification (TSA) is the most frequently used sequential multiplexing method. Two other multiplexing technologies use a combination of simultaneous and sequential staining methods. The CODEX platform[13] employs a cocktail of antibodies conjugated to unique DNA oligonucleotide sequences that are eventually labeled with

a fluorophore using an indexed polymerization step followed by imaging, stripping, and repeating the process to detect up to 50 markers. The MultiOmyx multiplex staining approach[14] is an iteration of staining with a cocktail of three to four fluorophore-conjugated antibodies, imaging, quenching the fluorophores, and repeating this cycle to detect up to 60 markers on a single section. Similar to the simultaneous multiplex staining method, while a broad range of markers can be detected, the time involved in staining, image acquisition, processing, and analysis is extensive. The stripping/quenching step involves heating and/or bleaching the tissue sample and thus, the sequential multiplex staining approach is commonly performed on FFPE tissues that maintain tissue integrity upon heating or bleaching.

Formalin fixation and subsequent paraffin embedding is readily performed in a clinical setting, tissue blocks are easy to store, and several multiplex staining protocols are available. However, the processing, embedding, and deparaffinization of FFPE tissues, as well as antigen retrieval[15], a process by which antibodies can better access epitopes, is time-consuming. Furthermore, the processing involved in FFPE tissues contributes to autofluorescence[16] and masks target epitopes, resulting in the variability and lack of antibody clone available to detect antigens in FFPE tissues[17-19]. An example is the human leukocyte antigen (HLA) class I alleles[20]. In contrast, snap freezing of tissues does not involve extensive processing steps prior to or after fixing, circumventing the need for antigen retrieval[21,22], and making it beneficial for detecting a wider range of targets. Therefore, using frozen tissues for multispectral fluorescence imaging can be valuable to detect targets for preclinical and clinical studies.

Given the abovementioned limitations when using FFPE tissues, we asked whether multispectral fluorescence imaging can be performed on frozen tissues. To address this question, we tested a simultaneous multiplex staining method using a panel of fluorophore-conjugated antibodies to detect multiple antigens and analyzed the staining using a semiautomated multispectral imaging system. We were able to simultaneously stain up to six markers in a single tissue section within 90 min.

PROTOCOL:

Mouse spleen and HLF16 mouse tumor tissues[23] were obtained from our laboratory. Human tonsil tissue was purchased from a commercial vendor. Details are provided in the **Table of Materials**.

1. Tissue embedding

1.1. Embed fresh tissue in OCT (optimal cutting temperature) solution and snap freeze using either dry ice or liquid nitrogen.

1.2. Store tissues at -80 °C.

2. Cryosectioning

2.1. Cut 8 µm sections in a cryostat with temperatures set at -25 °C.

NOTE: The preferred section thickness can be adjusted to generate crisper images.

2.2. Place sections on charged glass slides.

2.3. Air-dry the sections for 1 h at room temperature (RT) prior to fixing in histology grade ice-cold acetone for 10 min.

NOTE: Acetone causes coagulation of water-soluble proteins and extracts lipids but does not impact carbohydrate-containing components. In contrast, formalin preserves most lipids and has little impact on carbohydrates[24]. The choice of fixative is important depending on the choice of marker being detected.

2.4. Store the slides at -20 °C.

3. Selection of antibodies and fluorophores

NOTE: Before tissue staining, antibody clones that will robustly and specifically stain their antigens of interest within sequential sections from acetone fixed tissue must be validated. Some antibodies may require a different fixative, and their compatibility with other antibodies in the panel will also have to be empirically determined. The goal is also to identify fluorophores with minimal overlap that can be detected with the epifluorescence filters for DAPI, FITC, Cy3, Texas Red, and Cy5.

3.1. Confirm staining by conventional IHC or immunofluorescence (IF) detection in tissue sections with known expression target antigen.

3.2. Using the excitation and emission filter sets available on the semiautomated imaging system and after testing various combinations of fluorophore-conjugated primary antibodies, prepare fluorophores to be used that have minimal spectral overlap (e.g., see **Table 1**).

4. Staining

NOTE: The tissue rehydration and slide washes were performed in a Coplin jar. The blocking and antibody incubation steps were performed in a humidified slide box.

4.1. Allow the slides to warm to RT for 5–10 min.

4.2. Rehydrate in phosphate buffered saline (PBS) for 5 min.

4.3. Perform a blocking step prior to staining tissues with antibodies. For mouse sections, use specialized blocking solution (see **Table of Materials**) for 10 min at RT. For human sections, use 10% normal pooled human serum (NHS) diluted in PBS for 15 min at RT.

NOTE: Different blocking buffers may be tested as needed to preserve the specific properties of the sections depending on the follow-up procedures to be used.

4.4. Wash the slides for 5 min in PBS after blocking.

4.5. For multiplex staining, prepare a cocktail of antibodies with compatible fluorophores at predetermined optimal dilutions.

4.6. Add the cocktail of fluorophore-conjugated antibodies to the slides. For single-marker staining, add only the primary-conjugated antibody to the slide.

4.7. Include a control unstained slide that undergoes the same staining procedure without the addition of any primary-conjugated antibody.

4.8. Incubate the slides for 1 h at RT in the dark and then wash the slides 2x with PBS for 5 min each. From here on, the resulting slide is referred to as multiplex-stained.

4.9. To counterstain, add DAPI to the multiplex-stained slide, incubate for 7 min in the dark at RT, and wash the slides 2x with PBS for 5 min each. Do not counterstain single-stained and unstained slides.

4.10. To coverslip, add a drop of the mounting medium, and gently place the glass coverslip over the tissue.

5. Preparing a spectral library

NOTE: The following steps are performed in machine learning software (see **Table of Materials**), using the single stained and unstained slides to verify specific staining as well as to determine antibody cross talk.

5.1. Image acquisition

5.1.1. Set the lamp power to 100%. Usually the power is set to 10% because fluorescence detection on FFPE tissues includes a signal amplification step.

5.1.2. Begin by opening the microscope operating software (see **Table of Materials**).

5.1.3. Select **Edit Protocol** and then **New Protocol**.

5.1.4. Provide a "Protocol Name" and "Study Name" and select **Fluorescence**.

5.1.5. Place the single-stained slides on the stage and examine each marker in its corresponding fluorescence channel to ensure staining. Choose a region on the tissue expressing the strongest signal for the marker.

5.1.6. Adjust the exposure times using the **Autofocus** and **Autoexpose** options.

5.1.7. Acquire snapshots for the single-stained and unstained slides and save the protocol.

5.1.8. Under the **Build Libraries** tab in the software, load each single-stained slide image, choose the appropriate Fluor, and click **Extract**. The software will automatically extract the fluorescence signal chosen in the Fluor.

5.1.9. To save the extracted color, click on **Save to Store**. A "New Group" may be created or the extracted color can be stored to an existing group.

5.2. Verifying the spectral library

5.2.1. Check the emission spectral curve window, located to the right of the extracted image, for each filter set.

NOTE: The extracted signal is correct if the spectral curve is observed only in the filter sets where the fluorophore is detected. If a spectral curve is observed in the wrong filter set, it can mean that either the primary signal expected in the filter set is not strong enough, or the software is detecting another signal that is too high, possibly due to spectral overlap. In this case, first try using the **Draw Processing Regions** tool to draw regions around areas expressing the fluorescent marker and autofluorescence in the image. This trains the software to detect the true signal and remove any interfering signals. If this does not work, repeat the staining process for the single-stained slide to test different antibody titrations.

6. Multispectral imaging

NOTE: Once the spectral library is created and verified, perform the following steps for the multiplex-stained slide.

6.1. Whole slide scan

6.1.1. Adjust focus and exposure times on the multiplex-stained slide as mentioned under in section 5.1**.**

6.1.2. Under **Scan Slides**, create a **New Task** and choose the protocol saved above.

6.1.3. Perform a whole slide scan on the multiplex-stained slide.

6.1.4. Using the whole slide scan software, open the whole slide scan image. This image has not been spectrally unmixed.

6.1.5. Select regions of interest (ROI) across the whole slide scan image using the **Stamp** or **ROI** tool. These ROIs will be saved as an msi (multispectral image), for spectral unmixing and analysis.

6.1.6. Click **Process Slide** to acquire ROIs at 20x magnification

6.2. Spectral unmixing

6.2.1. Once the spectral library is created, under the **Manual Analysis** tab, load the multiplex-stained images by clicking **Open** under **File**.

6.2.2. In the **Spectral Library Source** dropdown menu click **Select Fluors**.

6.2.3. A new window will open. Here, choose the spectral library or group created above.

6.2.4. Load the unstained slide image. Click the **AF** ink marker icon located above the selected spectral library and draw a line or region on the unstained slide to identify autofluorescence in the tissue.

6.2.5. Under the **Edit Markers and Colors** tab, assign names for each marker. Pseudo colors can be assigned at this step.

