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## Automated Multiplex Immunofluorescence Panel for Immuno-Oncology Studies on Formalin Fixed Carcinoma Tissue Specimens. --Manuscript Draft--

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**TITLE:**

Automated Multiplex Immunofluorescence Panel for Immuno-oncology Studies on Formalin-fixed Carcinoma Tissue Specimens

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**KEYWORDS:**

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**SUMMARY:**

A detailed protocol for a six-marker multiplex immunofluorescence panel is optimized and performed, using an automated stainer for more consistent results and a shorter procedure time. This approach can be directly adapted by any laboratory for immuno-oncology studies.

## **ABSTRACT:**

Continued developments in immuno-oncology require an increased understanding of the mechanisms of cancer immunology. The immunoprofiling analysis of tissue samples from formalin-fixed, paraffin-embedded (FFPE) biopsies has become a key tool for understanding the complexity of tumor immunology and discovering novel predictive biomarkers for cancer immunotherapy. Immunoprofiling analysis of tissues requires the evaluation of combined markers, including inflammatory cell subpopulations and immune checkpoints, in the tumor microenvironment. The advent of novel multiplex immunohistochemical methods allows for a more efficient multiparametric analysis of single tissue sections than does standard monoplex immunohistochemistry (IHC). One commercially available multiplex immunofluorescence (IF) method is based on tyramide-signal amplification and, combined with multispectral microscopic analysis, allows for a better signal separation of diverse markers in tissue. This methodology is compatible with the use of unconjugated primary antibodies that have been optimized for standard IHC on FFPE tissue samples. Herein we describe in detail an automated protocol that allows multiplex IF labeling of carcinoma tissue samples with a six-marker multiplex antibody panel comprising PD-L1, PD-1, CD68, CD8, Ki-67, and AE1/AE3 cytokeratins with 4',6-diamidino-2-phenylindole as a nuclear cell counterstain. The multiplex panel protocol is optimized in an automated IHC stainer for a staining time that is shorter than that of the manual protocol and can be directly applied and adapted by any laboratory investigator for immuno-oncology studies on human FFPE tissue samples. Also described are several controls and tools, including a drop-control method for fine quality control of a new multiplex IF panel, that are useful for the optimization and validation of the technique.

## **INTRODUCTION:**

Immunoprofiling analysis of FFPE tumor tissue samples has become an essential component of immuno-oncology studies, particularly for the discovery and validation of novel predictive biomarkers for cancer immunotherapy in the context of clinical trials<sup>1,2</sup>. Chromogenic IHC, using chemical chromogens such as diaminobenzidine, remains the standard technique in diagnostic pathology for the immunolabeling of biopsy tissue<sup>3</sup>. Standard IHC can also be used for cancer tissue immunoprofiling, including the quantitation of subpopulations of tumor-associated lymphocytes and the assessment of expression levels of immune checkpoints such as programmed cell death ligand 1 (PD-L1)<sup>4,5</sup>. Standard IHC is limited, however, in that only one antigen can be labeled per tissue section. Because immunoprofiling studies typically require the analysis of the combined expression of several markers, the use of standard IHC would require the staining of multiple tissue sections, each stained with a single marker, and would, therefore, be substantially limited for the analysis of small tissue samples such as core needle biopsies. Standard IHC methods are also limited for the assessment of markers that are coexpressed by diverse cell populations, as is common with immune checkpoint markers such as PD-L1, which is expressed by both tumor-associated macrophages and cancer cells. This limitation has been reported in, for instance, the use of standard monoplex IHC by pathologists for the quantitative analysis of an IHC marker expressed by diverse cell types<sup>6</sup>. The development of multiplex chromogenic IHC techniques employing diverse colored chromogens on the same tissue section represents an advancement over the standard IHC monoplex method,<sup>7</sup> although they remain

limited by the immunolabeling of just a few markers and also present an important technical challenge for the proper evaluation of markers expressed in the same subcellular compartments of the same cell populations.

The aforementioned caveats regarding tissue availability from clinical samples, as well as the limitations of multiplex chromogenic IHC techniques, have given rise to the need to develop improved multiplex methods for immuno-oncology studies based on fluorescent labeling combined with imaging systems that can effectively separate the signals of multiple fluorophores from the same slide. One such technique is based on tyramide signal amplification (TSA) combined with multispectral microscopy imaging for efficient color separation<sup>8</sup>. A commercially available TSA-based kit employs fluorophores optimized for multispectral imaging<sup>8</sup> (see **Table of Materials**). A critical advantage of this system is its compatibility with the same unlabeled primary antibodies that have already been validated and optimized for standard chromogenic IHC<sup>9-11</sup>. This allows not only faster optimization but also flexibility in the optimization and panel modifications incorporating new targets. Furthermore, the multiplex immunofluorescence (mIF) TSA method can be optimized for commercially available automated IHC stainer systems, allowing for a straightforward transfer from monoplex chromogenic IHC to mIF.

Here we present a protocol for an mIF panel for immuno-oncology studies that is based on automated mIF TSA staining and uses a multispectral scanner for imaging. This protocol can be adapted and modified by any laboratory user with access to the described instrumentation and reagents. The protocol includes a panel of six primary antibodies for the immunoprofiling of carcinomas: PD-L1, PD-1, CD68 (as a pan-macrophage marker), CD8 (T-cytotoxic cells), Ki-67, and AE1/AE3 (pan-cytokeratin, used as an epithelial marker for the identification of carcinoma cells). A recent study describes the optimization of a manual TSA mIF protocol by using chromogenic IHC as a standard reference to validate the multiplex staining<sup>12</sup>. The updated method presented here has been developed by using a commercially available, seven-color TSA kit optimized in an automated stainer, drastically shortening the staining time from 3 - 5 days to 14 h, while also improving the consistency of the staining. In addition to the detailed main protocol presented here, a **Supplemental Materials** section includes the “drop-control” method, an additional quality control process to evaluate a new mIF panel, as well as technical notes for the optimization, troubleshooting, and development of new multiplex panels to help the laboratory user to set up and optimize the mIF TSA method for customized mIF panels.

## **PROTOCOL:**

**NOTE:** The protocol presented here describes how to perform immunoprofiling of an mIF panel by using TSA for six antibodies (CD68, ki67, PD-L1, PD-1, CD8, and AE1/AE3) on an automated stainer (see **Table of Materials**). The protocol also describes how to perform the drop controls for a quality control of a new mIF panel (see **Supplemental Materials**). In this protocol, staining is performed with eight unstained FFPE slides from human tonsil (positive control) and eight unstained slides from human lung adenocarcinoma. The first slide is used for full multiplex staining with all six markers, the second slide for the isotype control in which no primary antibodies are utilized, and the remaining six slides for the drop controls (see **Supplemental Materials**). An additional control for tissue autofluorescence is highly recommended and should

always be included in a multiplex study (see **Supplemental Materials**). However, investigators can employ other tumor types and controls according to their own project goals. Laboratory users without previous experience with the mIF TSA method and multispectral scanner techniques should read the Multiplex IHC Development Guide (available online at <http://info.perkinelmer.com/2016-lp-Opalassaydevelopmentguide-lp>). Although this guide describes a manual protocol, it also provides a good introduction to the mIF staining method. All tissue sections employed in this protocol were anonymized and approved according to the Declaration of Helsinki.

## **1. Tissue Samples**

1.1. Collect a paraffin block from an FFPE human tonsil sample, ideally with good lymphoid hyperplasia, as a positive tissue control. Also, collect a paraffin block from human lung adenocarcinoma tissue, preferably one known to be PD-L1 positive. Review the hematoxylin and eosin (H&E) slides of the selected lung cancer blocks to confirm the presence of a tumor. In addition, for technical optimization purposes, avoid tumors with abundant areas of necrosis.

NOTE: For this optimization, avoid using paraffin blocks that have been stored for 10 years or more and tissue with a dried appearance in the paraffin block (consult with a histology technician).

1.2. Using a microtome, cut 10 consecutive serial sections from each block, 4 µm thick. Mount the sections on a positively charged glass slide used for IHC, one section per slide.

NOTE: Sections must be mounted perfectly flat without wrinkles, as wrinkles may generate staining artifacts. Number the slides of the serial sections from 1 to 10.

1.3. Prepare a new H&E slide on the first section. Review the H&E slide with the help of a pathologist to confirm the presence of tonsil tissue or lung tumor remaining in the block.

NOTE: This H&E slide can help, later, with the selection of the regions of interest for multispectral analysis and phenotyping.

1.4. Store unstained sections in a plastic slide box at 4 °C for up to three months.

## **2. Creation of a New mIF Staining Program on an Automated Stainer: Registration of the Reagents**

NOTE: Reagents must be added to the reagent list in the automated stainer software before they are available for use in protocols. See the **Table of Materials** for information on the automated stainer model and software version.

2.1. Click on the **Reagents** tab within the automated stainer software. Click **Add** to designate a new reagent. Enter the name (*e.g.*, “520 TSA Reagent”) and the abbreviated name (*e.g.*, “520

TSA”), noting that there is a maximum of eight characters. Select **Ancillary** from the drop-down menu and set the supplier. Do not change **Single/Double Stain** from **Single/Sequential DS**. Set the default staining protocol to **Protocol F**. Set the default HIER protocol to **ER1 20**.

2.2. Save the new reagent and repeat step 2.1 for all reagents listed in **Table 1**.

### **3. Automated Stainer: Registration of the Containers**

3.1. Write the name of the reagent to be used on the side of each container. Scan the barcode on the side. In the pop-up window, select the correct reagent from the list. Select an expiration date and, then, save.

3.2. Repeat the procedure in step 3.1 for all reagents listed in **Table 1**.

3.3. Register the Open Research Kit. Scan both barcodes on the side of the kit and follow the on-screen prompts. Name the kit “Multiplex Research Kit 1.” Register the 1x tris(hydroxymethyl)aminomethane (Tris)-buffered saline as part of this kit (**Table 1**).

### **4. Automated Stainer: Creation of an mIF Protocol Program**

4.1. The base multiplex protocol is preloaded with the name **Opal 7 Multiplex** on new automated stainers (see the model and software version in the **Table of Materials**). Locate the protocol by filtering for “all” instead of “preferred” on the protocol screen. If the base protocol is not present, then update the automated stainer software with assistance from the manufacturer.

4.2. All modifications should be conducted on copies of the original protocol. Before making changes, save the original and create a copy by clicking the **Protocols** tab, then right-clicking the **Opal 7 Multiplex** protocol and selecting **Copy**.

4.3. Change the name to **7 Multiplex protocol 1** in the new window and select the tab associated with the correct device (**floor model or benchtop**). Match the protocol in **Supplemental Table S1**. Click **Add Reagent** to add a 10 min peroxidase inhibition step (not part of the standard protocol) and select the peroxidase inhibitor as the reagent.

4.4. Confirm that the blocking steps utilize a PKI blocking buffer. Ensure that each primary antibody refers to the appropriate reagent, that secondary antibody steps utilize an HRP polymer, and that spectral 4',6-diamidino-2-phenylindole (DAPI) provided with the multispectral automation kit is utilized. Confirm all choices by checking the reagent that has been designated for each respective step in the drop-down menu on the protocol screen.

### **5. Automated Stainer: Addition of Drop Controls, and Isotype Control Protocol**

5.1. Copy the name **7 Multiplex protocol 1** and change it to **7 Multiplex protocol 1 CD68 DROP**. Change the reagent **CD68 antibody** to **Mouse IgG** and, then, save the protocol.

5.2. Create six more new protocols, one for each drop control and one for the complete isotype control (changing all antibodies to the appropriate isotypes) in the same manner.

## 6. Automated Stainer: Slide Preparation Protocol

6.1. Copy the prestaining protocol **\*Bake and Dewax** and rename this protocol **Multispectral Bake and Dewax 2 h**.

6.2. Adjust the protocol to match the reagents shown in **Table 2**. Bake slides for 2 h at 60 °C. Dewax for 30 s at 72 °C.

