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Optimization, Design and Avoiding Pitfalls in Manual Multiplex Fluorescent Immunohistochemistry
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1 **TITLE:**
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3 **Immunohistochemistry**
4
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28 **KEYWORDS:**
29 cancer, metastasis, immunofluorescence, multiplex fluorescent immunohistochemistry,
30 microenvironment, cell-to-cell engagement, staining, fluorophore
31

32 **SUMMARY:**
33 Multiplex fluorescent immunohistochemistry is an emerging technology that enables the
34 visualization of multiple cell types within intact formalin-fixed, paraffin embedded (FFPE) tissue.
35 Presented are guidelines for ensuring a successful 7-color multiplex with instructions for
36 optimizing antibodies and reagents, preparing slides, design and tips for avoiding common
37 problems.
38

39 **ABSTRACT:**
40 Microenvironment evaluation of intact tissue for analysis of cell infiltration and spatial
41 organization are essential in understanding the complexity of disease processes. The principle
42 techniques used in the past include immunohistochemistry (IHC) and immunofluorescence (IF)
43 which enable visualization of cells as a snapshot in time using between 1 and 4 markers. Both
44 techniques have shortcomings including difficulty staining poorly antigenic targets and

45 limitations related to cross-species reactivity. IHC is reliable and reproducible, but the nature of
46 the chemistry and reliance on the visible light spectrum allows for only a few markers to be used
47 and makes co-localization challenging. Use of IF broadens potential markers but typically relies
48 on frozen tissue due to the extensive tissue autofluorescence following formalin fixation. Flow
49 cytometry, a technique that enables simultaneous labeling of multiple epitopes, abrogates many
50 of the deficiencies of IF and IHC, however, the need to examine cells as a single cell suspension
51 loses the spatial context of cells discarding important biologic relationships. Multiplex fluorescent
52 immunohistochemistry (mfIHC) bridges these technologies allowing for multi-epitope cellular
53 phenotyping in formalin fixed paraffin embedded (FFPE) tissue while preserving the overall
54 microenvironment architecture and spatial relationship of cells within intact undisrupted tissue.
55 High fluorescent intensity fluorophores that covalently bond to the tissue epitope enables
56 multiple applications of primary antibodies without worry of species specific cross-reactivity by
57 secondary antibodies. Although this technology has been proven to produce reliable and
58 accurate images for the study of disease, the process of creating a useful mfIHC staining strategy
59 can be time consuming and exacting due to extensive optimization and design. In order to make
60 robust images that represent accurate cellular interactions in-situ and to mitigate the
61 optimization period for manual analysis, presented here are methods for slide preparation,
62 optimizing antibodies, multiplex design as well as errors commonly encountered during the
63 staining process.
64
65 **INTRODUCTION:**
66 Visualization of an intact tumor microenvironment (TME) is essential in evaluating not only
67 cellular infiltration in solid malignancies but cell to cell interactions as well. Multiplex fluorescent
68 immunohistochemistry (mfIHC) has emerged as an effective tool for multi-antigen phenotyping
69 of cells in the study of cancer and associated diseases[1-7]. This, in combination with novel software
70 and programs designed to analyze large data sets, enables observation of complex interactions
71 between cells[1,2,4]. The rate limiting factor in data acquisition is often the quality of the stained
72 tissue prior to analysis.
73
74 Previous techniques used to phenotype cells in the TME include immunohistochemistry (IHC),
75 immunofluorescence (IF) and flow cytometry all of which present significant limitations. IHC uses
76 formalin fixed paraffin embedded (FFPE) tissue sections which are deparaffinized and rehydrated
77 before being stained by most often one antibody. Use of a horseradish peroxidase (HRP) bound
78 secondary antibody and a chemical reaction allows visualization of a single antigenic epitope[8].
79 While IHC is reliable and performed on FFPE tissue which is easy to work with, limitations to the
80 visible light spectrum means only one or two markers can be reliable distinguished and
81 colocalization of antigens quite difficult[8]. A way to expand available markers and therefore
82 antigens that can be probed on a single section is to change to fluorescence which allows for use
83 of a broader range of the visual spectrum. For IF, frozen or FFPE tissue is transferred onto slides
84 and antibodies used that are conjugated to various fluorophores. While this increases the
85 number of antigens that can be probed, there are several important limitations. First, because
86 each antibody typically only has one fluorophore attached, the brightness is often not strong
87 enough to overcome tissue autofluorescence. It is for this reason, most IF is performed on frozen
88 tissue which is expensive to store and difficult to work with. A limited number of fluorescent tags