NOTE: The color for the Autofluorescence image defaults to DarkSlateGray. Change this to Black.

6.2.6. Click **Prepare All**.

6.3. Verifying spectrally unmixed images

NOTE: When the spectral unmixing step is completed, a composite image consisting of all the colors is created.

6.3.1. Click the **Edit the View** eye icon. Here, each color in the "Component Display" can be turned off or on to view the staining of each individual marker.

6.3.2. Visually inspect the staining and the morphology of the cells to ensure that there is no overlapping of marker, unless it is biologically relevant. A pathologist can help verify the staining as well.

NOTE: The staining on the multiplexed slide should be validated by leaving out one fluorophore at a time and reviewing the staining pattern. In addition, the validation will also help to identify strong fluorophores that appear in adjacent spectra due to antibody cross talk or bleed-through.

6.3.3. For **Pathology Views**, which simulate brightfield images for each fluorescent marker, click the **Select a Component Image** button. Here, choose a marker to view a simulated brightfield image.

7. Analyzing multispectral images via cell segmentation

NOTE: After verifying the spectrally unmixed image, cell segmentation can be performed using the machine learning software, which will provide step-by-step instructions. Tissue segmentation was not performed here. If the panel includes one or more tissue specific marker and especially if the tissue is messy, tissue segmentation should be performed.

7.1. Select a marker from the panel. Configure the marker to detect either nuclei, cytoplasm, or membrane. For example, DAPI can be selected to detect nuclei.

7.2. Select the option to "**Use the Signal to Assist in Nuclear Splitting**".

7.3. The software automatically detects and labels the nucleus, cytoplasm, and membrane in the image and creates a cell segmentation map. Ensure all the cells are segmented. To adjust, use the configuration options in the software.

7.4. After cell segmentation, proceed with phenotyping cells. In this step, choose the markers needed for phenotyping and manually select at least five cells that are brightly stained with the chosen marker. This trains the software to then automatically detect all cells stained with the chosen marker in the image.

7.5. A phenotype map is created. Analyze to ensure that the cell stained with the marker is correctly phenotyped.

NOTE: Cell phenotyping can be an iterative process. If the software is unable to phenotype the cells correctly, it means that the training is inadequate or incorrect. In this case, the user has to manually select more cells and retrain the software and repeat this step until the user is satisfied with the training.

7.6. Create a group named "Others" and include cells that are not stained for any of the markers.

NOTE: This step is important to train the software to exclude all the unstained cells from phenotyping.

8. Exporting images and analysis tables

8.1. Click the **Export** button to view the **Export Settings** panel.

8.2. In the "Export Directory", click **Browse** to select a location to export the images.

8.3. In the "Image Export Options", choose the **Image Output Format**.

8.4. In the "Images to Export" list, select the images to be exported. "Composite image" is the final pseudocolored unmixed image. "Pathology Views" are the individual simulated brightfield images and the "Component Images (multi-image TIFF)" is a multi-image TIFF file of component data that can be used by third party analysis software.

8.5. Click "**Export for All**" button to export the images.

NOTE: Tables from analysis can also be selected and exported at this step.

REPRESENTATIVE RESULTS:

Detection of single-stained markers on frozen spleen sections

As the semiautomated imaging system uses a liquid crystal tunable filter (LCTF) system that allows for a wider range of wavelength detection[25], and because no signal amplification steps were performed here, we first optimized the detection of our primary-conjugated antibodies for each marker on the microscope. An example is shown in **Figure 1**, where each single-stained marker is pseudo-colored red. The Alexa Fluor conjugated antibodies used here have been validated by the companies for immunofluorescence and flow cytometry. However, the Per-CP-Cy5.5 fluorophore is only validated for flow cytometry. We were able to detect this color in our single-stained slides, supporting the suitability of flow cytometry validated antibodies for use in multispectral imaging and providing the benefit of using such antibodies to validate observations using two different techniques (i.e., flow cytometry and multispectral imaging). We then proceeded to perform multispectral imaging on frozen tissues.

Multicolor fluorescence detection on frozen mouse spleen

To test the multiplex staining method and spectral imaging, we used mouse frozen spleen tissue, which has an abundance of immune cells. **Figure 2** shows a spectrally unmixed image of different markers in a section of frozen mouse spleen. The antibodies, clones, and concentrations used for staining are described in **Table 2**. The captured image shows a portion of the T cell zone identified by the presence of CD3, CD4, and CD8 markers, surrounded by myeloid cells expressing CD11b mostly observed in the marginal zone. Regions of proliferating cells expressing Ki67 are observed primarily in germinal centers. The "Pathology Views" option for each marker showed its individual staining pattern within the tissue. Distinct staining patterns for CD11b, Ki67 and the CD3, CD4, and CD8 markers together were observed, suggesting that the multiplex staining method and spectral imaging worked on the frozen tissue.

Multi-color fluorescence detection on frozen human tonsil

After testing a staining panel suited for mouse tissue, we next assessed a separate panel for frozen human tonsil tissue. **Figure 3** shows a spectrally unmixed image of the different markers used. The antibodies, clones, and concentrations used for staining are described in **Table 2.** The captured image shows a follicular germinal center[26] expressing B-cells identified by the CD20 marker. Proliferating cells in the follicular germinal center were identified by Ki67. Some of these proliferating cells costained with CD20 and may be centroblasts[27], naïve B-cells that undergo active somatic hypermutation. The follicular germinal centers were surrounded by the interfollicular T cell region identified by the expression of CD3, CD4, and CD8. Again, distinct

staining patterns were observed here, confirming that the methodology worked on a frozen tissue.

Application of multispectral fluorescence imaging on frozen mouse tumor tissue

Multispectral imaging is a useful tool for monitoring immune cell infiltration in tumors as a prognosis for immunotherapies. To this end, we set out to stain a frozen mouse tumor tissue sample and detect immune cell infiltrates. The HLF16 cell line is used as a human papillomavirus $(HPV)^+$ tumor model of cervical cancer in HLA-A*0201 transgenic mice[28]. The transgenic cell line was developed by transfecting heart lung fibroblasts from HLA-A*0201 with HPV16 E6 and E7 oncogenes and H-Ras V12[28]. T cells and tumor associated macrophages are the most common immune infiltrates present in tumors[29]. **Figure 4** shows a spectrally unmixed image on a frozen HLF16 tumor section along with the pathology views; the individual staining patterns are shown in **Supplementary Figure 1**. The antibodies, clones, and concentrations used for staining are described in **Table 2.** The captured image shows regions of tumor-infiltrating T cells identified by the CD3 and CD8 markers along with the presence of other myeloid cell lineages identified by the CD11b marker. The captured region also shows tumor associated macrophages (TAMs), possibly of the M2 phenotype, detected by the CD206 marker[30], which is closely associated to several proliferating cells detected as $Ki67^+$.

The machine learning software[2] comes with features like tissue and cell analyses. These analyses are commonly performed on FFPE tissues stained with signal amplification. As our methodology does not use signal amplification, we wanted to test if the software could be used to analyze staining on frozen tissues. Using the adaptive feature of the software, we were able to segment cells based on a nuclear and membrane marker and to phenotype cells based on the markers used for staining. **Figure 5** shows the cell segmentation and phenotype maps and the number of stained cells for each marker analyzed by the software, demonstrating that the software can be used for quantification of multispectral staining on frozen tissues.

FIGURE AND TABLE LEGENDS:

Figure 1: Detecting primary-conjugated antibodies using a liquid crystal tunable filter microscope. Primary-conjugated antibodies to the indicated markers were stained on a frozen mouse spleen and detected using the Vectra 3.0 multispectral imaging system under 20x objectives. CD3 on Alexa Fluor 488, CD8 on Alexa Fluor 594, CD11b on Per-CP Cy5.5, CD206 on Alexa Fluor 647, and Ki67 on Alexa Fluor 555 were used. Each marker is pseudo-colored red. No counterstain was used on these slides. Scale bar = 20 µm.

Figure 2: Multispectral imaging on a frozen mouse spleen. (**A**) Whole slide scan of the multiplexed stained slide taken under 4x objectives. A 2 x 2 stamp across different regions of the tissue were chosen for multispectral imaging. Scale bar = 100 µm. (**B**) Composite image taken under a 20x objective after spectral unmixing. The pseudo-colored markers are indicated and a magnified image within the red box is shown next to the image. Scale bar = 20 µm. (**C**) Pathology views for each individual marker. A magnified image for each marker within the red box is shown next to the image. Scale bar = 20 µm.

Figure 3: Multispectral imaging on a frozen human tonsil. (**A**) Whole slide scan of the multiplexed stained slide taken under a 4x objective. A 1 x 1 stamp (669 µm x 500 µm) across different regions of the tissue was chosen for multispectral imaging. Scale bar = 100 µm. (**B**) Composite image after spectral unmixing taken under a 20x objective. The pseudo-colored markers are indicated and a magnified image within the red box is shown next to the image. Scale bar = 20 µm. (**C**) Pathology views for each individual marker. A magnified image for each marker within the red box is shown next to the image. Scale bar = 20 µm.