## 7. Automated Stainer: Preparation of the Reagents

7.1. Prepare one research detection kit. One reagent must be permanently associated with this kit, so fill the 30 mL open container marked for 1x Tris-buffered saline (TBS) with 1x TBS and locate this in position 1 (farthest from the handle) in the research detection kit designated **Multiplex Research Kit 1**.

7.2. Label two 30 mL open containers **Multispectral Block** and **Multispectral Secondary**. Fill the **Multispectral Block** container with 30 mL of blocking buffer/antibody diluent from the multispectral staining kit. Fill the **Multispectral Secondary** container with 30 mL of anti-mouse/anti-rabbit secondary antibody polymer from the multispectral staining kit.

7.3. Prepare ten 7 mL open containers. These containers have a dead volume of 600 µL, and each application of antibody requires 150 µL. The required volume in microliters is  $600 + (150 \times [\text{number of slides}])$ . Each antibody will be applied to 12 slides in this case (six tonsil and six lung). The labeling and volumes for all containers are denoted in **Table 3**.

7.4. For each antibody tube, first add the antibody diluent, followed by the concentrated primary antibody in the volumes indicated in **Table 3**. Mix by gentle pipetting.

7.5. Prepare a 7 mL open container for DAPI, using four drops of DAPI concentrate per milliliter of double-distilled water; a total of 16 will be stained with DAPI ( $600 + [150 \times 16] = 3,000 \mu\text{L}$ ). Add 3 mL of double-distilled water plus 12 drops of spectral DAPI into the container. Prepare fresh DAPI for each run.

7.6. Prepare a 7 mL open container for peroxidase inhibitor. All 16 slides will be treated with peroxidase inhibitor ( $600 + [150 \times 16] = 3,000 \mu\text{L}$ ). Add 3 mL of peroxidase block into the container.

7.7. Before preparing working concentrations of fluors, resuspend all lyophilized fluors by adding 75 µL of dimethyl sulfoxide to each fluor tube provided by the manufacturer. Mix by pipetting. This is the TSA fluor stock. Store at 4 °C.

265  
266 7.8. Prepare six titration containers, one for each fluor. These containers have a removable insert  
267 with a dead volume of 350 µL. Each application of fluor requires 150 µL. The required volume in  
268 microliters is  $350 + (150 \times [\text{number of slides}])$ . Each fluor will be applied to 16 slides in this case  
269 (eight tonsil and eight lung). The labeling and volumes for all containers are denoted in **Table 4**.

270  
271 7.9. When all reagents have been registered and prepared, place each container into any location  
272 on a rack and load all reagents onto the automated stainer. The automated stainer will detect  
273 the presence and confirm the volume of each reagent.

## 274 275 **8. Automated Stainer: Sample Setup and Multispectral Staining**

276  
277 8.1. In the automated stainer software, click **Slide Setup** at the top of the screen. Click **Add study**.  
278 Populate the pop-up window with the study ID, study name, and comments.

279  
280 • Select the name of the researcher conducting the staining run from the drop-down list.  
281 Leave the dispense volume at **150 µL**. Select **Multispectral Dewax** for **Preparation Protocol**. Click  
282 **OK** and, then, **Add slide**. Under **Comments**, type “Multiplex 1 Lung 123ABC.” Complete the  
283 following fields as indicated: **Tissue Type** = Test tissue; **Dispense volume** = 150 µL; **Staining mode**  
284 = Single, Routine; **Marker** = Negative; **Staining** = Type “7 Multiplex Protocol 1”; **Preparation** =  
285 Multispectral Bake, Dewax 2 h; **HIER** = HIER 20 min with ER1.

286  
287 8.2. Click **Add slide** and, then, change the comments and protocol to add the drop control slides  
288 and isotype control slide. When all slides have been added to the study, close the **Add slide**  
289 window and print labels. Apply the labels to the appropriate label area on each slide.

290  
291 8.3. Load the slides into racks, apply cover tiles in the correct orientation, insert the racks into  
292 the automated stainer, and lower the trays. The device will scan the slide labels.

293  
294 8.4. Open the **System status** window by clicking the small square in the upper left of the screen  
295 in the autostainer software. Once all slides are detected, all reagents are present, and protocols  
296 are correct, the triangular button below each loaded drawer will change from light to dark gray.

297  
298 8.5. Initiate staining immediately or schedule a delayed start. The protocol is approximately 14 h  
299 in duration. Time the protocol so that the slides can be removed immediately when complete.

## 300 301 **9. Coverslipping of the Multispectral Slides**

302  
303 9.1. Remove the slides from the automated stainer and store them in a rack submerged in 1x  
304 TBS.

305  
306 9.2. Mount the samples with no. 1.5 coverslips and 15 µL of the mounting media (see **Table of**  
307 **Materials**). Remove any bubbles by pressing gently on the coverslip with a pipette tip.



9.3. Allow the slides to cure overnight at room temperature on the laboratory bench. Uncured slides can be scanned immediately with careful handling.

## 10. Multispectral Scanner

10.1. Power on the multispectral scanner and its computer (see **Table of Materials** for model and software information). Start the microscope control software. Open the front door of the multispectral scanner by pressing the touch-sensitive button on the front of the device. Each spring-loaded carrier in the scanner holds four slides. Load all slides into carriers and record the order before loading the carriers into the device.

10.2. Select **Edit Protocol** and click **New...**; then, create the protocol name **Multiplex Panel 1 and Drop Controls**. Create the study name **Multiplex Panel Development**, click **Create Protocol**, and type **Overview Scan Filter DAPI**; then, select **Whole Slide Scan** and **Pixel Resolution 0.50  $\mu\text{m}$  (20X)**. All filters and bands should be represented. If not, click **Edit Filters and Bands**, select all five filters (DAPI, FITC, CY3, Texas Red, and CY5), and then, click **Edit Exposures**.

10.3. Load the carrier by selecting the carrier with the full multiplex of lung adenocarcinoma. Select the correct slide by using the graphic user interface. Focus the tissue either manually or by using autofocus. Navigate to any area with acceptable DAPI staining and click **Auto-expose** to set the exposure in milliseconds (0 - 2,000 ms).

10.4. Repeat the same procedure for all five filter sets in both the Whole Scan and MSI Region columns. Be sure to navigate to an area with acceptable staining and refocus the microscope before setting the exposure for each filter and magnification level. Auto-expose for all five filters in a 20X overall scan and 20X multispectral imaging (MSI) multispectral regions. Select a pixel resolution of 0.50  $\mu\text{m}$  (20X). Save the protocol and click **Back** to return to the home screen of the Vectra software; then, click **Scan Slides**.

10.5. Open the interface for each slide and set **Task** to **Scan**. Set **Study** to **Multiplex Panel Development**, set **Protocol** to **Multiplex Panel 1 and Drop Controls**, and set **Slide ID** to **Multiplex Lung ABC123**, **Multiplex CD68 Drop Lung ABC123**, etc. When all slides have been labeled and tasks are set, click **Scan**. The automated multispectral scanner will perform full-slide scans of all slides using all five filters.

## 11. Multispectral Scanner: Multispectral Imaging

11.1. Once the scans are complete, open the **QPTIFF** software and click **Load Slide** at the upper left. This will open a QPTIFF, which is a five-filter whole-slide scan. Each layer contains information from more than one fluor, so the utility is limited, but the tissue structure and broad expression patterns are apparent and can be used to select regions for MSI.

11.2. Navigate to and open the correct slide in the study by using the drop-down tree on the left. Log in (upper right) with user initials.

11.3. Select five regions for MSI by using the **Stamp** tool at the top of the screen. Consult a pathologist if one is available. Under **Select for**, select **Acquisition**; under **Size in fields**, select **1x1**; and under **Field restriction**, select **0.5 µm (20X)**. Based on cytokeratin (CK) staining (principally in Cy5), select several regions with both tumor (CK+) and stroma (CK-) to be imaged from the overall scan. Repeat this procedure for each slide.

11.4. Once all MSIs are selected, close the **Phenochart** software and return to the **Vectra** software. Change the task from **Scan** to **MSI** for each slide and, then, click **Scan** again to perform the MSI collection.

## 12. Spectral Unmixing

NOTE: The spectral unmixing step is required for channel separation and image analysis of the slides. Before spectral unmixing is performed in the inForm software, the spectral library must be built by using lung adenocarcinoma slides stained with each fluor alone and with DAPI alone. This procedure is also described in the aforementioned Multiplex IHC Assay Development Guide.

12.1. When the MSI acquisition is complete, use the spectral unmixing software to open files in the **MSI** folder. These files end in the .im3 file-type extension.

12.2. Under **Image format**, select **Multispectral**; under **Sample format**, select **Fluorescence**; and under **Spectral library source**, select **InForm**.

12.3. To select fluors, select and load all six fluors and DAPI from the library built with the lung adenocarcinoma library slides. Click **Prepare All** (lower left). Spectral unmixing software will perform spectral unmixing on all open images, using the provided spectral profiles.

12.4. With a pathologist, assess the staining pattern against chromogenic single stains of the same target in sequential slides of the same tissue.

## 13. Evaluation of Fluorescence Intensity and Signal Attenuation

NOTE: The counts tool, which appears as an arrow with a small brown box, is the most important tool for multiplex optimization and should be used frequently (see **Supplemental Materials**). Using the counts tool in the spectral unmixing software, assess the signal-to-noise ratio, which at a minimum should exceed 10:1 (see **Supplemental Materials** for troubleshooting and for the evaluation of the multiplex staining with the drop controls).

13.1. With an image open in the spectral unmixing software, engage the counts tool and survey images to assess the fluorescence intensity of each channel. The target intensity in lung tissue is between 10 and 20 normalized counts. The normalized counts tool is shown in **Figure 1**.

396 13.2. Exclude slides with maximum signal counts of less than 10 or normal signal area counts of  
397 less than five for any marker so that autofluorescence and off-target staining do not compete  
398 with the authentic signal.

400 13.3. Exclude slides with maximum counts greater than 30 for any channel to avoid spectral  
401 bleeding or inefficient spectral unmixing.

403 13.4. Address high or low normalized counts by adjusting the TSA concentration.

## 405 14. Multispectral Image Analysis

407 14.1. Open one or more MSI in the **spectral unmixing software** and only select fluors for  
408 unmixing that are represented in at least one open image. Click **Prepare All** to unmix all currently  
409 opened color images to yield spectrally unmixed images.

411 14.2. Select the counts tool to survey counts across each image by hovering over the area(s) of  
412 interest.

414 14.3. Select the eye icon to open the view editor. Select and adjust the display intensity of each  
415 independent channel.

417 14.4. Select **Configure** to open the tissue segmentation, cell segmentation, phenotyping, and  
418 scoring modules. These tools are required for the objective validation of multiplex staining  
419 against chromogenic single stains and can be used to generate quantitative phenotypic data  
420 (**Figure S1**).

422 14.5. Use the **Export** tab to save grayscale, fluorescent, pathview-style uncompressed TIFF, and  
423 data files from the phenotypic analysis modules for further analysis, archiving, and presentations  
424 (**Figure S1**).