89   are available for use due to spectral overlap and cross species reactivity particularly when non-
90   conjugated antibodies are used. Flow cytometry, which consists of fresh tissue processing into a
91   single cell suspension and labeling with fluorescent antibodies has been the gold standard for
92   immunophenotyping for decades[9,10]. A benefit of flow cytometry is the ability to label multiple
93   antibodies without concern for species cross reactivity as most are conjugated. Because the cells
94   are "visualized" by a machine and not human eyes, there are far more fluorophores available but
95   this comes with a cost. Compensation must be done manually which can significantly alter results
96   producing false positive and negative populations. The most significant limitation of flow
97   cytometry is that tissue architecture and subsequently all spatial information is lost by the
98   necessity for single cells suspension.
99
100 Multiplex fluorescent immunohistochemistry (mfIHC) using an automated fluorescent
101 microscope in combination with novel software combines the benefits of IHC, IF and flow
102 cytometry by allowing multi-antigen tissue staining with signal amplification and retention of
103 spatial relationships without the need for compensation. FFPE tissue is placed on charged slides
104 which, after antigen retrieval, undergo a round of primary antibody application to the target
105 antigen of interest followed by a secondary antibody with an HRP chemical tag, similar to IHC.
106 After placement of the secondary antibody, an HRP specific reaction results in a fluorophore
107 covalently binding to the epitope of interest[11]. Once the tissue is labeled, another round of
108 heating the slides is completed removing the previously applied primary and secondary antibody
109 complex leaving only the fluorescent tag bound to the tissue epitope[11]. This allows for multiple
110 antibodies of any species to be reapplied without concern for cross-reactivity[11,12]. To minimize
111 any need for manual compensation of multiple fluorescent dyes, a collection of fluorophores with
112 little spectral overlap including a nuclear counter stain is used to complete the mfIHC. To account
113 for the autofluorescence encountered with FFPE tissue, software subtracts the autofluorescence
114 from the final image using an image from a blank slide which is possible because of the strength
115 of antigen specific fluorescence following fluorophore signal amplification. Using novel programs
116 designed for large data sets, cell locations can be identified and analyzed for spatial context[1,2,4].
117 The most significant limitation of this technique is optimization time. A detailed methodology
118 with instructions for experimental design and staining and imaging strategy is found here. mfIHC
119 will be useful for laboratories that do not currently have an optimized automated staining system
120 that would like to better understand the spatial context of cell-to-cell interactions in intact FFPE
121 tissue using the manual technique.
122
123 **PROTOCOL:**
124 All work has been approved by the University of Michigan's internal review board.
125
126 **1. Optimizing primary antibodies and slide preparation**
127
128 1.1. Determine ideal concentration of antibodies for multiplex using conventional IHC.
129
130 1.1.1. Test the antibodies for the multiplex by manual conventional immunohistochemistry
131 (IHC)[13].
132

133   1.1.2. Use specific tissues that have an abundant cell type for each antibody tested such as using
134   tonsil tissue for CD3 antibody testing.

135

136   1.1.3. Use the reference concentration for IHC recommended by the company and then complete
137   IHC with antibody concentrations at the recommended concentrations as well as concentrations
138   above and below the recommended concentration.

139

140   1.2. Prepare formalin fixed paraffin embedded tissue onto slides.

141

142   1.2.1. Using a microtome, cut blocks of FFPE tissue at a thickness between 4 and 6 μm and apply
143   to charged slides.