Figure 4: Multispectral imaging on a frozen mouse tumor. Multispectral imaging was performed on a 1 x 1 region of a frozen mouse HLF16 tumor taken under a 20x objective. The composite image (above). The pseudo-colored markers are indicated and the pathology views (below) for each individual marker is depicted. A magnified image for each marker within the red box is shown next to the original image. Scale bar = 20 µm

Figure 5: Analysis of a frozen mouse tumor section. (**A**) Cell segmentation map. Adaptive cell segmentation using inForm was performed. The software was trained to identify nuclei (green) and membrane (red). (**B**) Phenotype maps for each stained marker. The software was trained to identify phenotypes based on the staining. Colored dot represents the indicated marker. "Other" (black) refers to cells that were not stained for the indicated marker. (**C**) Bar plot (mean ± STDEV) depicting the number of stained cells for each marker in two multispectral images. The number indicates cells phenotyped in each image but does not indicate if the markers are coexpressed.

Table 1: List of fluorophores with their maximum excitation and emission wavelengths and expected detection in appropriate filter sets.

Table 2: List of antibodies, clones, and concentrations used.

Supplementary Figure 1: Individual staining patterns of the frozen HLF16 tumor after spectral unmixing. Scale bar = 20 µm

DISCUSSION:

Frozen tissues have extensively been used for mIF imaging to traditionally detect three to four markers[31] on a tissue using the direct and indirect method[32]. In the direct method, antibodies are conjugated to fluorescing dyes or quantum dots[33] to label the tissue, whereas in the indirect method, an unconjugated primary antibody is used to label the tissue followed by a fluorophore-conjugated secondary antibody that specifically recognizes the primary antibody. Some of the recent simultaneous multiplex staining approaches discussed earlier can also be used to stain frozen tissues and detect more than four markers. But the cost for the reagents and the time taken for staining become intensive depending on the number of markers being detected. Another multiplex technique for frozen tissues is the multi-epitope-ligand-cartography (MELC)[34]. The technique involves staining the sample with fluorophore-conjugated antibodies, imaging, and photobleaching of the fluorophore. A major caveat of this technique is that only one field or region of the tissue can be multiplexed. In order to multiplex other fields or regions the technique needs to be performed manually, which is time-consuming, or requires automation, which is

costly. One group[35] was able to detect six colors on frozen tissues using a combination of direct, indirect, and TSA staining. However, the tissue staining took 2 days. In comparison, our methodology uses a simultaneous multiplex staining method involving the application of a cocktail of five directly conjugated antibodies plus DAPI to stain frozen tissues within 90 min. Furthermore, using a semiautomated multispectral fluorescence imaging system, we were able to spectrally separate and detect six markers in frozen spleen, tonsil, and tumor tissues using this simplified multiplex staining technique. The Cy3, Texas Red, and the Cy5 filter sets available on the microscope provide opportunities for detecting additional fluorophores, thereby potentially increasing the number of markers that can be detected simultaneously in frozen tissues.

Multiplex staining using the TSA approach on FFPE tissues requires an antigen retrieval step followed by sequential labeling, washing, and stripping steps. Performing the procedure ranges from 1–2 days depending on the incubation times used for the antibodies[6]. Recently, using a microfluidic tissue processor, a four-plex staining using the TSA approach was performed on FFPE tissues under 90 min. Other multiplex techniques for FFPE tissues can be performed under 4-5 h using an automated stainer. However, the appropriate antigen retrieval steps need to be optimized to ensure epitope availability for the antibodies[36], which in turn relies on the careful consideration of the antibody clones as well as the order of markers being detected. For example, some antibody clones of CD3 cannot be used, because subsequently CD4 and CD8 antibodies are not detected[37]. Similarly, there is variability of antigen detection among the available antibody clones[17,18]. Therefore, multiplex staining of FFPE tissues requires optimization of antibody clones, their concentrations, and the order in which they are stained, all of which is time-consuming. In contrast, the lack of extensive tissue processing of frozen tissues allows for the use of various antibody clones with high specificity. Moreover, antibody clones available for frozen tissues can also be used in flow cytometry and ELISA, allowing simultaneous validation across various assays. Tissue architecture is also a concern in frozen tissues[38]. The multiplex staining shown here on frozen tissues is significantly faster than the TSA approach. The fluorophore combinations require a careful selection of markers to ensure that antibodies do not sterically hinder each other, especially when different antigens expressed in the same cellular location are detected. We chose markers that stain different cells on fluorophores that are spectrally separate, enabling better detection. The antibody concentrations also require optimization, but because the staining protocol is quick, the overall time taken for optimization is not time-consuming. A caveat to our method may be the inability to detect low expressing markers in tissues. Some of the multiplex staining approaches on FFPE tissues involve a signal amplification step that is useful to detect low expressing markers. However, the use of secondary and tertiary antibodies can be employed to boost the signal. In such cases, cross reactivity to antibodies in the panel should be avoided to prevent incorrect interpretation of the results.

In addition to mouse spleen and human tonsil tissues, which are rich in immune cells, we have used tumor tissue as an example for the proposed multispectral fluorescence imaging of frozen tissues. Multispectral fluorescence imaging in tumors has provided valuable information, such as characterizing the tumor microenvironment (TME)[39], predicting the success of adoptive T cell transfers in malignant melanoma[40], and characterizing proteins in tumor signal transduction pathways[41]. Using markers specific to immune cells commonly found in tumors, we were able to

detect infiltrating CD8 T cells and TAMs in the HLF16 tumor tissue used in this study. We used the machine learning software to successfully segment and phenotype cells. Multiplex staining for FFPE tissues employs a signal amplification step to enhance signal-to-noise (SNR) ratio that helps image processing and quantification[42,43]. The machine learning software has been used for analyses on FFPE tissues stained with signal amplification[2]. The methodology present here does not use signal amplification, but we were able to successfully segment and phenotype cells using the software. For further analysis (e.g., phenotype coexpression and spatial relationships) and accurate interpretation of the quantitation, the software training requires validation using a training set, test set, and validation set. In an iterative process, one set of images (i.e., the training set) is used to train the machine learning software to identify the phenotypes until the model's predictions for a separate set of images (i.e., the test set) are accurate. After training is complete, a final set-aside batch of images (i.e., the validation set) is analyzed to see if there has been over-fitting.

In conclusion, the methodology presented here is a rapid way of performing multispectral fluorescence imaging using frozen tissues. The method is useful to detect markers to which either antibodies are not available or cannot be detected in FFPE tissues. In conjunction with the machine learning software, the time taken to perform quantitative analyses can significantly facilitate rapid preclinical and clinical diagnoses and may be applied in the field of high-resolution spatial transcriptomics that utilizes frozen tissues[44].