## 426 REPRESENTATIVE RESULTS:

427 The protocol described here will provide results like those shown in **Figure 2**. Start with an  
428 evaluation of the staining in the tonsil control, beginning with the surface squamous cell  
429 epithelium. The histology of the tonsil sample can be reviewed with a pathologist, using the H&E  
430 slide as a reference. If chromogenic IHC sections are performed with the same markers on the  
431 same tissue block, then these can be used to confirm the density and distribution of each marker  
432 on the mIF slide. As shown in **Figure 2A**, the tonsil tissue should provide clearly defined AE1/AE3  
433 cytokeratin staining in the tonsil surface squamous epithelium (**Figure 2A**; labeled as red  
434 pseudocolor), without background in the lymphoid tissue. The staining should be cytoplasmic,  
435 occasionally with membrane accentuation, and the nuclei must be negative. The reticulated  
436 epithelium in the crypts should be positive for both cytokeratins (red) and PD-L1 (**Figure 2A**;  
437 green pseudocolor). The follicular germinal centers of the tonsil should, then, be identified. The  
438 germinal centers should be easily recognizable and should be rich in Ki-67-positive lymphocytes  
439 (**Figure 2A**; yellow pseudocolor). Ki-67 staining should always be nuclear. The macrophages

present in the germinal centers should also be easily recognizable, sometimes as larger cells (also called “tingible bodies”). They will show positive staining for CD68 as a granular cytoplasmic stain (**Figure 2A**; orange pseudocolor). A variable proportion of CD68 cells outside the germinal centers is also to be expected. Macrophages may show membrane staining for PD-L1. This is typically paler in intensity than staining for PD-L1 in the reticulated epithelium. There should be no nuclear CD68 staining. CD8 should stain lymphocytes with a T-cell distribution (fewer in the germinal centers and a greater proportion in the interfollicular areas; see **Figure 2A**, cyan pseudocolor). CD8+ lymphocytes are usually small cells with scant cytoplasm, so it is practically impossible to distinguish membrane *versus* cytoplasm in the lymphocytes. PD-1 will strongly stain small lymphocytes in the germinal center area, usually clustered at the periphery of the germinal centers (**Figure 2A**; magenta pseudocolor), as well as scattered lymphocytes in the interfollicular region, usually showing a lower staining intensity than those in the germinal center area. As with CD8, it is not possible to clearly distinguish membrane and cytoplasmic staining in small lymphocytes stained with PD-1. Because a variable proportion of CD8 cells coexpress PD-1, it is recommended for quality-control purposes that the staining pattern and distribution of each marker that is stained in the same tissues be compared with chromogenic IHC reference slides, as suggested in previously published protocols<sup>12</sup>.

After the expected staining pattern has been confirmed in the tonsil tissue control, proceed to evaluate the staining in the lung cancer sample (**Figure 2B**). Because tumor tissues are expected to show a variable and heterogeneous expression of the markers, it is very important during the optimization of a new mIF panel to compare the staining pattern for each individual marker observed in the mIF method and its expression observed in the standard chromogenic IHC slide, evaluating the quantity and distribution of the positive cells as previously described<sup>12</sup>. Nevertheless, the basic staining pattern should be preserved; for example, cytokeratins should be observed in the cytoplasm of epithelial cells and never in a nucleus; CD68 should appear as granular cytoplasmic staining in macrophages, *etc.* Importantly, in some markers, the staining intensity may change from the tonsil control to the cancer tissue; for example, PD-1 and PD-L1 can show lower-intensity staining in the tumor tissue as compared with the very high expression observed in tonsil control. It is, therefore, important to perform the evaluation and optimization with the counts tool in the inForm software, using examples of tumor tissues rather than the tonsil controls.

Finally, isotype-negative controls and drop controls need to be evaluated, not only for the detection of background and autofluorescence but also for umbrella effects and spectral bleed (**Figure 3** and **Figure 4**). Isotype controls should not demonstrate any immunostaining across the slide; if any staining is observed, then the imaging or staining procedure must be revisited. Drop controls are important to evaluate potential artifacts in the staining, such as spectral bleed or umbrella effects, due to the mIF method during the optimization of a new multiplex panel (see **Figure 5**, **Supplementary Figure S2**, **Supplementary Figure S3**, and **Supplemental Materials**). Fluorescent monoplex and drop controls should be evaluated and compared with the full multiplex panel. Each drop control should show the same staining pattern except for the single primary antibody, which should be removed on each specific control. Further details are provided in the **Supplemental Materials** section.

## FIGURE AND TABLE LEGENDS:

**Figure 1: inForm Counts Tool.** The inForm software counts tool is activated by selecting the beige box icon. Normalized counts are corrected for bit depth, exposure time, gain, and binning. The images were taken at 20X magnification; the scale bar = 50  $\mu\text{m}$ .

**Figure 2: Representative results.** (A) Tonsil and (B) nonsmall-cell lung carcinoma were stained with PD-L1 (520 TSA, green), CD8 (540 TSA, cyan), Ki67 (570 TSA, yellow), CD68 (620 TSA, orange), cytokeratin (650 TSA, red), and PD-1 (690 TSA, magenta). Individual channels are represented separately below in small panels. The marker is indicated in the upper right of each image. The images were taken at 20X magnification; the scale bar = 50 or 250  $\mu\text{m}$ . Indicated in panel A are 1) lymphoid follicle, 2) germinal center, 3) interfollicular area, and 4) stratified squamous epithelium.

**Figure 3: TSA blocking/umbrella effect.** This is an example of 690 TSA deposited by a PD-1 antibody-blocking CD3 signal in a manner dependent on the amount of TSA present. The brightest 690 TSA signal blocks the 620 TSA signal most effectively (nonsmall-cell lung carcinoma stained with anti-PD-1 D4W2J for 30 min at 0.52  $\mu\text{g}$  or isotype followed by a 10 min 690 TSA detection at 1:100; then, anti-CD3 F7.2.38 for 30 min at 0.14  $\mu\text{g}$  followed by a 10 min 620 TSA detection at 1:100). The images were taken at 20X magnification; the scale bar = 50  $\mu\text{m}$ .

**Figure 4: Spectral bleed.** Nonsmall-cell lung carcinoma tissue was stained with the Ki-67 drop-control protocol (complete multiplex protocol with Ki-67 antibody omitted). The 540 TSA channel (CD8) and the 570 TSA channel (Ki-67) are shown. No nuclear staining pattern is apparent, yet a diminished copy of the CD8 staining pattern is observed in the 570 TSA channel. This is best addressed by ensuring comparable staining intensities in all channels. In this case, the addition of a robust Ki-67 signal (normalized counts between 10 and 20) will correct the spectral bleed. The images were taken at 20X magnification; the scale bar = 50  $\mu\text{m}$ .

**Figure 5: Drop controls.** This figure shows a composite image of a full multiplex compared with the same region of sequential slides of lung adenocarcinoma stained with drop controls in which the indicated primary antibody was omitted. The images were taken at 20X magnification; the scale bar = 50  $\mu\text{m}$ .

## DISCUSSION:

The ongoing cancer immunotherapy revolution is opening novel and promising therapeutic options for cancer patients<sup>13</sup>. Advances in the field of immuno-oncology will require increased knowledge of the inflammatory tumor microenvironment, not only to understand the biology of the immunological mechanisms involved in carcinogenesis but also to find predictive biomarkers for new immunotherapy-based treatments<sup>1,2</sup>. Due to the complex biology of cancer immunology, interrogation of tumor tissue samples is usually required to develop a growing list of immune markers, which interact with each other and are frequently coexpressed by diverse cell populations in the tissue<sup>1,2,5</sup>. Clinical trials typically employ small biopsies, such as core needle or

endoscopic biopsies, which are collected at different points of therapy to evaluate and monitor patient response. Such biopsies provide limited amounts of tissue, which in turn limits the number of tissue sections that can be employed for cancer immunoprofiling. This limitation is particularly onerous when traditional techniques, such as standard monoplex IHC, are used. There is, therefore, a clear need for new methods for cancer immunoprofiling that make use of multiplex techniques with the capacity to simultaneously evaluate the coexpression of different biomarkers and to make more efficient use of valuable tissue specimens.

In the multiplex staining and multispectral imaging methods described in detail here, an mIF panel is used for immuno-oncology studies on FFPE tissues such as biopsies from a clinical trial. This method has been previously described, validated, and successfully applied to cancer immunoprofiling<sup>8-12,14,15</sup>. Previously published protocols have described similar mIF staining methods with TSA-based systems, such as the seven-color kit, which is time-consuming and requires approximately 4 days of work by a dedicated laboratory scientist<sup>12,15</sup>. In response to these shortcomings, this protocol has been optimized by using a commercially available automated stainer, dramatically shortening the staining time to 14 h and eliminating the variability introduced by manual operators. Imaging is conducted on a multispectral scanner capable of separating the spectra of the seven or more fluorescent signals in the multiplex slides, including autofluorescence, which can be efficiently removed without degrading the signal quality. This protocol can be replicated, modified, and performed by any laboratory user with access to the instruments and reagents detailed in the **Table of Materials**.

Critical steps in this protocol are sections 7, 10, 11, 12. Section 7 pertains to the preparation of reagents. A careful preparation of the reagents for multiplex staining is essential, requiring the focused attention of a laboratory scientist or technician to avoid operator-dependent variation. For instance, one of the major issues with the multiplex method is staining variability, which can be reduced by the careful preparation of the dilutions of the reagents, specifically the TSA dyes. Sections 10 and 11 are related to image acquisition. An important risk is the overexposure or oversaturation of the images, which may lead to spectral bleed, an artifact in which the signal from another channel interferes with the channel being imaged (see **Supplemental Materials**). Carefully regulating the exposure times can help to prevent oversaturation and spectral bleed. Spectral unmixing is conducted in section 12. This relies on the preparation of the spectral library (described in the Multiplex IHC Assay Development Guide, available online). In this step, the inForm software is “trained” to specifically recognize the colors for each TSA dye. The spectral library is created by using sections from a control tissue, such as human tonsil, stained with a uniformly expressed marker, such as CD20, coupled with each individual TSA dye on individual slides. In addition, autofluorescence slides (also described in the Multiplex IHC Assay Development Guide) are needed to train the inForm software to recognize and subtract tissue autofluorescence. Autofluorescence control slides should be prepared by using samples from the same tissues being studied (such as lung cancer, *etc.*), treated in the same way as the mIF slides, and incubated with primary and secondary antibodies but without the TSA dye. Only a single autofluorescence spectral profile can be selected, so care must be taken to select a representation of the observable autofluorescence which may come from collagen, fibrosis, red blood cells, endogenous pigments, and other sources. Creating a new spectral library at the

beginning of each new project and using an autofluorescence spectrum which is representative of the tissue study specimens, as well as running the same tissue blocks as the positive control for each batch within the same project, are valuable methodological steps that will help to improve the consistency of the spectral unmixing across different samples within the same project.

The multiplex method described in this protocol offers several troubleshooting tools, including a signal-to-noise evaluator (counts tool), drop controls (**Supplemental Materials**), and a pathology view for quality control of the staining. The counts tool helps to evaluate the intensity of the staining and the level of background. If the intensity and/or background levels are high, the first approach is to reduce the dilution of the TSA dye. If the problem persists, the chromogenic IHC slides used for the optimization of that particular marker should be reviewed with a pathologist, looking for the presence of background. The pathology view is generated by the inForm software, using a diaminobenzidine-like pseudocolor representation of the fluorescence from the TSA-linked fluor, as well as a faux hematoxylin representation generated from all fluorescent signals, including DAPI. Images from the pathology view help with the visual evaluation of the staining patterns by a pathologist for improved staining. These tools should always be employed during the optimization of a new panel and as quality control steps during the staining workflow.

The mIF method requires the use of primary antibodies that have been properly validated and optimized for standard chromogenic IHC on formalin-fixed tissues. For instance, one of the major advantages of the mIF TSA method is its compatibility with the use of primary antibodies that have been validated and optimized in the anatomic pathology laboratory for clinical use<sup>12</sup>. This advantage means that the multiplex panels can be customized by the laboratory user to answer specific cancer immunology questions without the need for preconjugated antibodies, providing rapid and dynamic panel development. In this regard, the actual workflow for the optimization of a new panel begins with the validation and optimization of the antibodies with standard chromogenic IHC, looking for the best dilution and staining conditions that will provide clean and consistent staining. The next step, using the same dilutions optimized for IHC, is to transfer the staining by using TSA fluors instead of diaminobenzidine and to compare the results of monoplex immunofluorescent TSA with the chromogenic IHC as reference. Finally, the antibodies can be combined in mIF staining, always using the chromogenic IHC staining pattern as a control reference to adjust the mIF TSA staining parameters, mainly the TSA dye dilutions and the staining sequence. During this process, collaboration with a pathologist is instrumental to evaluate the quality of the staining.