144

145   NOTE: Using distilled water ensures proper tissue mounting and adherence throughout the
146   multiplex.

147

148   1.2.2. Place the slides flat, tissue side up, and allow to dry at 37 °C overnight.

149

150   1.2.3. Store slides in a slide box away from extreme temperature until ready to complete the
151   multiplex.

152

153   **2. General staining method**

154

155   2.1. Preparation of wash and antigen retrieval solutions

156

157   2.1.1. Prepare the wash solution of 0.1% TBST by mixing 9 L of deionized water, 1 L of tris buffered
158   saline (TBS) and 10 mL of polysorbate 20.

159

160   2.1.2. Prepare both antigen retrieval solutions (pH 6 and pH 9; **Table of Materials**) by diluting to
161   1x with deionized water.

162

163   2.2. Deparaffinization and rehydration

164

165   2.2.1. Bake slides, lying flat, tissue side up at 60 °C for 1 h in a hybridization oven[1]. Remove slides
166   from the oven and allow to cool for at least 5−10 min then place in a vertical slide rack.

167

168   2.2.2. Treat the slides in the following solutions sequentially: xylene in triplicate, followed by a
169   single treatment of 100% ethanol, 95% ethanol, and lastly 70% ethanol for 10 min each using a
170   slide staining set[1].

171

172   2.2.3. Wash the rack of slides for 2 min with deionized water by submersion in a plastic slide box
173   followed by fixation by submersion in a plastic slide box filled with neutral buffered formalin for
174   30 min[13]. Wash the slides in deionized water for 2 min then proceed to antigen retrieval.

175

176   2.3. Antigen retrieval

177

2.3.1. Place the rack of slides into a heat resistant box filled to the internal line (approximately 300 mL) with pH 6 or pH 9 antigen retrieval buffer.

NOTE: Antigen retrieval buffer can be either pH 6 or pH 9 which may need to be optimized per epitope. However, start by using pH 9 for antibodies that bind to nuclear epitopes and pH 6 for all other antibodies.

2.3.2. Cover the box with plastic wrap and use a rubber band to secure. Place the box into the microwave at the edge of the rotating plate (microwave must be equipped with inverter technology for even heating). Heat the slides for 45 s at 100% power followed by 15 min at 20% power[13].

NOTE: Microwave treatment may need optimization depending on the microwave used.

2.3.3. Let the slides cool for approximately 15–20 min.

2.4. Prepare working solutions of antibodies and fluorophores while slides cool.

2.4.1. Prepare the primary antibody diluent by dissolving 0.5 g of bovine serum albumin granules into 50 mL of TBST. Alternatively, use 50 mL of 1x phosphate buffered saline (PBS) to dissolve granules.

2.4.2. Make each primary antibody working solution to the optimized concentration determined in section 1, in the primary antibody diluent. Estimate approximately 200 µL per slide.

2.4.3. Prepare the fluorophore working solution by diluting each fluorophore in the fluorophore diluent at a concentration of 1:100.

NOTE: This may need to be optimized depending on the antibody. Estimate approximately 100 µL per slide.

2.4.4. On the last day of staining, prepare the 4',6-diamidino-2-phenylindole (DAPI) working solution by adding 3 drops of DAPI into 1 mL of TBST.

2.5. Slide staining (washing and blocking)

2.5.1. Remove the box from the microwave after cooling and take off the plastic wrap, wash the slides with deionized water for 2 min followed by a 2 min wash in a plastic slide box filled TBST[13].

2.5.2. After drying each slide around the tissue with a delicate task wipe careful not to let the tissue dry out, trace around the outside of the tissue using a hydrophobic barrier pen. Do not touch the tissue with the delicate task wipe or pen.

221 2.5.3. Place each slide in the humidified chamber and add blocking solution (approximately 4
222 drops) to the tissue. Incubate for 10 min at room temperature (RT).

224 2.6. Slide staining (primary antibody application)

226 2.6.1. Take each slide and remove the blocking solution by tapping the side of the slide on a stack
227 of paper towels and using a delicate task wipe to remove excess blocking agent from around the
228 tissue.