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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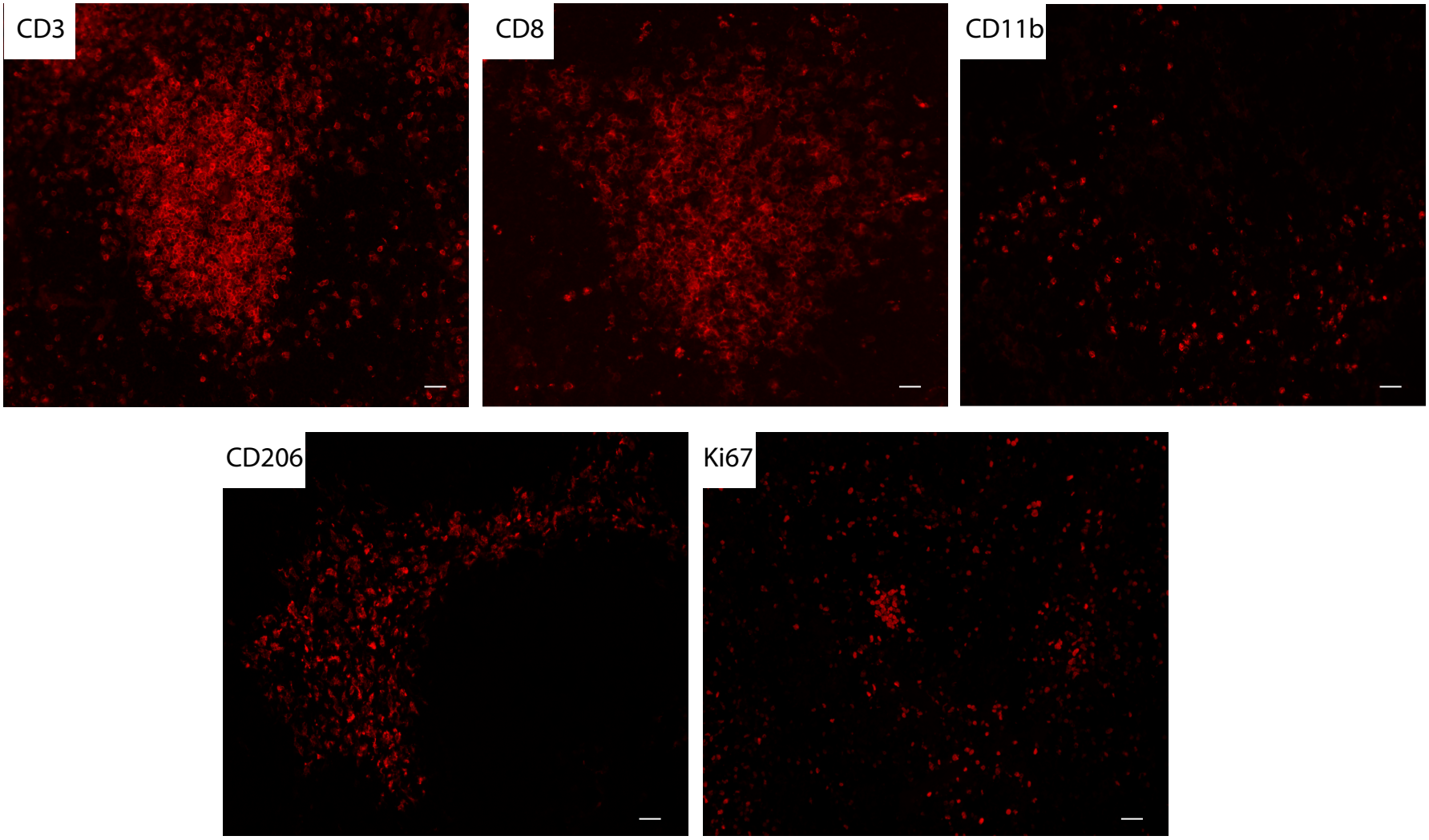
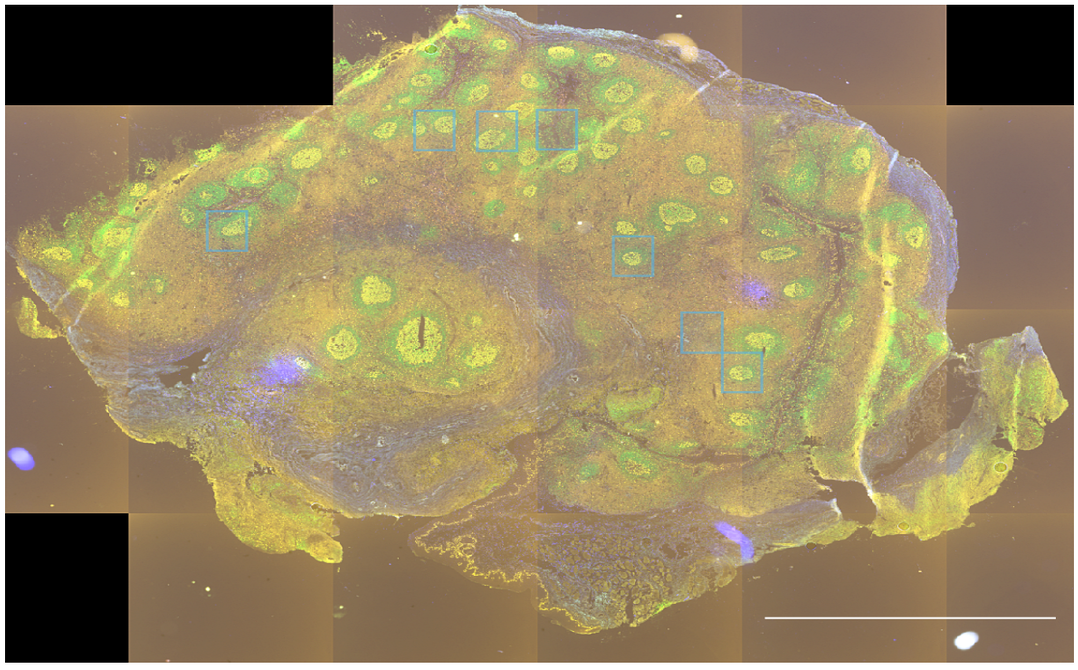
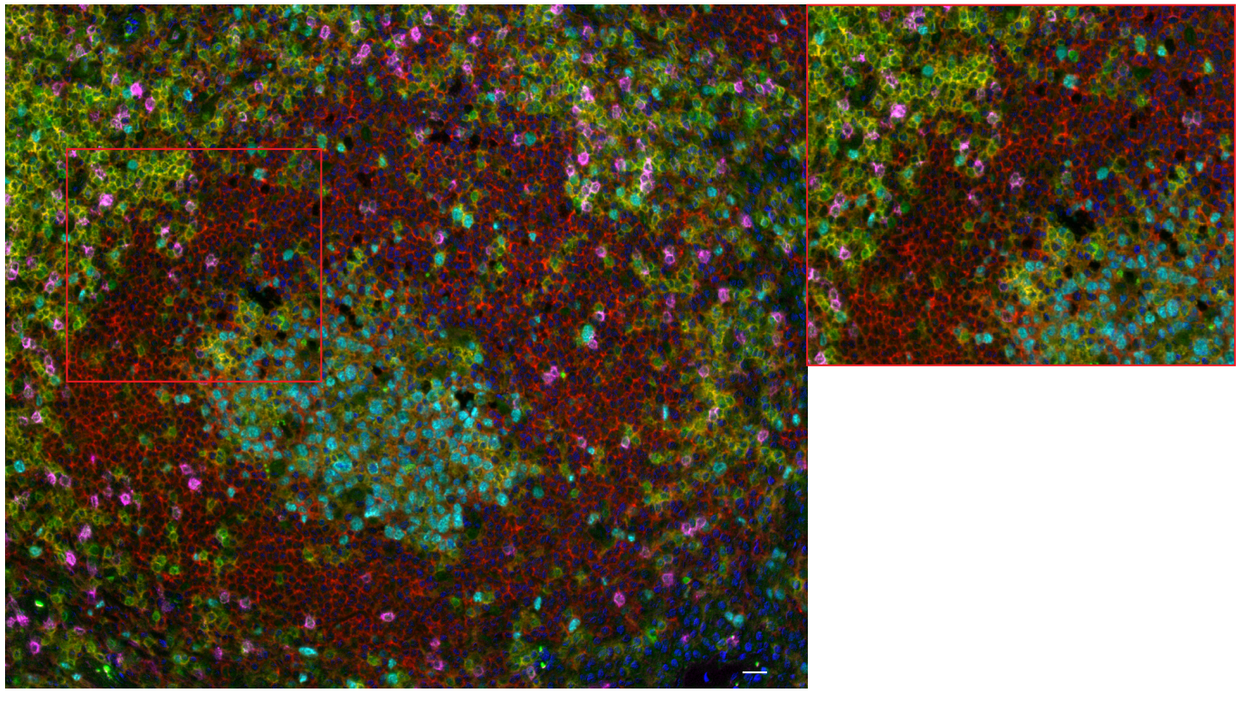