Limitations of this technique include the time and effort required for the optimization and validation of new panels and the immunophenotyping analysis of mIF panels. The main concern, however, is the proper validation of mIF methods. The lack of proper validation of these techniques carries the risk of generating invalid results, which may add confusing data to the literature, thereby hindering attempts to gain a better understanding of the biology of cancer immunology<sup>16</sup>. It is, therefore, incumbent on the investigator to make every effort to properly validate multiplex methods, including ensuring close participation and evaluation by pathologists with training and experience in validation and evaluation of tissue histopathology and IHC-based

techniques<sup>17-20</sup>. The protocol presented here includes some of the tools that can help with the validation of a new multiplex panel, including the pathology evaluation of H&E slides before staining and analysis, the use of chromogenic IHC as a reference to optimize the mIF TSA staining, the drop control method for quality control of a new multiplex panel, and the use of isotype, autofluorescence, and drop controls. Despite the technical complexity of mIF methods, technologies such as multispectral imaging with spectral unmixing, as well as other novel multiplex platforms, are opening a new era for the analysis of tissue specimens from patients and the generation of new forms of data that is not possible with standard IHC alone. Therefore, the development and application of mIF techniques will continue to grow, becoming powerful tools for the cancer immunoprofiling of biopsies for clinical trials and helping to identify new multiparametric predictive biomarkers for immunotherapy, benefiting, above all, the cancer patient.

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Figure 1

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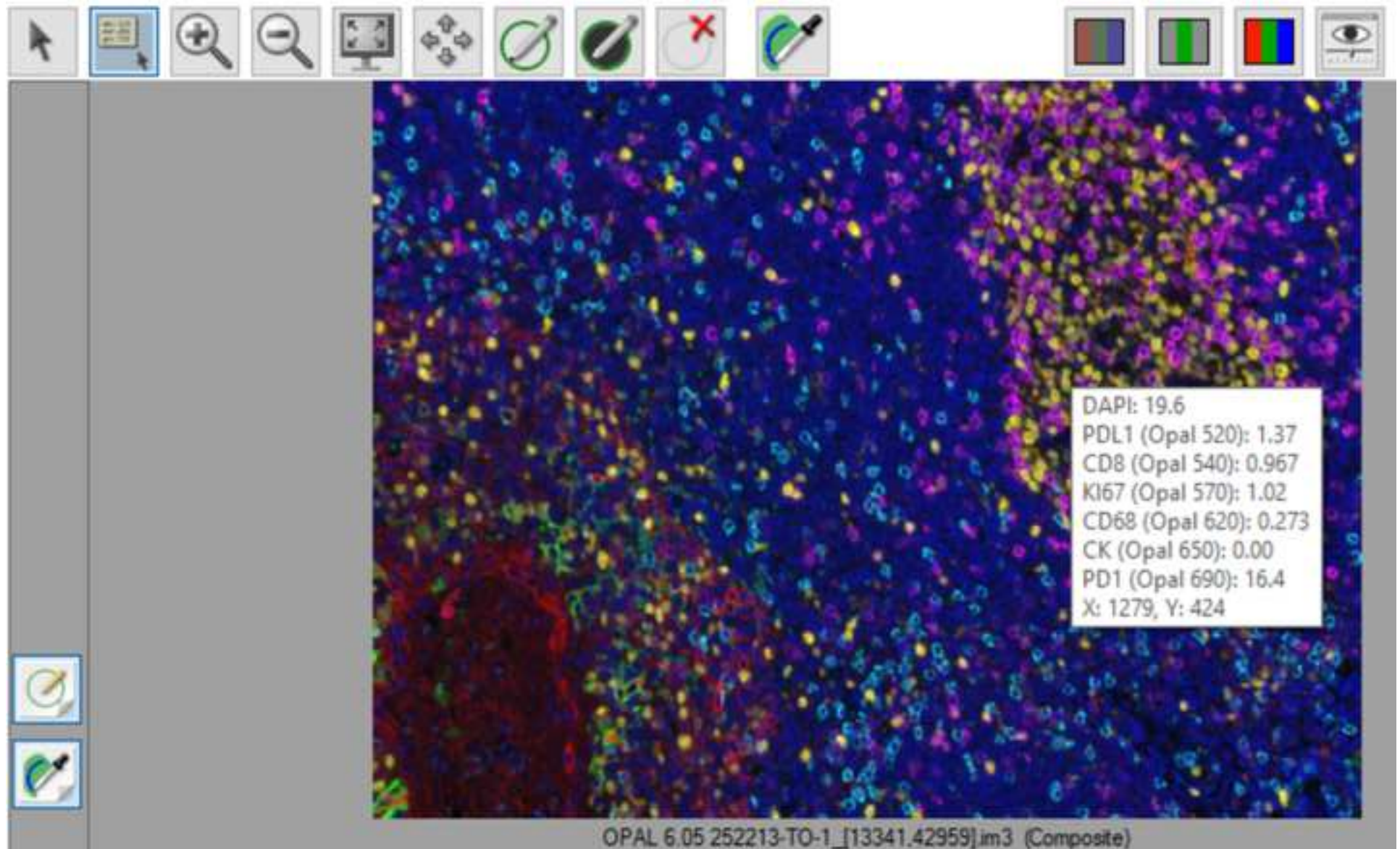
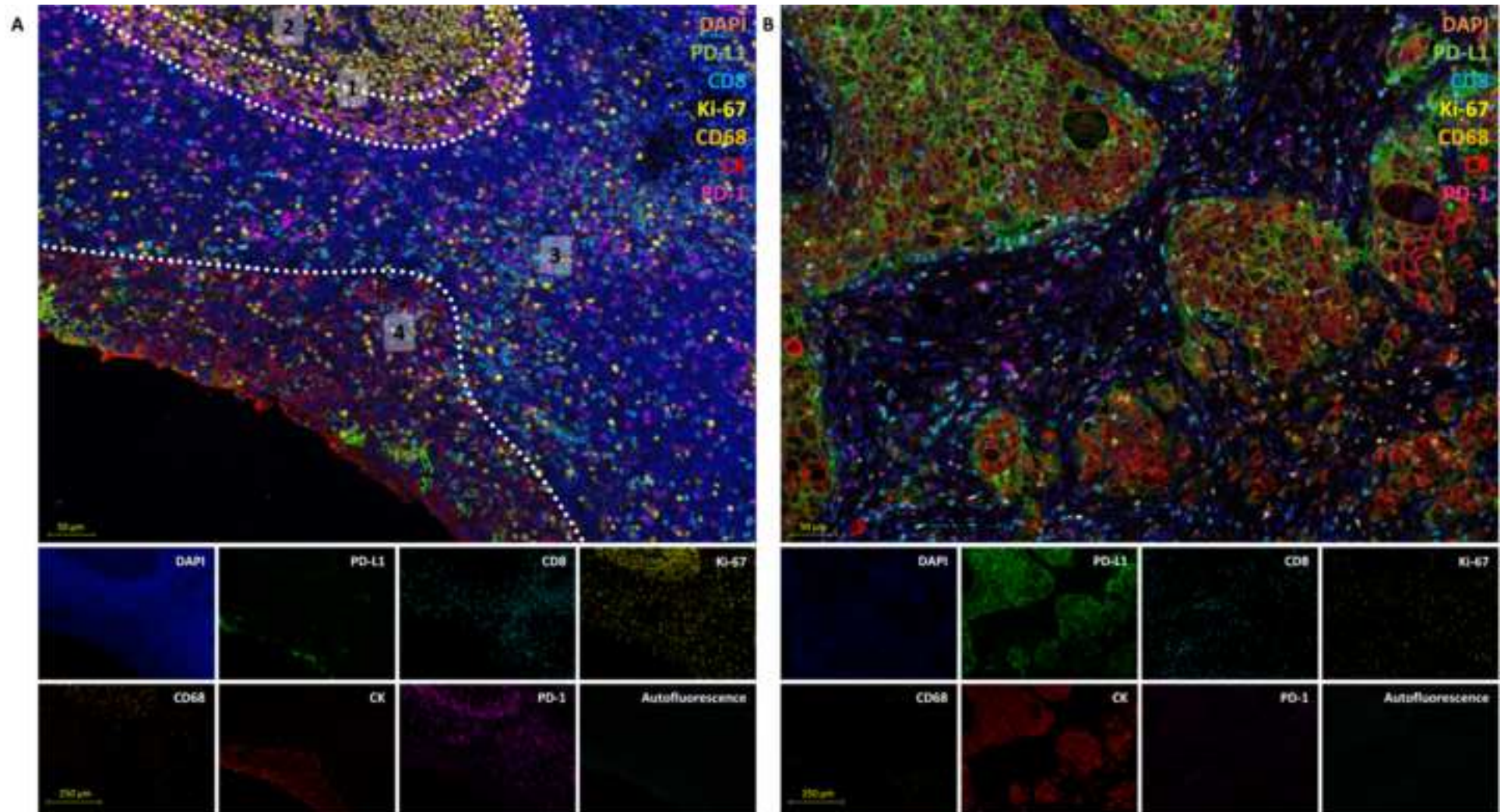




Figure 2

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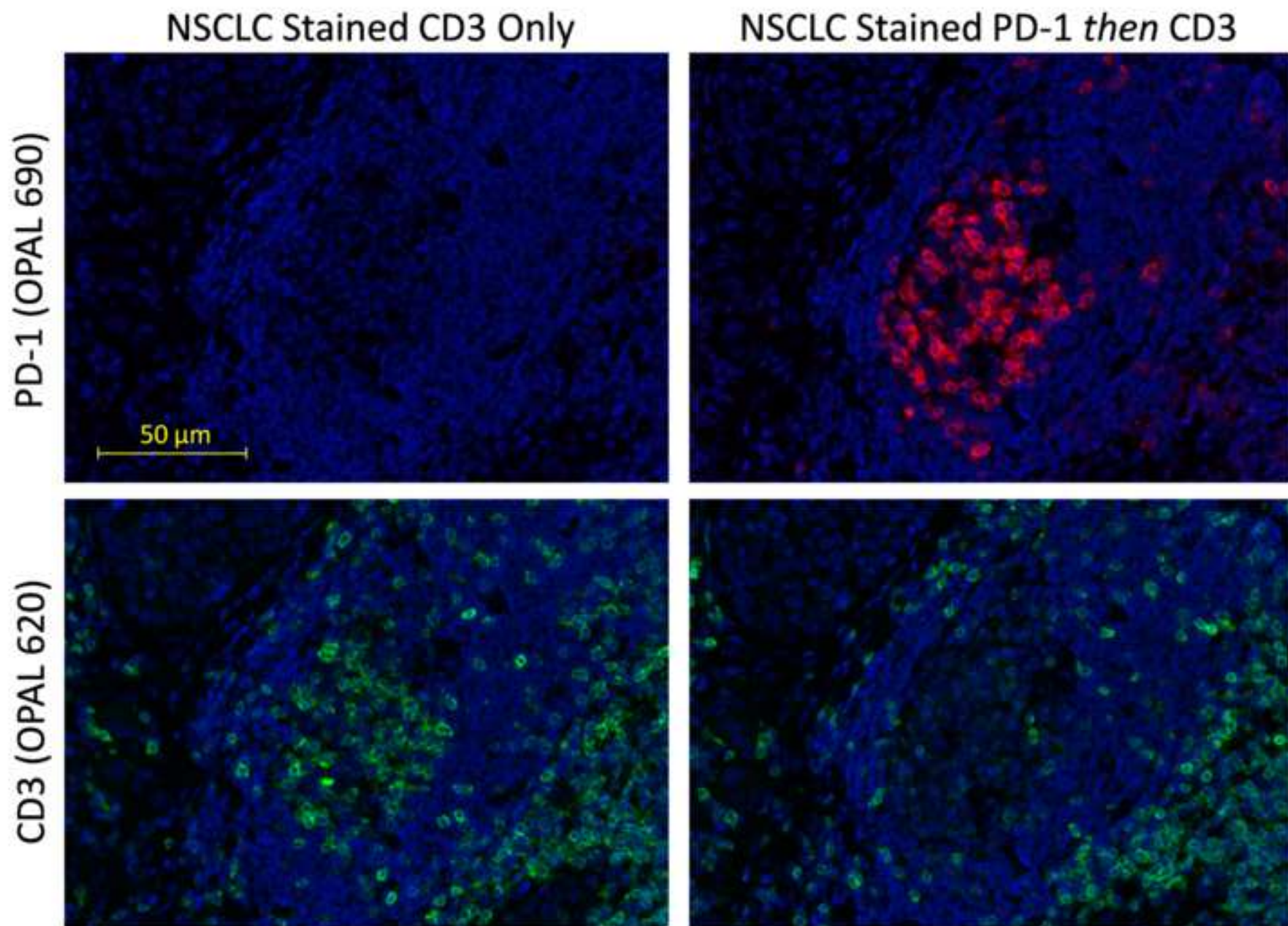