230 2.6.2. Lay the slide back into the humidified chamber and add approximately 200 µL of the
231 working primary antibody. Incubate in the humidified chamber for 1 h at RT.

233 2.6.3. Wash with TBST for 2 min by submersion in triplicate[13].

235 NOTE: After each washing step, changing the TBST will help ensure a cleaner image.

237 2.7. Slide staining (secondary antibody application)

239 2.7.1. Dab off the remaining TBST from each slide and apply approximately 3 to 4 drops of
240 secondary antibody (mixture of rabbit and mouse secondary HRP conjugated antibodies).
241 Incubate for 10 min in the humidified chamber at RT[13].

243 NOTE: If planning to use a primary antibody that requires a secondary antibody other than mouse
244 or rabbit or choosing to use an alternative secondary antibody, apply the appropriate HRP
245 conjugated secondary to the slides and incubate at RT for 45 min. Optimization of incubation
246 time may be needed for the alternative secondary[14].

248 2.7.2. After incubation, wash with TBST for 2 min in triplicate[13].

250 2.8. Slide staining (fluorophore application)

252 2.8.1. Apply approximately 100 µL of the fluorophore working solution and incubate in the
253 humidified chamber at RT for 10 min[13].

255 2.8.2. Wash with TBST for 2 min in triplicate[13].

257 2.9. Removal of antibodies

259 2.9.1. Microwave slides (45 s at 100% then 15 min at 20%) in the heat resistant box (see step
260 2.3.2) for removal of antibodies[13,14].

262 NOTE: This completes one round of the multiplex. This is the only point at which the protocol can
263 be paused. To pause the protocol, leave the slides submerged in the antigen retrieval buffer
264 overnight at room temperature.

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3.1. Choose slides for building a fluorophore library.

284

285

3.1.1. For a 7-color multiplex gather 7 immune cell rich tissue slides (i.e., tonsil, spleen, etc.) for the library.

286
287

288

NOTE: Choose control slides to be used for a fluorophore library with each slide representing each unique fluorophore that will be used in the multiplex. Control slides should have an abundant epitope of choice and may be the same type of tissue for each fluorophore.

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292

3.2. Stain and image library slides for use with monoplexes and multiplexes.

293

294

3.2.1. Follow steps 2.1–2.9.1 using the control slides and control tissue slides of choice. Place a different fluorophore on each slide at a concentration of 1:100; one slide should contain only DAPI.

295
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3.2.2. Proceed to section 2.11 except omit DAPI staining and instead use TBST and mount as described.

299
300

301

3.2.3. After letting slides dry overnight, image with all channels DAPI, CY3, CY5, CY7, Texas Red, semiconductor quantum dots, and fluorescein isothiocyanate (FITC) setting the exposure to 250 ms with the saturation protection feature[1].

302
303
304

305

NOTE: The exposure times can be set at 50–250 ms[13].

306

307

3.2.4. Upload the library images onto the software and use in the analysis of the monoplexes and

308

309   multiplex.

311   3.3. Choose slides for monoplexes.

313   3.3.1. Choose control slides that have an abundant epitope of choice based on optimized
314   antibodies (section 1). For every round of staining to be done in the multiplex, select that number
315   of control slides to create monoplexes.

317   NOTE: The purpose of the monoplex is to determine the best position (order) for each antibody
318   to be used in the multiplex.

320   3.4. Stain monoplexes.

322   3.4.1. Follow sections 2.1–2.11 using the primary antibody at a different order (position) for each
323   of the slides. Each slide should have only one primary antibody applied at an order (position)
324   different than the other slides.

326   NOTE: For each slide where the round calls for no primary antibody or fluorophore, instead use
327   primary antibody diluent and fluorophore diluent[13].

329   3.5. Image slides and analyze the monoplex.

331   3.5.1. Using the microscope and setting the exposure time to 250 ms for all channels capture
332   each image utilizing DAPI to focus.

334   NOTE: The exposure times can be set at 50–250 ms[14].

336   3.5.2. Using the analysis software evaluate each monoplex slide by looking at the fluorescent
337   intensity of the stained marker.