Figure 3





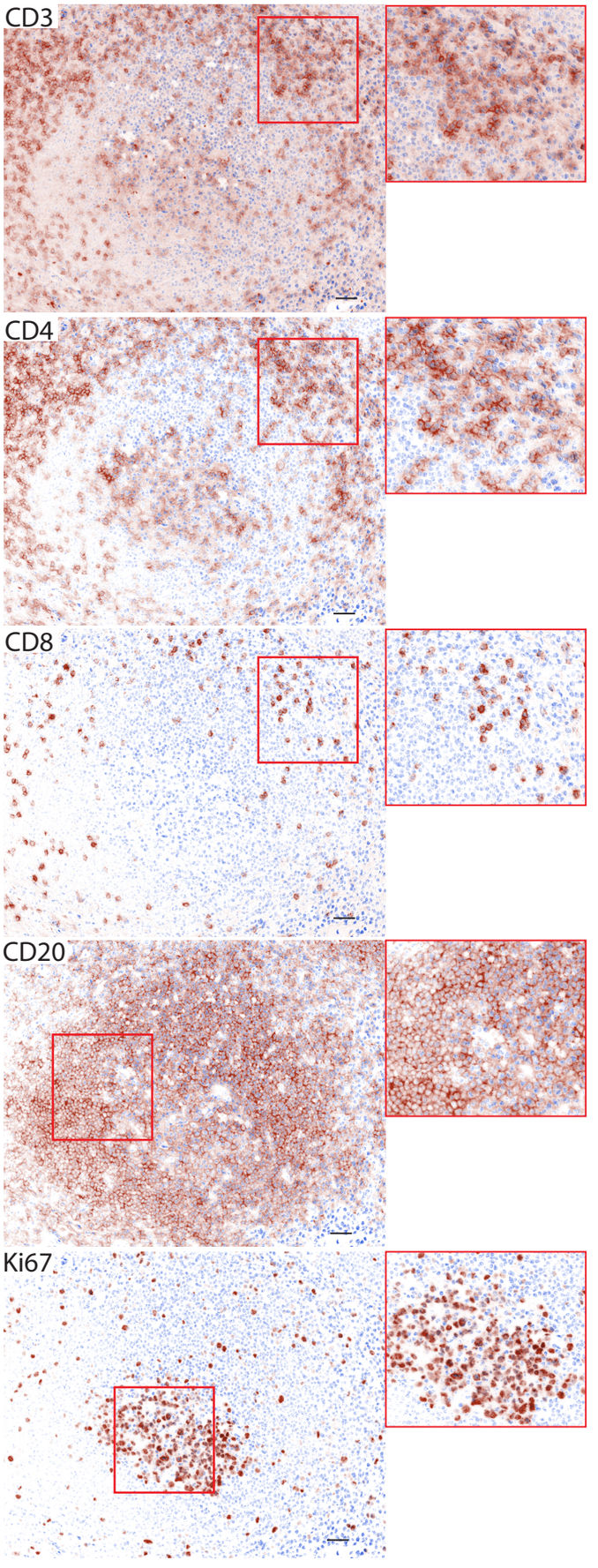


Figure 4

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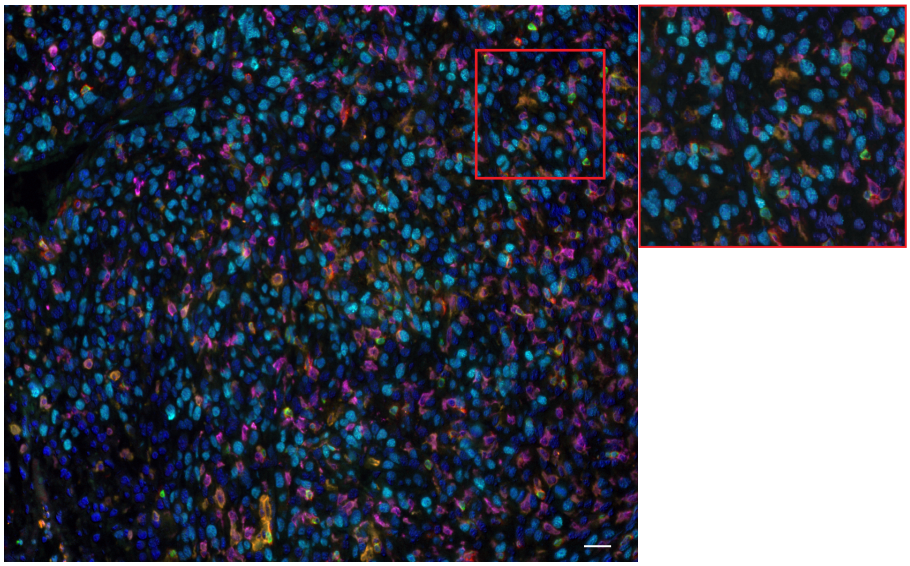

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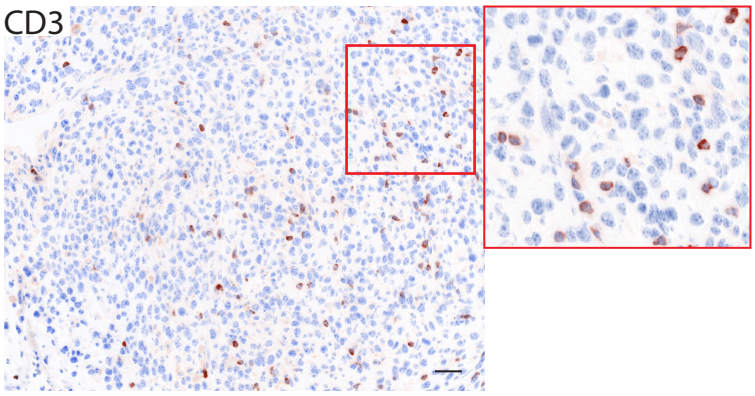
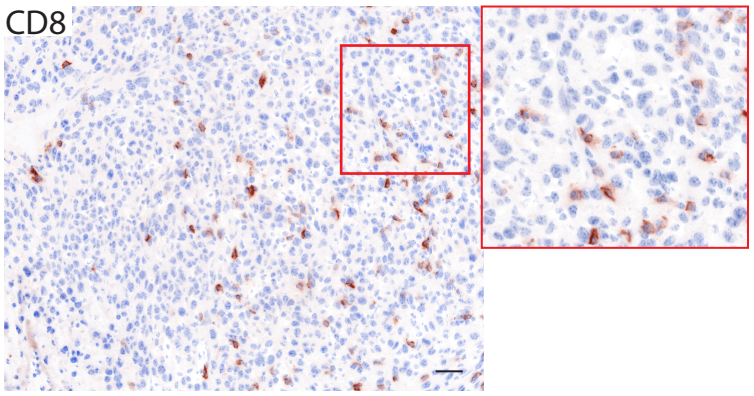
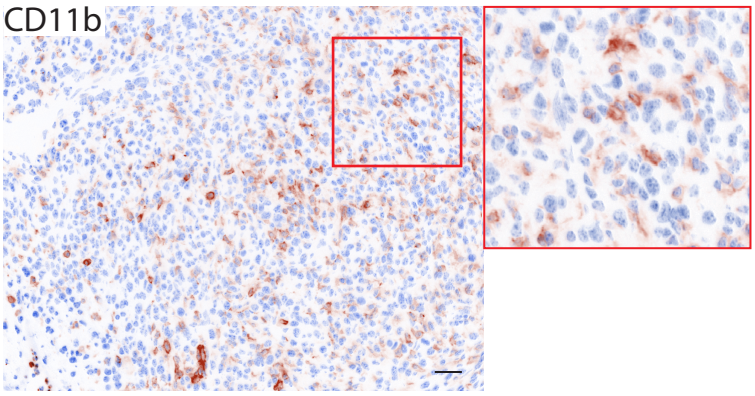
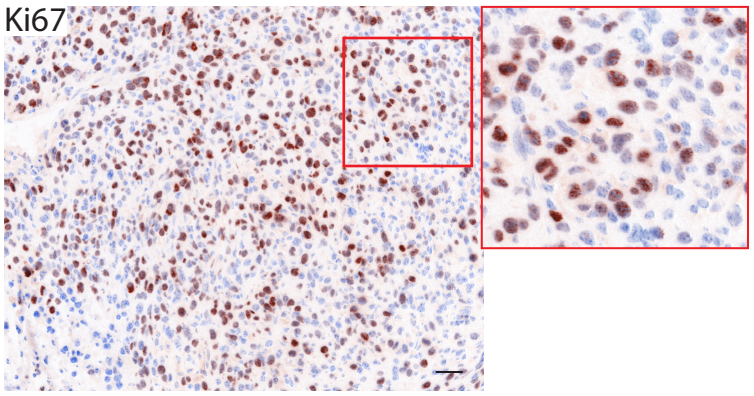








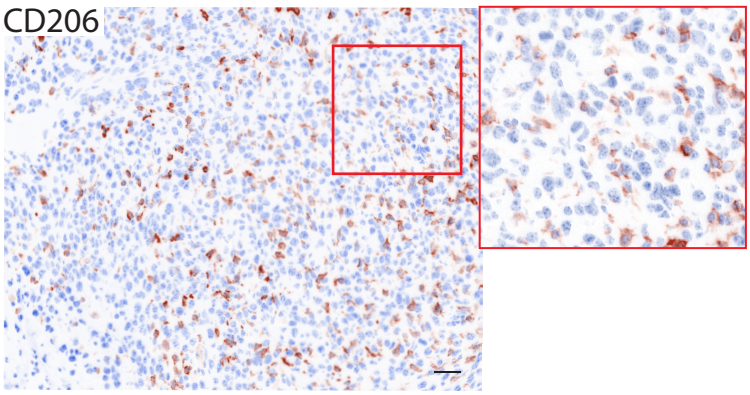
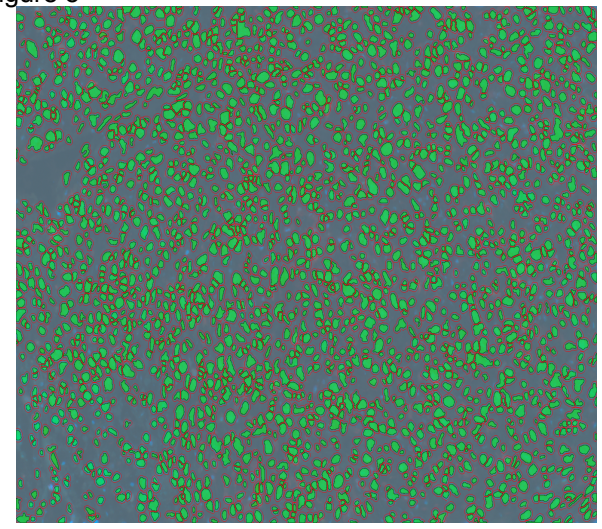