Figure 4

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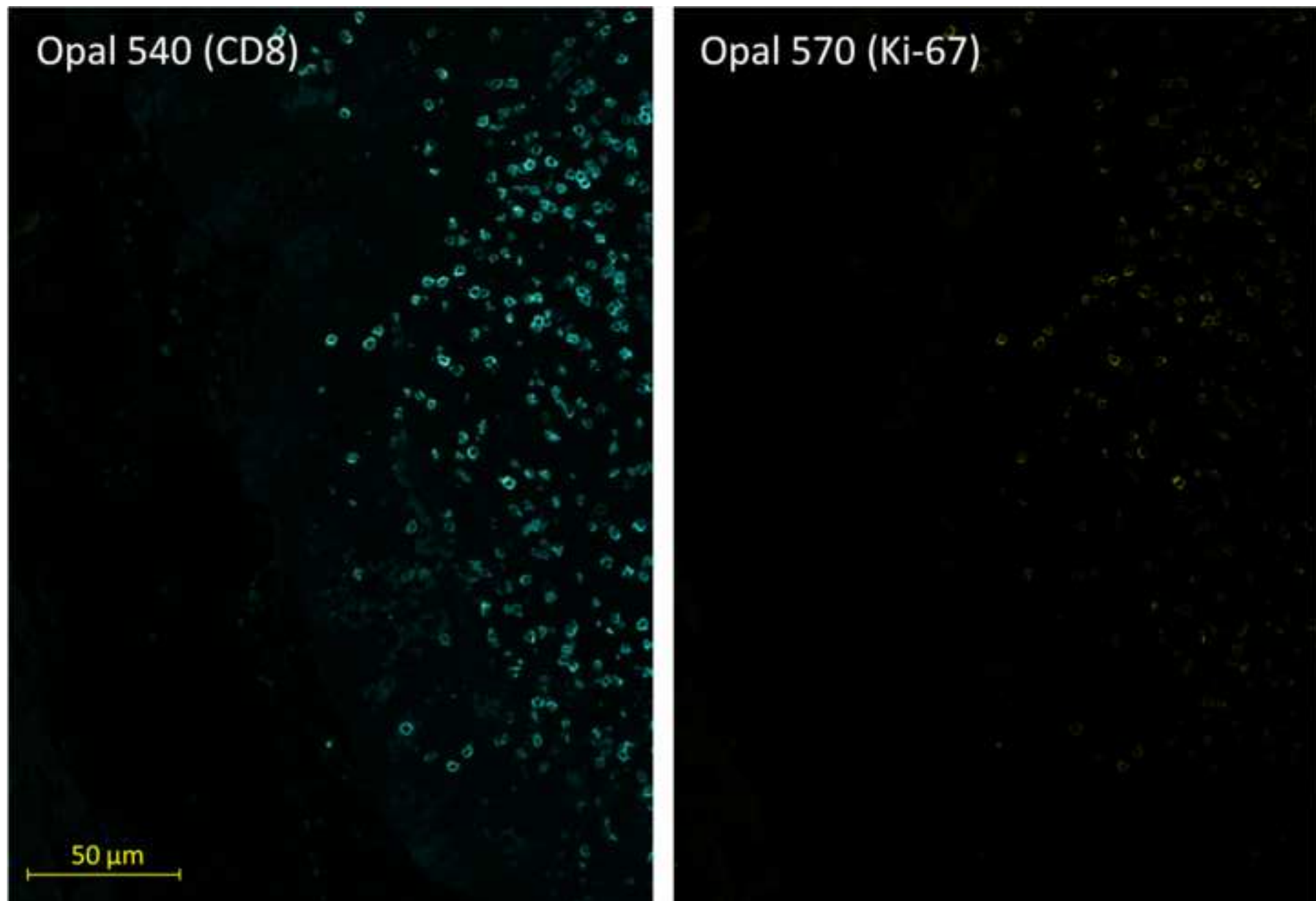
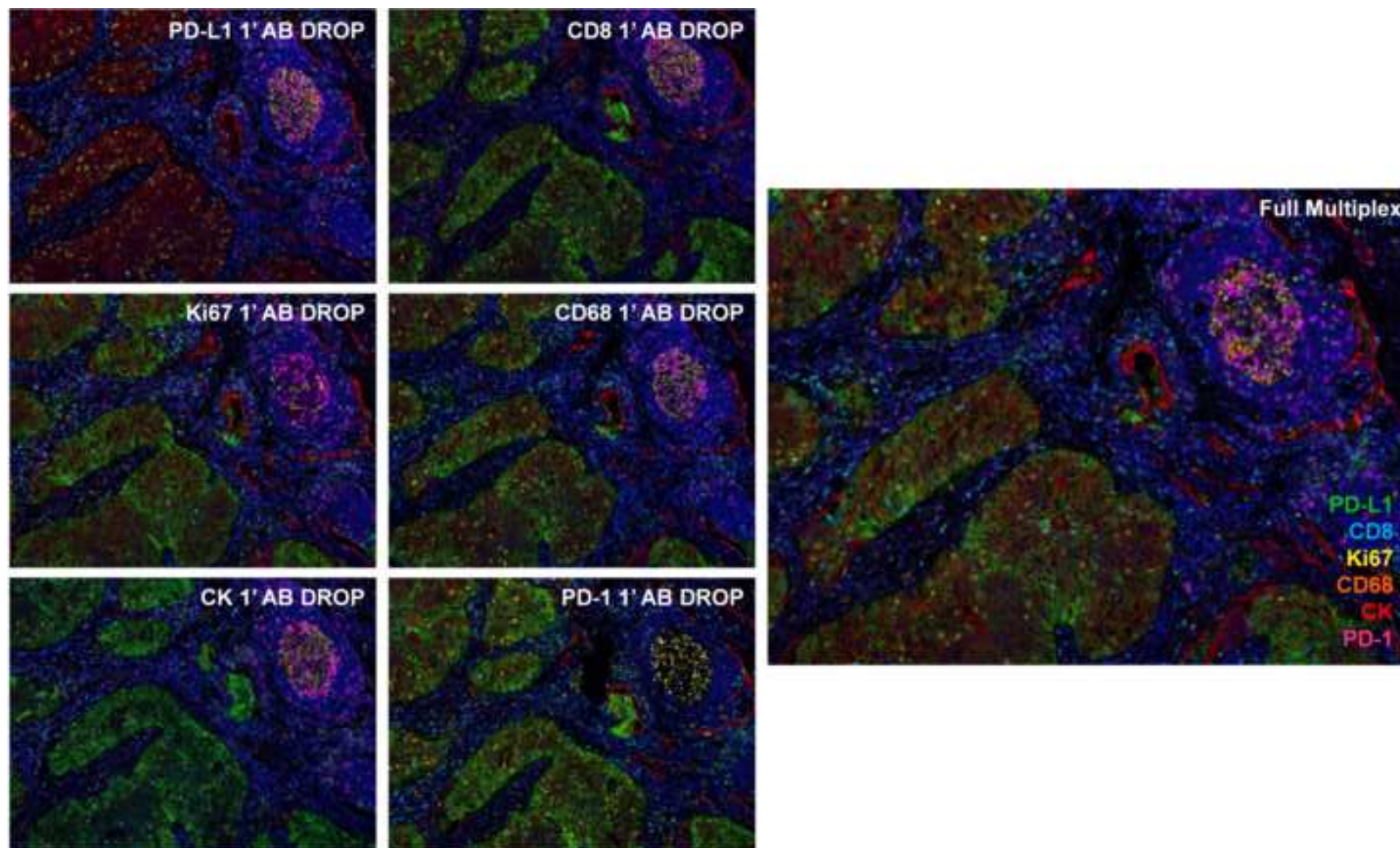


Figure 5

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**Table 1: Leica Bond RX Protocol, Opal Reagents**

<b>Name</b>	<b>Abbr. Name</b>	<b>Type</b>	<b>Container</b>	<b>Group</b>
PKI Blocking Buffer	OPALBLOK	Ancillary	Open 30 mL	General
Opal Polymer HRP	OPAL 2AB	Ancillary	Open 30 mL	General
OPAL 1x TBS	OPAL1TBS	Ancillary	Open 30 mL	Det. Kit
CD68 0.30 µg PG-M1 Mus	OPALCD68	Ancillary	Open 7 mL	General
ki67 0.61 µg MIB-1 Mus	OPALki67	Ancillary	Open 7 mL	General
PD-L1 0.20 µg SP263 Rab	OPALPDL1	Ancillary	Open 7 mL	General
PD-1 0.52 µg D4W2J Rab	OPAL PD1	Ancillary	Open 7 mL	General
CD8 0.70 µg SP239 Rab	OPAL CD8	Ancillary	Open 7 mL	General
CK 0.24 µg AE1/AE3 Mus	OPAL CK	Ancillary	Open 7 mL	General
Mouse IgG	OPAL MUS	Ancillary	Open 7 mL	General
Rabbit IgG	OPAL RAB	Ancillary	Open 7 mL	General
OPAL Peroxidase Block	OPALPERX	Ancillary	Open 7 mL	General
Spectral DAPI	OPALDAPI	Ancillary	Titration 6 mL	General
Opal 520 Reagent	OPAL 520	Ancillary	Titration 6 mL	General
Opal 540 Reagent	OPAL 540	Ancillary	Titration 6 mL	General
Opal 570 Reagent	OPAL 570	Ancillary	Titration 6 mL	General
Opal 620 Reagent	OPAL 620	Ancillary	Titration 6 mL	General
Opal 650 Reagent	OPAL 650	Ancillary	Titration 6 mL	General
Opal 690 Reagent	OPAL 690	Ancillary	Titration 6 mL	General



**Table 2: OPAL Preparation Protocol**

Reagent	Time (h)	Temperature (degrees Celsius)
1 No Reagent	120:00	60
2 Bond Dewax Solution	0:30	72
3 Bond Dewax Solution	0:00	72
4 Bond Dewax Solution	0:00	Ambient (room temperature)

**Table 3: Primary Antibodies Preparation**

Target	µg/mL	Clone	Source	Lot	Stock µg/mL	µL Diluent	µL AB
CD68	0.3	PG-M1	Dako	20043031	30	2376	24
ki67	0.61	MIB-1	Dako	20049476	46	2368.17	31.83
PD-L1	0.2	SP263	Vent.	F09591	1.61	2101.86	298.14
PD-1	0.52	D4W2J	CST	1	100	2387.52	12.48
CD8	0.7	SP239	Ventana	160318	70	2376	24
CK	0.24	AE1/AE3	Dako	10129428	179.5	2396.79	3.21
Mus IgG	0.61						
Rab IgG	0.7						