339   3.5.3. Compare this intensity to the background. If it is at least 5–10 times higher than the
340   background, the position of that slide is an optimal position to use in the final multiplex.

342   3.6. Choose slides for the multiplex.

344   3.6.1. Choose the slides of interest and add an extra slide to be used as a blank slide for
345   subtraction of autofluorescence after staining and imaging.

347   3.7. Stain the multiplex.

349   3.7.1. Based on the monoplex results, choose the appropriate order (positions) for each antibody
350   based on step 3.5.3. Assign each fluorophore to an antibody.

352   NOTE: This may need optimization; however, plan to use bright antibodies for less abundant

353 markers[1], co-localizing antibodies should be matched with fluorophores at far spectrums from
354 each other[13], and fluorophores with the spectrum 540 and 570 should not be co-localized due to
355 spectral overlap issues[1].

356

357 3.7.2. Proceed with sections 2.1–2.11 ensuring the blank slide does not receive primary antibody,
358 fluorophore, or DAPI, instead use antibody diluent, fluorophore diluent, and TBST respectively in
359 its place.

360

361 **3.8. Image multiplex and analyze for integrity of staining**

362

363 3.8.1. Using the microscope and setting the exposure time to 250 ms for all channels, capture
364 each image utilizing DAPI to focus.

365

366 3.8.2. Using the analysis software (**Table of Materials**), evaluate each multiplex by fluorescent
367 false color.

368

369 NOTE: A clear image should demonstrate each marker clearly. If a marker looks diffuse and grainy
370 or is nonexistent, it is likely that the marker didn't work and needs to be re-optimized.

371

372 3.8.3. Using the analysis software, evaluate each multiplex by pathology view. Pathology view
373 will confirm the specificity of each marker. Compare to IHC previously optimized (section 1).

374

375 **REPRESENTATIVE RESULTS:**
376 The overall process of obtaining a 7-color multiplex assay follows a repetitive pattern. **Figure 1**
377 describes the process in a diagrammatic form. Once slides are cut and dried or are received from
378 the laboratory and baked in a hybridization oven at 60 °C for 1 h, then proceed to
379 deparaffinization and rehydration, fix the slides in formalin again followed by antigen retrieval.
380 Each round of multiplexing starts at antigen retrieval and finishes at antibody removal (**Figure 1**).

381

382 Optimization of each step in the process is paramount to achieving a clear and useable image.
383 Antibodies should be tested by IHC first using antigen retrieval buffers with pH 6 and pH 9 for
384 each antibody to visualize which antigen retrieval buffer works best with that particular antibody.
385 Generally, nuclear targets respond to antigen retrieval with buffer pH 9. For example, FOXP3
386 works best when antigen retrieval buffer of pH 9 is used as opposed to CD3 which works best
387 with antigen retrieval buffer of pH 6. Images taken from the IHC process should be used to
388 validate the specificity of the multiplex analysis.

389

390 A monoplex is necessary to determine the order of each antibody in the multiplex. When using
391 either a 4- or a 7-color kit, each antibody will have a preferred order in the array. For the number
392 of antibodies that will be used, gather the appropriate control slides (i.e., for a 7-color multiplex
393 where one color is DAPI, select 6 tissue specific representative control slides for the 6 other
394 fluorophores). **Figure 2A-C** is a schematic representation of a monoplex assay using 3 slides with
395 FFPE colon cancer tissue. Each slide has FOXP3 in a different position in the array using
396 fluorophore 570 as its tag. DAPI is used as a counter stain to adequately visualize nuclei. In **Figure**