Figure 5

A



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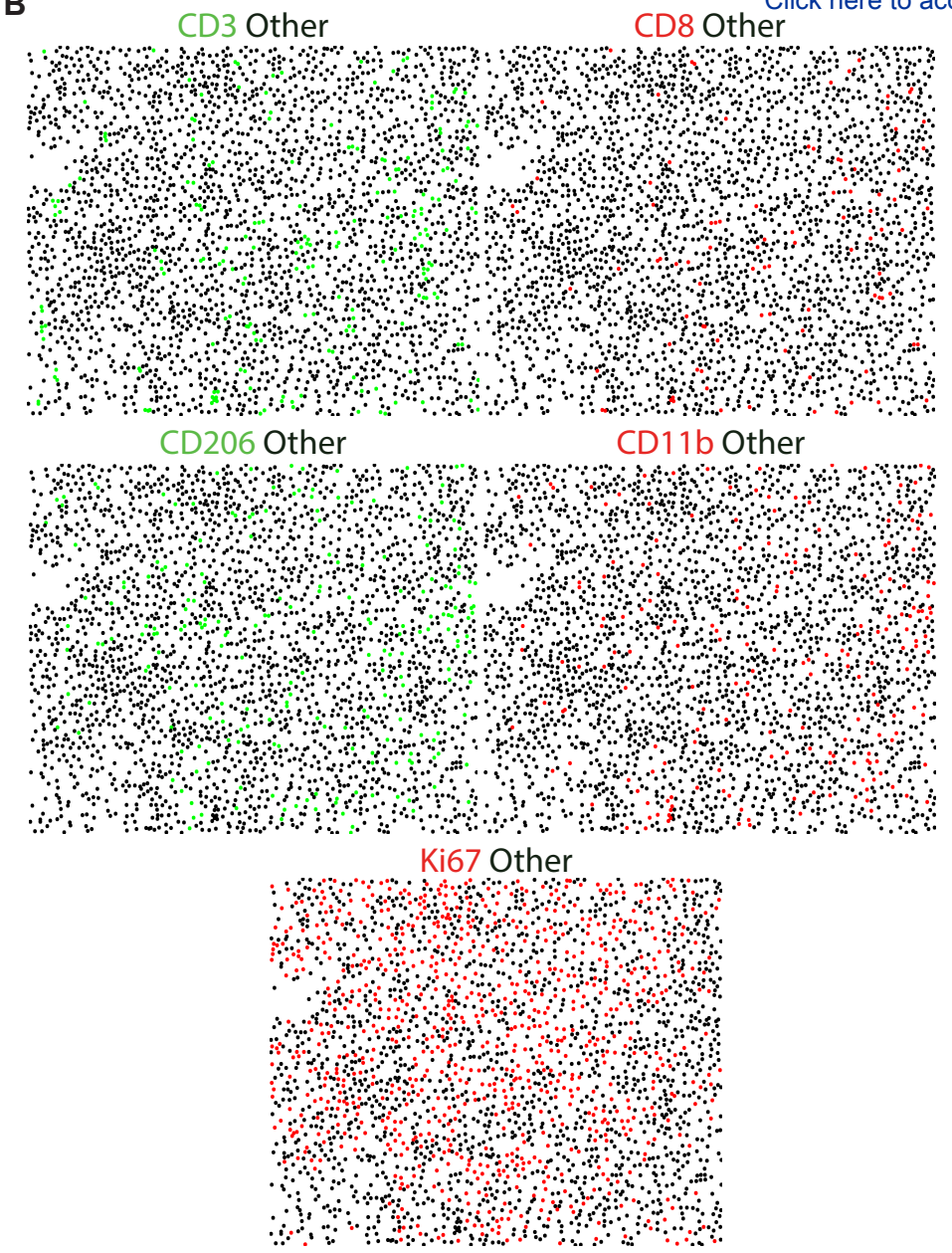

C

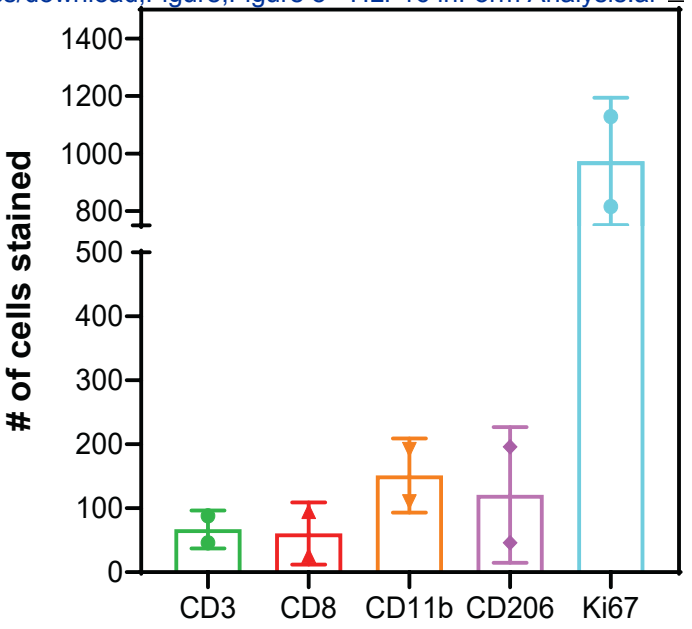

Table 1

Fluorophore	Excitation maximum (nm)	Emission maximum (nm)	Expected Detection in Filter Set (name)
Alexa Fluor 488	488	519	FITC
Alexa Fluor 555	555	580	Cy3 and Texas Red
Alexa Fluor 594	590	617	Texas Red
Alexa Fluor 647	650	668	Texas Red and Cy5
DAPI	350	470	DAPI
PerCP-Cy 5.5	482	690	Cy3, Texas Red and Cy5

Table 2

Frozen Mouse Spleen

Antibody/Dye	Clone	Concentration (µg/mL)
Alexa Fluor 594 anti-mouse CD8a	53-6.7	10
Alexa Fluor 488 anti-mouse CD3	17A2	20
Alexa Fluor 647 anti-mouse CD4	GK1.5	10
PerCP-Cy 5.5 Rat Anti-CD11b	M1/70	2
Alexa Fluor 555 Mouse anti-Ki-67	B56	10
DAPI		0.1

Frozen Mouse Tumor

Antibody/Dye	Clone	Concentration (µg/mL)
Alexa Fluor 594 anti-mouse CD8a	53-6.7	20
Alexa Fluor 488 anti-mouse CD3	17A2	20
Alexa Fluor 647 anti-mouse CD206 (MMR)	C068C2	5
PerCP-Cy5.5 Rat Anti-CD11b	M1/70	1
Alexa Fluor 555 Mouse anti-Ki-67	B56	0.25
DAPI		0.1

Frozen Human Tonsil

Antibody/Dye	Clone	Concentration (µg/mL)
Alexa Fluor 594 anti-human CD3	UCHT1	10
PerCP/Cyanine5.5 anti-human CD4	RPA-T4	4
Alexa Fluor 647 anti-human CD8a	C8/144B	10
Alexa Fluor 488, eBioscience anti-human CD20	L26	10
Alexa Fluor 555 Mouse anti-Ki-67	B56	1
DAPI		0.1

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetone (histological grade)	Fisher Scientific	A16F-1GAL	Fixing tissues
Alexa Fluor 488 anti-mouse CD3	BioLegend	100212	Clone - 17A2; primary conjugated antibody
Alexa Fluor 488, eBioscience anti-human CD20	ThermoFisher Scientific	53-0202-82	Clone - L26; primary conjugated antibody
Alexa Fluor 555 Mouse anti-Ki-67	BD Biosciences	558617	Primary conjugated antibody
Alexa Fluor 594 anti-human CD3	BioLegend	300446	Clone - UCHT1; primary conjugated antibody
Alexa Fluor 594 anti-mouse CD8a	BioLegend	100758	Clone - 53-6.7; primary conjugated antibody
Alexa Fluor 647 anti-human CD8a	BioLegend	372906	Clone - C8/144B; primary conjugated antibody
Alexa Fluor 647 anti-mouse CD206 (MMR)	BioLegend	141711	Clone - C068C2; primary conjugated antibody
Alexa Fluor 647 anti-mouse CD4 Antibody	BioLegend	100426	Clone - GK1.5; primary conjugated antibody
C57BL/6 Mouse	Charles River Laboratories	27	Mouse frozen tissues used for multispectral training
Coplin Jar	Sigma Aldrich	S6016-6EA	Rehydrating and washing slides
DAPI Solution	BD Biosciences	564907	Nucleic Acid stain
Diamond White Glass Charged Slides	DOT Scientific	DW7590W	Adhering tissue sections
Dulbecco's Phosphate Buffered Saline 1x (without Ca and Mg)	Fisher Scientific	MT21031CV	Washing and diluent
Gold Seal Cover Slips	ThermoFisher Scientific	3306	Protecting stained tissues
Human Normal Tonsil OCT frozen tissue block	AMSBio	AMS6023	Human frozen tissue used for multispectral staining