**Table 4: OPAL Fluor Preparation**

Opal Fluor	Slides	Dilution	µL Diluent	µL Opal Fluor
Opal 520	16	1:200	2736.3	13.8
Opal 540	16	1:700	2746.1	3.9
Opal 570	16	1:150	2731.7	18.3
Opal 620	16	1:100	2722.5	27.5
Opal 650	16	1:350	2742.1	7.9
Opal 690	16	1:150	2731.7	18.3

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
"InForm 2.4.2" Software for Spectral Unmixing and Image Analysis	PerkinElmer	CLS151066	Called "spectral unmixing software" in text
"Phenochart 1.0.9" QPTIFF Software for Selection of MSI and Overall Slide Scan Viewing	PerkinElmer	CLS151067	Called "QPTIFF software" in text
#1.5 Coverslips	Sigma Aldrich	2975246	
200 Proof Ethanol	Koptec	V1001	
20x Tris-Buffered Saline	VWR	J640-4L	
Antibody Diluent	DAKO	S2203	
Anti-CD68 Mouse Monoclonal	DAKO	M087601-2	Clone PG-M1
Anti-CD8 Rabbit Monoclonal	Ventana	M5392	Clone SP239
Anti-CK Mouse Monoclonal	DAKO	M351501-2	Clone AE1/AE3
Anti-ki67 Mouse Monoclonal	DAKO	M724001-2	Clone MIB-1
Anti-PD-1 Rabbit Monoclonal	Cell Signaling	#86163	Clone D4W2J
Anti-PD-L1 Rabbit Monoclonal	Ventana	790-4905	Clone SP263
Bond Dewax Solution	Leica	AR9222	Called "dewax solution" in text
Bond Epitope Retrieval Solution 1	Leica	AR9961	Called "ER1" in text
Bond Epitope Retrieval Solution 2	Leica	AR9640	Called "ER2" in text
Bond Open Containers, 30 mL	Leica	OP309700	Called "30 mL open containers" in text
Bond Open Containers, 7 mL	Leica	OP79193	Called "7 mL open containers" in text
Bond Polymer Refine Detection	Leica	DS9800	Called "chromogenic detection kit" in text
Bond Research Detection Kit	Leica	DS9455	Called "research detection kit" in text
Bond Titration Kit	Leica	OPT9049	Called "titration kit" in text
Bond Universal Covertile Novocastra	Leica	S21.2001	Called "covertiles" in text
Bond Wash Solution 10X Concentrate	Leica	AR9590	Called "10x wash solution" in text
BondRX Autostainer	Leica		Called "automated stainer" in text
BondRX Software Version 5.2.1.204	Leica		Called "automated stainer software" in text
Opal 7-Color Automation IHC Kit	PerkinElmer	NEL801001KT	Called "multispectral staining kit" in text
Peroxidase Block	Leica	RE7101	
ProLong Diamond Antifade Mountant	Thermo	P36965	Called "slide mountant" in text
Starfrost Slides	Fisher	15-183-51	

Vectra Polaris Multispectral Microscope  
with "Vectra 3.0.5" Software for  
Multispectral Microscope Control

PerkinElmer

CLS143455

Called "microscope control software" in text



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Article Title:

Optimization of an Automated Multiplex IF Assay

Signature:



Date:

05/04/2018

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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.

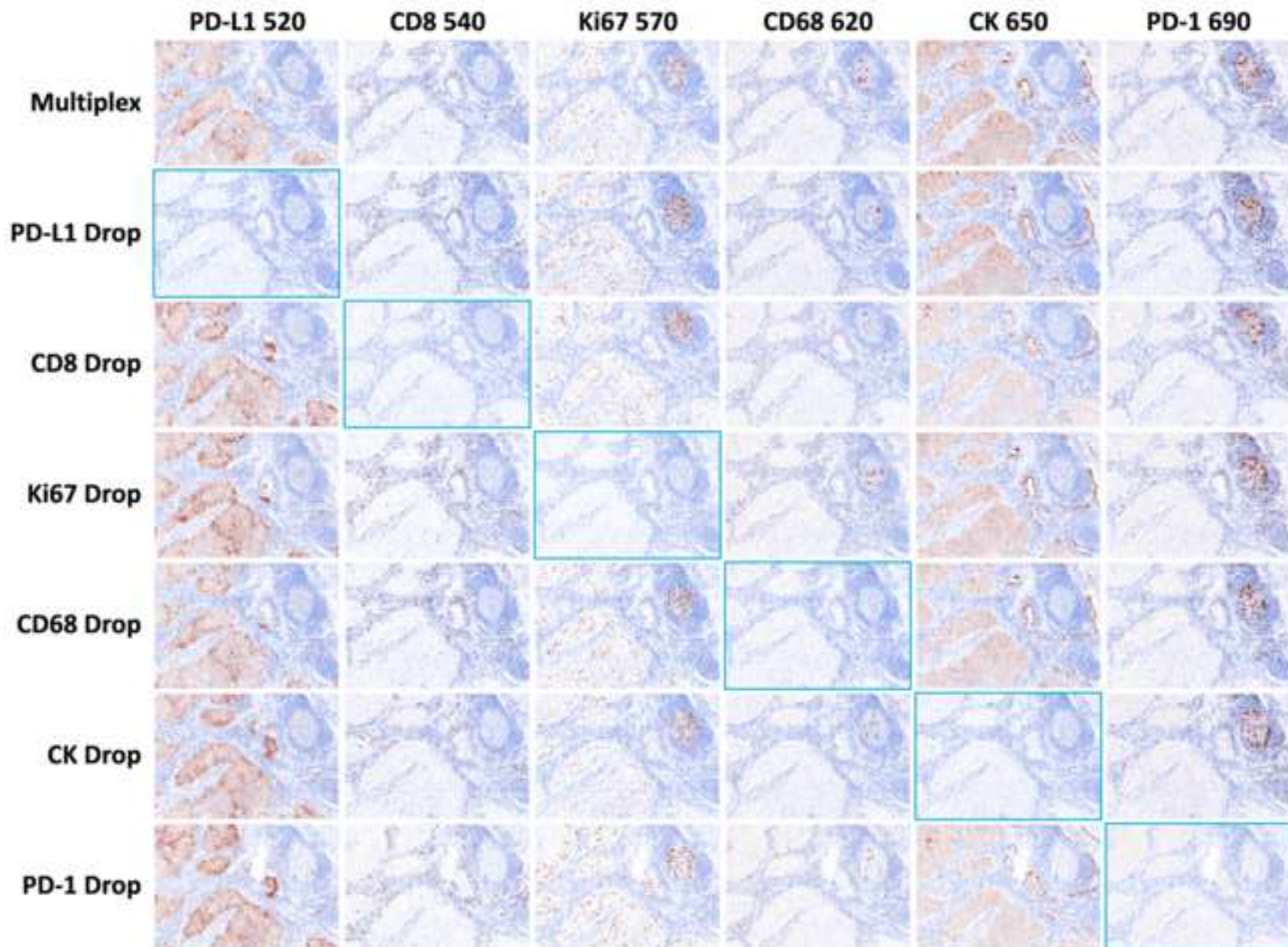
**Answer:** We thank the reviewer for this comment. Because JoVE is focused on the presentation of protocols for laboratory investigators, we have included important details in the main body of the manuscript.

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

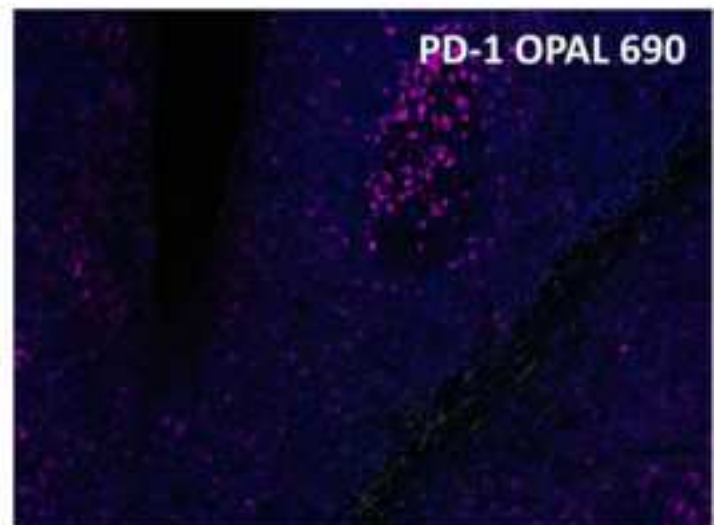
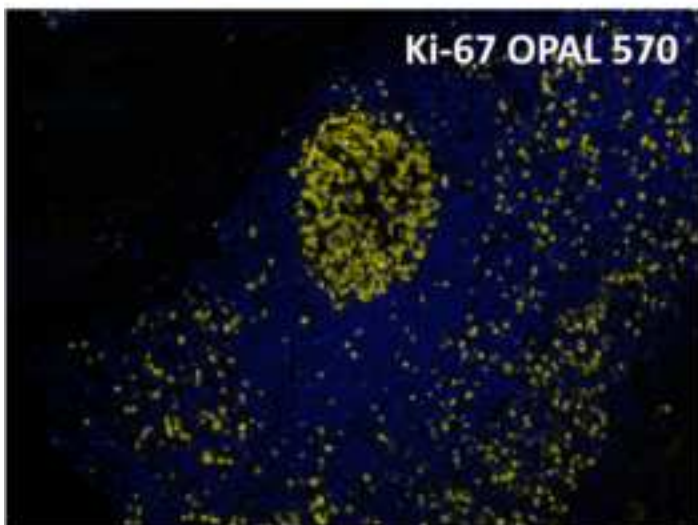
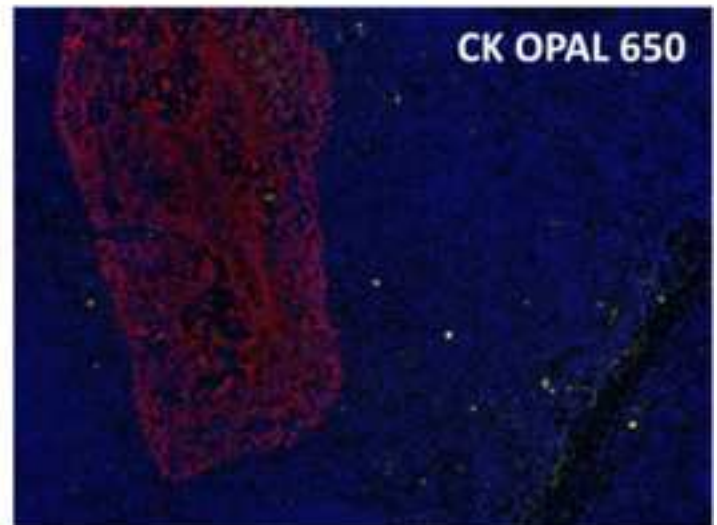
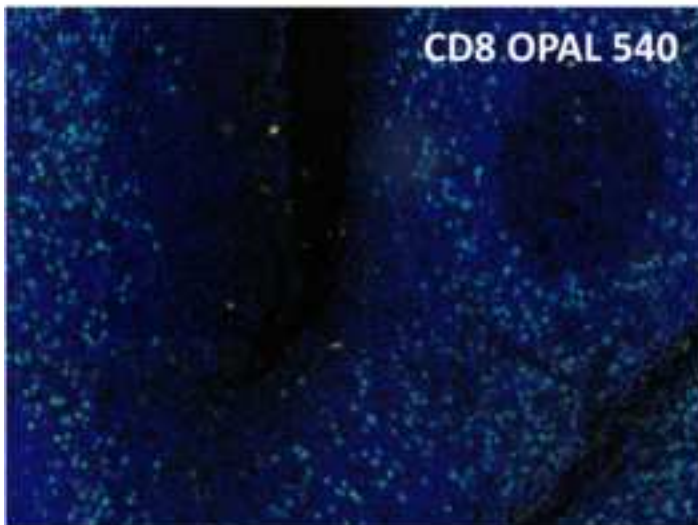
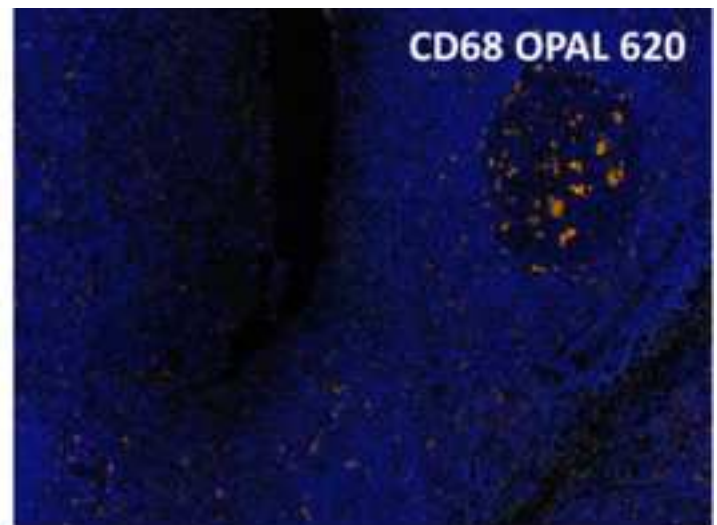
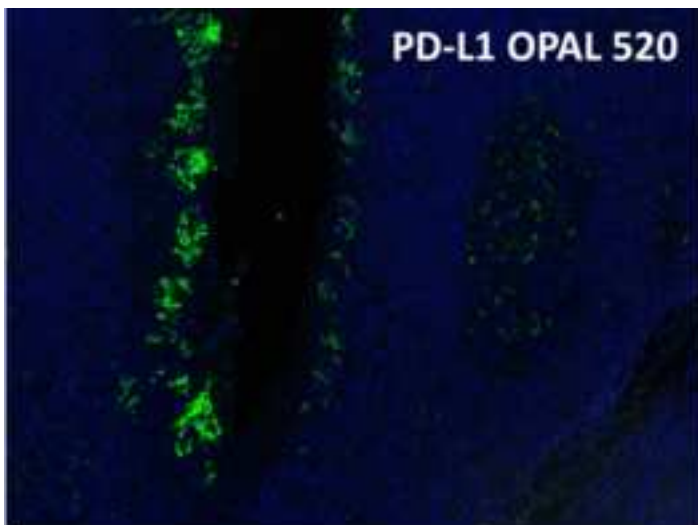
**Answer:** We agree with the reviewer. Due to the length of the manuscript, we kept the Discussion section focused on the main protocol and moved part of the troubleshooting and drop controls to the Supplemental Material.