397 **3**, representative images of a monoplex and a multiplex with varying FOXP3 position are shown.
398 In **Figure 3A,B** where FOXP3 is at the third position (corresponding to the order shown in **Figure**
399 **2C**), specific and robust staining of FOXP3 (red) is seen as demonstrated by bright nuclear staining
400 **Figure 3A** and co-localization with CD3 **Figure 3B**. The fluorophore signal intensity confirms the
401 specificity of FOXP3 without nonspecific staining elsewhere in the tissue **Figure 3A**. In **Figure 3C,**
402 where FOXP3 is at the first position (corresponding to the order in **Figure 2A**), there is non-
403 specific staining of FOXP3. Grainy diffuse areas of red cover most of the slide and fluorescent
404 intensity at these areas are less than 1. Attempting to do a multiplex without completing a
405 monoplex first to test the order of the staining results in a similar outcome and will waste
406 reagents. Another critical step that cannot be overlooked in the multiplex process is DAPI
407 staining. A working DAPI counterstain is the basis for cell identification and further spatial analysis
408 using the analysis software. An important contrast can be seen in the composite image with
409 working DAPI (blue) in **Figure 3E** and in the non-working DAPI in **Figure 3F**. The image in **Figure**
410 **3F** was not able to be used to identify cells in the analysis software. An attempt to rescue the
411 multiplex and the tissue analysis, the coverslip maybe removed by soaking the slide in TBST
412 followed by a microwaving step in pH 6 antigen retrieval buffer with an additional application of
413 DAPI.
414
415 Once the monoplex assay has been completed and optimal antibody order confirmed, a multiplex
416 strategy can be constructed **Figure 4**. For every target a different fluorophore must be chosen.
417 The number associated with each fluorophore roughly represents the spectral fluorescent
418 wavelength emitted after excitation, similar to flow cytometry. Care should be taken when
419 matching proposed antibodies with fluorophores to limit spectral overlap and potential bleeding
420 of markers. A good strategy is to choose wavelengths far from each other for co-localizing
421 antibodies to reduce excess spectral overlap providing a crisp image and more reliable
422 phenotyping[13]. For example, in the multiplex strategy displayed (**Figure 4**), CD8 is paired with
423 fluorophore 570 while CD3 is paired with fluorophore 520. One should avoid use of fluorophore
424 540 and 570 in co-localized targets as these tend to be the most undiscriminating and can impair
425 results. Fluorophore 620 tends to be very bright and should be used for antibodies that are not
426 abundant in the tissue. A recent library used as a reference for each fluorophore also aids in
427 reducing spectral overlap of fluorophores. A blank slide that undergoes all rounds of antigen
428 retrieval is necessary to ensure autofluorescence extraction from the composite image. This
429 blank slide will undergo the same treatment as the multiplex slides except in place of a working
430 primary antibody and fluorophore, the antibody diluent and fluorophore diluent will be used
431 respectively **Figure 4B**.
432
433 To ensure the multiplex design worked properly and the markers seen in the composite image
434 are specific, use the "pathology image" in combination with the "brightfield" setting option using
435 microscope software which creates a faux IHC image which can then be compared to the previous
436 IHC assays discussed in section 1. Unfortunately, beautiful composite images may not necessarily
437 reflect an accurate stain and this is an important step to quality control the process and prevent
438 false positive results. **Figure 5A** demonstrates a composite image without initial concern. CD3 is
439 visualized and shows specific staining (green) and is confirmed by the pathology brightfield image
440 in **Figure 5B**. CD163 (orange) is seen in the composite image (**Figure 5A**); however, in evaluating