Human Serum 1X	Gemini Bio-Products	100-512	Blocking and diluent for human tissues
inForm	Akoya Biosciences	Version 2.4.1	Machine learning software
PerCP/Cyanine5.5 anti-human CD4	BioLegend	300529	Clone - RPA-T4; primary conjugated antibody
PerCP-Cy 5.5 Rat Anti-CD11b	BD Biosciences	550993	Clone - M1/70; primary conjugated antibody
Phenochart	Akoya Biosciences	Version 1.0.8	Whole slide scan software
ProLong Diamond Antifade Mountant	ThermoFisher Scientific	P36965	Mounting medium
Research Cryostat	Leica Biosystems	CM3050 S	Sectioning tissues
Superblock 1X	ThermoFisher Scientific	37515	Blocking mouse tissues
Tissue-Tek O.C.T Solution	Sakura Finetek	4583	Embedding tissues
Vectra 3.0 Automated Quantitative Pathology Imaging System, 6 Slide	Akoya Biosciences	CLS142568	Semi-automated multispectral imaging system
Vectra Software	Akoya Biosciences	Version 3.0.5	Software to operate microscope

Chicago, November 8th, 2019

RE: Manuscript# JoVE60806

TITLE: A rapid method for multispectral fluorescence imaging of frozen tissue sections

Dear Editor,

We would like to thank the Editor and the Reviewers to take time out to provide suggestions to strengthen our manuscript. We have addressed all the points stated by the Reviewers. Changes in the revised manuscript are highlighted in red. Please see below for a point-by-point response.

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: The manuscript has now been revised to incorporate the Reviewers' comments and has been proofread

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Response: The revised manuscript now adheres to the formatting guidelines.

3. Please provide at least 6 key words or phrases.

Response: We have now included 6 keywords: Multispectral imaging; frozen tissues; cancer; quantitative pathology; multiplexing; immune profiling

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of 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: Vectra, ProLong, Phenochart, inForm

Response: This has now been addressed in the revised manuscript and all products are referenced in the Table of Materials

Protocol:
1. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: This is now addressed in the revised manuscript.

Figures:
1. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Response: Scale bar for all images are now included and have been defined in the figure legend in our revised manuscript.

2. Figure 5C: Please define the error bars.

Response: The error bars are mean±STDEV. This is now mentioned in the revised manuscript, page 15, line 578

References:
1. Please do not abbreviate journal titles.

Response: Full name of the journal titles have now been incorporated in the revised manuscript

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Response: Information of materials and reagents and equipment used in the study are all mentioned in the Table of Materials and is provided along with the revised manuscript.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

This manuscript has two parts: 1) a method using a cocktail of fluorescently labeled primary antibodies to perform multiplexed staining of frozen sections; and 2) using a Vectra multispectral imaging system to image and analyze the samples. A JOVE video on both parts would be of interest. Multiplex fluorescent staining is becoming more popular and the Vectra system is one of the more common systems used to image and analyze such samples.

The manuscript goes into (mostly) sufficient detail on both parts (see below for comments). All in all, I think it would make a good JOVE video and there is a good framework to work from in this manuscript. However, there are some gaps in the specific methods (detailed below) that will

need to be addressed. The biggest concern with the manuscript is the introduction. The comparison of FFPE to frozen sections is not well done, and there is not enough information on existing alternate methods for multiplexed staining (which can easily be found through a good literature review, and then highlights added to the intro). But I feel that with some major revisions this could be a good JOVE video.

Major Concerns:

- The introduction does not review alternate strategies for multiplexing, nor does it mention that there have been many publications of using "cocktails" for both frozen and FFPE sections. Readers would appreciate having some citations to follow so they can review methods.

- The introduction needs a better discussion of the relative merits of FFPE vs frozen sections. They each have their strengths and weaknesses, but the discussion is one-sided as written (see below).

- There are some steps missing in the training and validation of the Vectra analysis.

- Tissue segmentation. This is mentioned after the image analysis section, but needs its own step in the video

- Validation. There is no mention of the iterative methods (using a Training Set, Test Set, and Validation Set). More on that below.

Response: We thank the reviewer for providing their detailed insights. Responses to their specific comments are provided below.

Minor Concerns:

* Line 55: this is far from true and is rather misleading.

Response: The Introduction section has now been revised and this line has been removed.

* There are two different companies who have commercialized methods to do this (Ultivue and Cell IDx) and there are lots of publications showing simultaneous staining (often using haptens, but there are many other methods). Some of these methods can be performed on an autostainer in under 4 hours.

Response: We have now mentioned these technologies and other technologies in the Introduction section of our revised manuscript; pages 2-3, lines 57-92

* There are also lots of papers involving direct fluorescence labeling using fluors (often QDots) dating back to the early 2000s.

Response: We have now mentioned and referenced (#s 31, 32 & 33) papers in our revised manuscript that perform multiplex fluorescence labeling on frozen tissues:

Au - Granier, C. et al. Multiplexed Immunofluorescence Analysis and Quantification of Intratumoral PD-1+ Tim-3+ CD8+ T Cells. Journal of Visualized Experiments. (132), e56606, doi:doi:10.3791/56606, (2018).

Odell, I. D. & Cook, D. Immunofluorescence Techniques. Journal of Investigative Dermatology. 133 (1), 1-4, doi:https://doi.org/10.1038/jid.2012.455, (2013).

Xing, Y. et al. Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry. Nature Protocols. 2 (5), 1152-1165, doi:10.1038/nprot.2007.107, (2007).

* Please do a literature search and become familiar with other methods for multiplexing (in FFPE and frozen tissue) if you are going to compare yourself to them.

Response: We have now included different 'simultaneous' and 'sequential' multiplex staining methods in the Introduction section of our revised manuscript; pages 2-3, lines 57-92

* Line 59: True for sequential methods involving heating/bleaching (like TSA), but not true for directly labeling.

Response: This line is now corrected in the revised manuscript and is mentioned only for the TSA multiplex approach; page: 3, lines: 90-92.

* Line 63: Antibody retrieval is the process of opening up the FFPE tissue to allow better penetration by antibodies and reagents. You should probably describe since you are going to compare yourself to it.

Response: We have now mentioned about antigen retrieval on page 2, lines 95-96.

* You mention the limitations of FFPE, and point out some advantages of frozen tissue. However, to be fair, you should mention some advantages of FFPE (storage, many suitable staining methods developed by the pathology community for both brightfield and FL) and some of the disadvantages of frozen (poor tissue architecture, difficulty with making 2-4 um sections, etc.).

Response: This is now mentioned on page 3, lines 93-94 and page 10, lines 376-377.

* You forgot one big disadvantage of FFPE: tissue autofluorescence. It is present in frozen tissues, but is far less than in FFPE.

Response: Tissue autofluorescence on FFPE is now mentioned in the revised manuscript on page 2, line 98 along with a reference (#16).

* Line 89: 8 um sections are quite thick, especially for higher magnification microscopy - there is considerable out-of-focus tissue even at 20x, and at 40x it pretty much requires a confocal. Most good pathology imaging is done on 3 or 4 um sections.

Response: Indeed a laser light source would provide a crisper image, however, the Vectra instead is designed to use a metal halide lamp. We advise against cutting thinner sections, which will reduce the signal of the least abundant antigen (or for the lowest affinity antibody) and thus limit the information that can be gauged from the image. In our experience, meaningful quantification requires an amount of signal that is more reliably captured at a standard section thickness of 8 um. We now mention on page 4, line 121 of the revised manuscript that the 'preferred section thickness can be adjusted to generate crisper images'.

* Line 96: You mention the acetone step in the methods, and state that it is an "acetone fixation", but there is no background material on that, especially vis-a-vis formalin fixation (i.e., FFPE). Please weigh their pros and cons.

Response: We have now included a note in the 'Cryosectioning' section (page 4, lines 125-128) comparing acetone to formalin and also mention that the choice of fixative is important factor to consider.

* Line 100: The Vectra is great for autofluorescence removal (which isn't a huge problem in frozen tissue) and removing cross-talk between fluorophores, but there are other systems that could be used with your samples. Have you tried any? This would be a much more interesting and general method if you could show it worked on more than just one specialized imaging system.

Response: We have not tried any other system apart from the Vectra and we agree that it will be interesting to try this with other systems. The advantage of the Vectra system is that it is a stand-alone system that can be used for imaging, unmixing as well as quantitation analyses.

* Line 101: I am not sure this is true. Please give some proof that antibodies that work for cells in flow cytometry labeling methods also work for frozen tissue. For instance, Cell Signaling, who does a LOT of validation of each antibody for each tissue/fixation combination, has different methods for cultured cells than frozen tissues.

Response: We apologize for the confusion. This sentence has been removed in the revised manuscript.