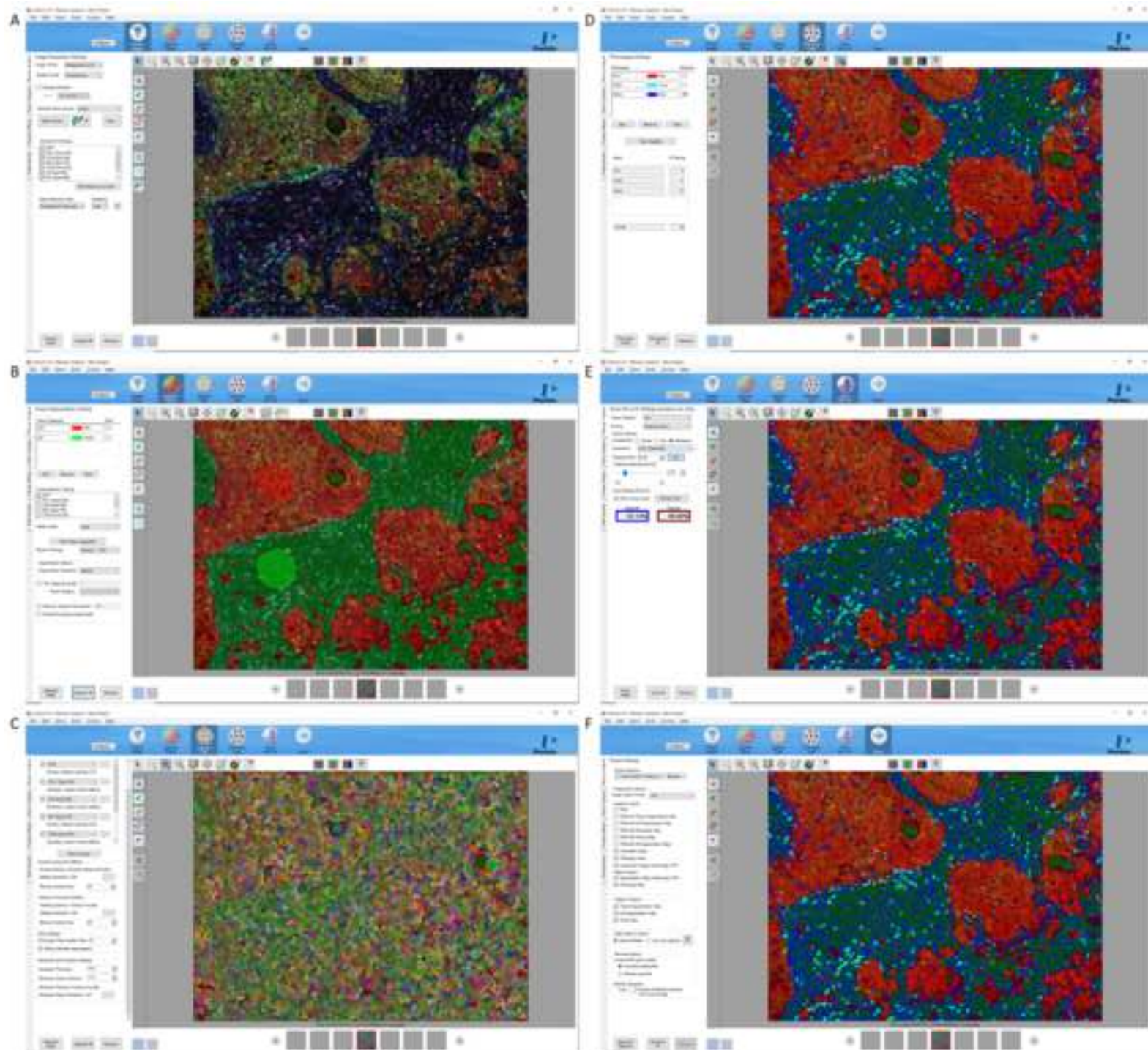
# 7 Different Slides – 1 Dropped Antibody

## Each Channel Rendered as Brightfield









**TITLE:** Automated Multiplex Immunofluorescence Panel for Immuno-Oncology Studies on Formalin-Fixed Carcinoma Tissue Specimens.

**AUTHORS:** Michael Surace, Karma Dacosta, Anna Huntley, Weiguang Zhao, Christopher Bagnall, Charles Brown, Chichung Wang, Kristin Roman, Jennifer Cann, Arthur Lewis, Keith Steele, Marlon Rebelatto, Edwin R. Parra, Clifford C. Hoyt, Jaime Rodriguez-Canales

## **SUPPLEMENTAL MATERIAL:**

### **Troubleshooting Common Problems Observed with Opal**

The most common Opal staining artifacts are spectral bleeding and signal attenuation or cancelation by the umbrella effect of tyramide signal amplification (TSA). These artifacts can be resolved principally by adjusting the TSA dilution for the affected markers; however, it may also be necessary to revisit earlier steps of the optimization. Multiple rounds of fine-tuning of TSA are not uncommon during the optimization of a new Opal panel. Once the major issues are addressed and the multiplex is optimized, the unmixed multiplex channel can be assessed by channel by comparing the number of cells detected and the staining pattern in sequential sections of multiplex with chromogenic immunohistochemistry single stains done in serial sections from the same tissue. This comparison can be done in InForm by selecting the multispectral imaging of the same tissue area in scans of both the chromogenic single stains and the multiplex and then using tissue segmentation, cell segmentation, and cell phenotyping to identify and quantify cells for each marker. If substantial overcounting or undercounting of cells occurs in the Opal multiplex vs the chromogenic single-stain references, re-optimization



should be performed, first using TSA concentration and then other conditions as necessary to match the chromogenic staining pattern and quantitation in the Opal multiplex.

**1. Spectral Bleed.** Spectral bleed is a common artifact in all multiplex immunofluorescence methods, but the risk is higher with Opal staining due to the high density of fluors in limited visual spectra (see Figure 4 in the main text). In spectral bleeding, the signal of a lower-wavelength marker manifests in a higher-wavelength channel. Spectral bleed can be detected by using the Counts tool in InForm. Overall, we have found that with the Vectra Polaris scanner, spectral bleed is most often observed in the 540/570 and 650/690 spectral interfaces. The interface between 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)/520, 520/540, 570/620, and 620/650 are, in practice, more robust and less subject to spectral bleed. Therefore, it may be advisable to avoid detecting exceptionally strong targets or targets with highly variable expression in Opal 540 and Opal 650, and inadvisable to detect exceptionally weak targets using Opal 570 and Opal 690. Using this protocol, we have also found occasional intensity-driven spectral bleed from Opal 650 (AE1/AE3) to Opal 690 (PD-1) channel. For instance, we have found that PD-1 expression is variable in tumors, whereas AE1/AE3 is more consistent. Uniformly low PD-1 expression in the context of robust AE1/AE3 expression will result in contamination of the PD-1 (Opal 690) channel with signal from the AE1/AE3 (Opal 650) channel. When this occurs, it manifests as a light cytokeratin pattern in the PD-1/Opal 690 channel. This artifact can be confirmed with the Counts tool, particularly if the maximal Opal 690 in signal is lower than 10 normalized counts. In this case, the concentration of Opal 690 can be increased by 1.5× or 2×. Another example of spectral bleeding in this Opal panel can be observed if the normalized counts are above 30 for CD8+ cells in the Opal 540 channel. The

following protocol describes the steps to detect spectral bleeding in the Opal 540 and Opal 570 channels as an example.

**1.1.** Using the channel selector, turn off all channels other than OPAL 540 and OPAL 570. A well-designed panel will detect a target with a different cellular and subcellular distribution (such as ki67) in the OPAL 570 channel.

**1.2.** Toggle the CD8/ OPAL 540 signal on and off while leaving the ki67/OPAL 570 signal on. If there is spectral bleed, then there will be a “phantom” of the CD8/ OPAL 540 staining pattern in the ki67/ OPAL 570 channel.

**1.3.** The phantom signal will be low in the ki67/ OPAL 570 channel, will have a membranous expression pattern, and will correlate in intensity with the CD8/ OPAL 540 signal. The solution to this problem is to equalize the CD8/ OPAL 540 signal and the ki67/ OPAL 570 signal by changing the TSA concentration of one or both fluors.

**2. Signal Attenuation or Cancellation due to TSA Blocking/ Umbrella Effect.** This artifact can be observed when two targets colocalize in the same cell compartment from the same cell (Figure 3). The TSA deposition from the target detected first may block the access to the second epitope, reducing or even ablating the second signal. To detect this problem, evaluate one target at a time.

**1.1.** First, stain two sequential lung adenocarcinoma slides. The first will be stained with the full multiplex and the second will be stained with only the fluorescent monoplex for the target in question (maintain all antigen retrieval conditions).

**1.2.** Unmix images from the same region of each slide in a single InForm project. Observe the staining pattern visually and count cells using InForm. If a change in expression pattern



(subjective) or a significant reduction in cell counts is observed in the optimized multiplex as compared to the monoplex (Figure S2), then TSA blocking may be occurring. If TSA blocking effect is suspected, then the solution to this problem is to:

**1.3.** Alternate the order of staining to stain the blocked target before the blocking target. Test for blocking again.

**1.4.** Reduce the TSA concentration of the offending fluor as low as possible keeping the normalized values in the range of 10-20.

**1.5.** If neither of these approaches solves the problem, then primary antibody clone and conditions, target/fluor combinations, and/or staining order may have to be revisited.

