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

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**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. 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.
   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.
   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. 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).
   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:
   3. Alternate the order of staining to stain the blocked target before the blocking target. Test for blocking again.
   4. Reduce the TSA concentration of the offending fluor as low as possible keeping the normalized values in the range of 10-20.
   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.