* Line 113: Is this an optimal blocking? It differs from other published protocols for frozen sections. Please provide proof that it is optimal.

Response: Yes. The blocking buffers mentioned in our study have been used in other studies as well (reference #35 and Gilbert ER et al., Positioning ganglioside D3 as an immunotherapeutic target in lymphangioleiomyomatosis. *Am J Pathol*, 2013). We have also included a note on page 5, lines 146-147 'different blocking buffers may be tested as needed to preserve specific properties depending on follow-up procedures'.

* Section 4: Staining. This describes a cocktail method of using antibodies that have been pre-conjugated to fluorophores and is a non-amplified method (i.e., there is no enzymatic amplification primary/secondary step). There is no discussion in the introduction about antigen expression and how this impacts the ability to use a non-amplified staining method for weakly expressing antigens. The detection limits for a non-amplified staining method are very different than for an amplified one, and some antigens may be too weakly expressing to be measured this way. Please discuss this in the introduction.

Response: We agree with the reviewer and this was mentioned as a caveat of our method in the Discussion section during our initial submission. Because we have revised our Discussion section now, this is now mentioned on page 10, lines 384-388 of our revised manuscript.

* In addition, there can be a lot of problems even on a multispectral microscope like Vectra with intra-scene dynamic range, i.e., when one fluorophore is very, very strong, it can make it difficult to see a spectrally adjacent fluorophore because of bleed-through. The Vectra does improve this compared to non-multispectral systems, but it is not immune.

Response: We agree with the reviewer. We have mentioned this as a note under the 'Multispectral Staining' section on page 7, lines 236-239 in the revised manuscript.

* Line 130: Preparing a spectral library

* There is no mention of the absolute necessity to use singly stained samples to develop a spectral library! One needs an unstained sample (all steps exactly the same but no antibody deposition) and one sample for each fluorophore. This is important and should be in the video.

Response: The 'Staining' section describes how to prepare single stained and unstained slides. We have also included a sentence in the 'Preparing a Spectral library' section on page 5, lines 168-169 in the revised manuscript.

* Line 149: Verifying Spectral Library

* There is no mention of validating the multiplexed sample by leaving out one fluorophore at a time (i.e., using only 4 of 5 fluorophores on a slide and leaving them out one at a time). This is an important validation step in any multiplexed assay development. There is also no mention of testing for antibody cross-talk, which can be a big problem.

Response: Thank you for pointing this out. We have now mentioned this as a note in the 'Multispectral Staining' section on page 7, lines 236-239 in the revised manuscript.

* Tissue Segmentation

* There is no mention at all about the tissue segmentation step of image analysis on Vectra. This is a very important step and cannot be left out of the video. It is mentioned later in the manuscript (line 285) but needs to be a part of the training steps.

Response: We did not perform tissue segmentation as our panel did not include a tissue specific marker. As per the scientists from Akoya Biosciences, the tissue segmentation can be skipped. However, for phenotyping, it is essential to perform cell segmentation. Hence, we skipped the tissue segmentation step and proceeded to cell segmentation. We do agree with the reviewer that tissue segmentation is important when there is a tissue marker or especially when the tissue is messy. In the latter instance, tissue segmentation is a must as it becomes easy to train the software for phenotyping. We have now included a note in the 'Analyzing multispectral image' section on page 7, lines 246-247 in the revised manuscript.

* Line 211: Cellular phenotyping

* Line 214. It is not clear from this description that cellular phenotyping is an iterative process. Some cells are chosen as starter cells, and then the system is trained. The user then goes back through the phenotyped cells and checks if the training is correct. If it is not, then more cells are selected for training and the steps repeated until the user is satisfied with the training

Response: This has now been revised (page 7, lines 261-264) to confirm that phenotyping is an iterative process.

* Training and Validation. There is very little mention of the process one needs to follow in order to fully validate that the tissue segmentation and cellular phenotyping are correct. Because they utilize machine-learning, they are both prone to over-fitting, and care must be taken to ensure that there is no over-fitting. This is done by dividing the data into three portions: 1) Training set; 2) Test set; 3) Validation set. The user trains using samples from the Training Set, then iteratively looks at the Test Set samples (which are not used in training) until the Test Set is

accurate. Then as a final test, the user analyses the Validation Set as a blind set to check if there has been over-fitting.

Response: We agree with the reviewer and we have now mentioned this in the Discussion section of our revised manuscript on page 10, lines 401-406.

* Line 308: another failure to mention that there are other ways to multiplex than Opal!

Response: We have now included different multiplex approaches in the Introduction section, lines 57-92, of our revised manuscript.

* The entire section on mass-spec imaging probably should be moved to the introduction as a part of a larger, expanded comparison between methods for multiplexed staining of tissues.

Response: We have now moved the entire section of imaging mass spectrometry to the Introduction section (pages 1-2, lines 65-77) in the revised manuscript.

Reviewer #2:

Manuscript Summary:
Jaishankar and colleagues present a method for multispectral imaging of frozen tissues that is proposed to be rapid and carry several advantages over currently available approaches.

Response: We thank the reviewer for their comments and providing suggestions to improve our manuscript. Please see individual responses below.

Major Concerns:

1. The approach is not completely novel and in that respect the authors have failed to acknowledge the work of other colleagues which have in fact published in this same journal (see Granier et al. 2018 JOVE). Further, another group has described a methodology to avoid the use of stripping and Opal methodology for multispectral imaging (see Ijsselsteijn et al. J Pathol Clin Res 2019). That same group has applied that methodology on frozen sections(see de Vries et al. Gut 2019). This reviewer suggests that while referring to these works, the authors exploit the niche between the fact that Granier et al. have only explored 3 markers while de Vries et al. does not provide a detailed protocol for frozen tissues. In addition, the staining of sequential sections should also be referred as an option in the introduction (see Tsujikawa et al. Cell Rep 2017).

Response: We have now referenced (# 31 & 35) these studies and discussed the advantage of our methodology over these published studies in the Discussion section, page 10, lines 338-360, in

the revised manuscript. In fact, de Vries et al. mention their staining protocol which takes 2 days to stain the tissue. As per Reviewer 1's comments, we have now discussed about 'simultaneous' and 'sequential' multiplex staining in the Introduction section of our revised manuscript (pages 2-3, lines 57-92).

2. The statement that HLA class I cannot be detected on FFPE tissues is incorrect. Total HLA class I expression is easily detectable, namely with clones such as HCA2 or HC10. Perhaps the authors wanted to refer to the detection of specific alleles which is indeed the case, the reference should be adjusted in this respect. They could also complement this example with several markers for which there is no IHC-p option available (e.g. CD25)

Response: Yes, we wanted to refer to the detection of alleles. Thank you pointing this out. This sentence is now corrected in our revised manuscript on line 100. The reference we have cited compares HCA2 and HC10 antibodies to a pan-anti HLA 1 antibody to detect specific alleles.

3. The time span for the whole procedure is exaggeratedly short. No way that freezing, cutting, staining, and detection takes only 90 minutes. Just the freezing, cutting, air drying and fixation would surpass this time. Please provide a realistic estimation.

Response: We apologize for the confusion. We meant that the staining time including washes was done within 90 minutes. This line is now re-worded in the revised manuscript on page 3, lines 109-110.

4. Why did the authors choose to cut 8 micron sections, didn't this compromise nuclear segmentation?

Response: Please see the response to Reviewer 1 above. The section thickness is a compromise between meaningful antigen detection for each target molecule under study, and crispness of the resulting image.

5. At some point the authors discuss the possibility to use BD dyes but no data is presented. The fact that their detection is possible is less important than whether their signal separation is possible from other markers and this will be highly dependent on their emission spectra. Thus, either the authors present data with these antibodies in combination with others or i would suggest to remove such reference.

Response: We have now removed this sentence in our revised manuscript.

6. Quality of figure 2 was not enough to inspect staining patterns and was not able to download figure 3 (received an AI file with missing links to TIFFs).

Response: We apologize for the inconvenience. We are not sure why this reviewer received a broken AI file.

7. I praise the authors to include IHC images next to the multispectral images but I would also like to see the individual marker patters after spectral unmixing of the 6 markers included. IHC images could be moved to supplementary data.

Response: As pathologists prefer IHC images, we would appreciate if the reviewer will allow us to keep the stimulated IHC images in the main figure for each frozen tissue. We have now included the individual marker expression in fluorescence staining of the frozen HLF16 tumor in the supplementary figure 1.

8. The authors should justify the choice for their panels in terms of composition. And possibly acknowledge that the staining and/or separation of markers is made easier when employing a number of markers that stain different cells.
Response: We have now included the following in the Discussion section on page 10, lines 378-382: The fluorophore combinations require a careful selection of markers to ensure that antibodies that do not sterically hinder each other, especially when different antigens are detected that are expressed in the same cellular location. We chose markers that stain different cells on fluorophores that are spectrally separate enabling better detection.