### **Evaluation of a New Opal Panel using Drop Controls**

The drop controls are identical to the full multiplex except for the absence of one primary antibody in each control slide. We recommend processing the images from drop controls in a single project with a full multiplex panel. The purpose of the drop controls is to confirm that the entirety of a particular signal after spectral unmixing is in fact derived from the epitope targeted by the primary antibody associated with that channel. This ensures that co-staining and spectral unmixing are robust and free of interference. Each drop control should generate no signal in the dropped channel. If a signal is present, this may be due to spectral bleed or to high autofluorescence in that channel. If this is observed, re-optimization is required.

Drop controls can also identify the umbrella effect. If any target increases in intensity or cell counts when a colocalized and earlier-stained target is dropped, then this may indicate that the TSA deposited to detect the earlier-stained target may be blocking binding of the later-stained primary antibody.

Successful drop controls will provide assurance that each signal is derived faithfully from binding of the appropriate primary antibody and that spectral unmixing for the multiplex at large is not affected by the presence or absence of a particular signal. See Figure 5, Supplemental Figures S2 and S3, and Supplemental Table S2 for a summary and comparison of fluorescent monoplex, full multiplex, drop controls, and full isotype controls.

**Supplemental Table 1: Leica Bond RX Opal Multiplex Protocol**

Reagent	Time (h)	Temperature
1 Bond Wash Solution	0:00	Ambient
2 Opal Peroxidase Block	20:00	Ambient
3 Bond Wash Solution	0:00	Ambient
4 PKI Blocking Buffer	5:00	Ambient
5 Opal CD68 0.3 µg PG-M1 Mus	30:00	Ambient
6 Bond Wash Solution	0:00	Ambient
7 Bond Wash Solution	1:00	Ambient
8 Bond Wash Solution	0:00	Ambient
9 Opal Polymer HRP	10:00	Ambient
10 Bond Wash Solution	0:00	Ambient
11 Bond Wash Solution	1:00	Ambient
12 Bond Wash Solution	0:00	Ambient
13 Bond Wash Solution	0:00	Ambient
14 Bond Wash Solution	0:00	Ambient
15 Opal 620 Reagent	10:00	Ambient
16 Bond Wash Solution	0:00	Ambient
17 Bond Wash Solution	1:00	Ambient
18 Bond Wash Solution	0:00	Ambient
19 Bond Wash Solution	0:00	Ambient
20 Bond ER Solution 2	0:00	Ambient
21 Bond ER Solution 2	0:00	95°C
22 Bond ER Solution 2	40:00	95°C
23 Bond ER Solution 2	0:00	Ambient
24 Bond Wash Solution	0:00	Ambient
25 Bond Wash Solution	1:00	Ambient
26 Bond Wash Solution	0:00	Ambient
27 PKI Blocking Buffer	5:00	Ambient
28 Opal KI67 0.61 µg MIB-1 Mus	30:00	Ambient
29 Bond Wash Solution	0:00	Ambient
30 Bond Wash Solution	1:00	Ambient
31 Bond Wash Solution	0:00	Ambient
32 Opal Polymer HRP	10:00	Ambient
33 Bond Wash Solution	0:00	Ambient
34 Bond Wash Solution	1:00	Ambient
35 Bond Wash Solution	0:00	Ambient
36 Bond Wash Solution	0:00	Ambient
37 Bond Wash Solution	0:00	Ambient
38 Opal 570 Reagent	10:00	Ambient
39 Bond Wash Solution	0:00	Ambient
40 Bond Wash Solution	1:00	Ambient
41 Bond Wash Solution	0:00	Ambient
42 Bond Wash Solution	0:00	Ambient
43 Bond ER Solution 1	0:00	Ambient
44 Bond ER Solution 1	0:00	95°C
45 Bond ER Solution 1	20:00	95°C

46 Bond ER Solution 1	0:00	Ambient
47 Bond Wash Solution	0:00	Ambient
48 Bond Wash Solution	1:00	Ambient
49 Bond Wash Solution	0:00	Ambient
50 PKI Blocking Buffer	5:00	Ambient
51 Opal PD-L1 0.20 µg SP263 Rab	30:00	Ambient
52 Bond Wash Solution	0:00	Ambient
53 Bond Wash Solution	1:00	Ambient
54 Bond Wash Solution	0:00	Ambient
55 Opal Polymer HRP	10:00	Ambient
56 Bond Wash Solution	0:00	Ambient
57 Bond Wash Solution	1:00	Ambient
58 Bond Wash Solution	0:00	Ambient
59 Bond Wash Solution	0:00	Ambient
60 Bond Wash Solution	0:00	Ambient
61 Opal 520 Reagent	10:00	Ambient
62 Bond Wash Solution	0:00	Ambient
63 Bond Wash Solution	1:00	Ambient
64 Bond Wash Solution	0:00	Ambient
65 Bond Wash Solution	0:00	Ambient
66 Bond ER Solution 1	0:00	Ambient
67 Bond ER Solution 1	0:00	95°C
68 Bond ER Solution 1	20:00	95°C
69 Bond ER Solution 1	0:00	Ambient
70 Bond Wash Solution	0:00	Ambient
71 Bond Wash Solution	1:00	Ambient
72 Bond Wash Solution	0:00	Ambient
73 PKI Blocking Buffer	5:00	Ambient
74 Opal PD-1 0.52 µg D4W2J Rab	30:00	Ambient
75 Bond Wash Solution	0:00	Ambient
76 Bond Wash Solution	1:00	Ambient
77 Bond Wash Solution	0:00	Ambient
78 Opal Polymer HRP	10:00	Ambient
79 Bond Wash Solution	0:00	Ambient
80 Bond Wash Solution	1:00	Ambient
81 Bond Wash Solution	0:00	Ambient
82 Bond Wash Solution	0:00	Ambient
83 Bond Wash Solution	0:00	Ambient
84 Opal 690 Reagent	10:00	Ambient
85 Bond Wash Solution	0:00	Ambient
86 Bond Wash Solution	1:00	Ambient
87 Bond Wash Solution	0:00	Ambient
88 Bond Wash Solution	0:00	Ambient
89 Bond ER Solution 1	0:00	Ambient
90 Bond ER Solution 1	0:00	95°C
91 Bond ER Solution 1	20:00	95°C
92 Bond ER Solution 1	0:00	Ambient

93 Bond Wash Solution	0:00	Ambient
94 Bond Wash Solution	1:00	Ambient
95 Bond Wash Solution	0:00	Ambient
96 PKI Blocking Buffer	5:00	Ambient
97 Opal CD8 0.70 µg SP239 Rab	30:00	Ambient
98 Bond Wash Solution	0:00	Ambient
99 Bond Wash Solution	1:00	Ambient
100 Bond Wash Solution	0:00	Ambient
101 Opal Polymer HRP	10:00	Ambient
102 Bond Wash Solution	0:00	Ambient
103 Bond Wash Solution	1:00	Ambient
104 Bond Wash Solution	0:00	Ambient
105 Bond Wash Solution	0:00	Ambient
106 Bond Wash Solution	0:00	Ambient
107 Opal 540 Reagent	10:00	Ambient
108 Bond Wash Solution	0:00	Ambient
109 Bond Wash Solution	1:00	Ambient
110 Bond Wash Solution	0:00	Ambient
111 Bond Wash Solution	0:00	Ambient
112 Bond ER Solution 1	0:00	Ambient
113 Bond ER Solution 1	0:00	95°C
114 Bond ER Solution 1	20:00	95°C
115 Bond ER Solution 1	0:00	Ambient
116 Bond Wash Solution	0:00	Ambient
117 Bond Wash Solution	1:00	Ambient
118 Bond Wash Solution	0:00	Ambient
119 PKI Blocking Buffer	5:00	Ambient
120 Opal CK 0.24 µg AE1/AE3 Mus	30:00	Ambient
121 Bond Wash Solution	0:00	Ambient
122 Bond Wash Solution	1:00	Ambient
123 Bond Wash Solution	0:00	Ambient
124 Opal Polymer HRP	10:00	Ambient
125 Bond Wash Solution	0:00	Ambient
126 Bond Wash Solution	1:00	Ambient
127 Bond Wash Solution	0:00	Ambient
128 Bond Wash Solution	0:00	Ambient
129 Bond Wash Solution	0:00	Ambient
130 Opal 650 Reagent	10:00	Ambient
131 Bond Wash Solution	0:00	Ambient
132 Bond Wash Solution	1:00	Ambient
133 Bond Wash Solution	0:00	Ambient
134 Bond Wash Solution	0:00	Ambient
135 Bond ER Solution 1	0:00	Ambient
136 Bond ER Solution 1	0:00	95°C
137 Bond ER Solution 1	20:00	95°C
138 Bond ER Solution 1	0:00	Ambient
139 Bond Wash Solution	0:00	Ambient

140 Bond Wash Solution	1:00	Ambient
141 Bond Wash Solution	0:00	Ambient
142 Spectral DAPI	10:00	Ambient
143 Bond Wash Solution	0:00	Ambient
144 Bond Wash Solution	1:00	Ambient
145 Bond Wash Solution	0:00	Ambient

**Supplemental Table S2: Summary and comparison of full multiplex, drop controls, and full i**

<b>Full Isotype</b>	<b>Multiplex</b>	<b>CD68 Drop</b>	<b>ki67 Drop</b>	<b>PD-L1 Drop</b>	<b>PD-1 Drop</b>	<b>CD8 Drop</b>	<b>CK Drop</b>
Mouse IgG	CD68 Primary	Mouse IgG	CD68 Primary	CD68 Primary	CD68 Primary	CD68 Primary	CD68 Primary
Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	OpalL 2AB	Opal 2AB	Opal 2AB	Opal 2AB
Opal 620	Opal 620	Opal 620	OpalL 620	Opal 620	Opal 620	Opal 620	Opal 620
Mouse IgG	ki67 Primary	ki67 Primary	Mouse IgG	ki67 Primary	ki67 Primary	ki67 Primary	ki67 Primary
Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB
Opal 570	Opal 570	Opal 570	Opal 570	Opal 570	Opal 570	Opal 570	Opal 570
Rabbit IgG	PD-L1 Primary	PD-L1 Primary	PD-L1 Primary	Rabbit IgG	PD-L1 Primary	PD-L1 Primary	PD-L1 Primary
Opal 2AB	Opal 2AB	Opal 2AB	OpalL 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB
Opal 520	Opal 520	Opal 520	Opal 520	Opal 520	Opal 520	Opal 520	Opal 520
Rabbit IgG	PD-1 Primary	PD-1 Primary	PD-1 Primary	PD-1 Primary	Rabbit IgG	PD-1 Primary	PD-1 Primary
Opal 2AB	Opal 2AB	Opal 2AB	OpalL 2AB	Opal 2AB	Opal 2AB	Opal 2AB	OpalL 2AB
Opal 690	Opal 690	Opal 690	Opal 690	Opal 690	Opal 690	Opal 690	Opal 690
Rabbit IgG	CD8 Primary	CD8 Primary	CD8 Primary	CD8 Primary	CD8 Primary	Rabbit IgG	CD8 Primary
Opal 2AB	Opal 2AB	Opal 2AB	OpalL 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB
Opal 540	Opal 540	Opal 540	Opal 540	Opal 540	Opal 540	Opal 540	Opal 540
Mouse IgG	CK Primary	CK Primary	CK Primary	CK Primary	CK Primary	CK Primary	Mouse IgG
Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB	Opal 2AB
Opal 650	Opal 650	Opal 650	Opal 650	Opal 650	Opal 650	Opal 650	Opal 650
Spectral DAPI	Spectral DAPI	Spectral DAPI	Spectral DAPI	Spectral DAPI	Spectral DAPI	Spectral DAPI	Spectral DAPI

CK = cytokeratin; DAPI = 4',6-diamidino-2-phenylindole dihydrochloride; IgG = immunoglobulin

**sotype controls**

in G; PD-L1 = programmed cell death ligand 1.