441 the pathology brightfield view of CD163 (**Figure 5C**), the antibody is nonspecific. An example of
442 an optimal multiplex image with specific staining is demonstrated in a composite image (**Figure**
443 **5D**). CD3 and CD163 specificity is confirmed using pathology brightfield view (**Figure 5E** and
444 **Figure 5F,** respectively) and compares favorably to previous IHC assays performed using these
445 antibodies (not shown).
446
447 **FIGURE LEGENDS:**
448
449 **Figure 1: Staining workflow diagram.**
450
451 **Figure 2: Monoplex development.** (**A**) Colon cancer slide with FOXP3 at position 1. (**B**) Colon
452 cancer slide with FOXP3 at position 2. (**C**) Colon cancer slide with FOXP3 at position 3.
453
454 **Figure 3: Successes and pitfalls of monoplex and multiplex.** (**A**) Monoplex assay of primary colon
455 cancer with FOXP3 at position 3, fluorescent intensity label shown. (**B**) Multiplex assay of primary
456 colon cancer with FOXP3 at position 3 and CD3 at position 2. (**C**) Monoplex assay of primary colon
457 cancer with FOXP3 at position 1, fluorescent intensity label shown. (**D**) Multiplex assay of primary
458 colon cancer with FOXP3 at position 1 and CD3 at position 2. (**E**) Multiplex assay of primary colon
459 cancer with order of antibodies as follows: CD8 (yellow), CD3 (green), CD163 (orange),
460 pancytokeratin (white), PD-L1 (magenta), FOXP3 (red), working DAPI (blue). (**F**) Multiplex assay
461 of primary colon cancer with order of antibodies as follows: CD8 (yellow), CD3 (green), CD163
462 (orange), pancytokeratin (white), PD-L1 (magenta), FOXP3 (red), non-working DAPI (blue).
463
464 **Figure 4: Multiplex development.** (**A**) Multiplex slide of colon cancer in a 7-color multiplex. (**B**)
465 Blank slide in a 7-color multiplex using colon cancer tissue.
466
467 **Figure 5: Analyzing specificity of a 7-color multiplex.** (**A**) Multiplex composite image with
468 antibodies as follows: CD8 (yellow), CD3 (green), CD163 (orange), pancytokeratin (white), Ki67
469 (red), DAPI (blue). (**B**) Pathology brightfield view of image in panel A on CD3 only view. (**C**)
470 Pathology brightfield view of image in panel A on CD163 only view. (**D**) Multiplex composite
471 image with antibodies as follows: CD8 (yellow), CD3 (green), CD163 (orange), pancytokeratin
472 (white), PD-L1 (magenta), FOXP3 (red), DAPI (blue). (**E**) Pathology brightfield view of image in
473 panel D on CD3 only view. (**F**) Pathology brightfield view of image in panel D on CD163 only view.
474
475 **DISCUSSION:**
476 Intact tissue specimens from solid tumor biopsy and surgical resection remain important
477 diagnostic and predictive tools for disease analysis as well as patient prognosis. Multiplex
478 fluorescent immunohistochemistry (mfIHC) is a novel technique that combines the benefits of
479 immunohistochemistry (IHC), immunofluorescence (IF) and flow cytometry. Previous methods to
480 probe cells in situ have allowed for evaluation of cell-to-cell arrangements in a tissue
481 environment[8], however, the low number of epitopes that can be probed limits complex analysis.
482 Flow cytometry, although useful in analyzing many cell types in a specimen, loses spatial context
483 by virtue of preparing samples as a single cell suspension[9,10]. mfIHC bridges these techniques by
484 retaining the spatial context of the cellular microenvironment and increasing the number of

485 epitopes that can be labeled while amplifying the signal per labeled epitope[11]. Previous research
486 has used mfIHC for phenotyping the cellular infiltrate in cancer and non-cancer tissue specimens[1-
487 5,15]. Post-image analysis has also been used to analyze spatial relationships of cells and structures
488 within the tissue microenvironment[1,2,4]. For reliable analysis, a specific and accurate image must
489 first be produced. The manual staining technique, as opposed to an automated staining system,
490 enables smaller and cost-conscious laboratories access to this technology. Optimization time and
491 staining time are among the largest barriers to achieving a reliable image for further spatial
492 analysis.
493
494 Several general rules enhance the likelihood of successful generation of a reliable composite
495 multiplex image and will save time and resources. Antibodies to be used in the multiplex should
496 be chosen based on success with manual conventional IHC. Antigen retrieval buffers with pH 6
497 and pH 9 should be used for IHC in order to investigate the appropriate antigen retrieval for use
498 in the multiplex. Monoplex development is necessary to identify the placement of antibodies
499 within the multiplexing protocol. The rationale for this is complicated and varies between tissue
500 specimens and epitopes. In some instances, the epitope can degrade with multiple microwaving
501 steps and marker visualization is lost, as is often the case with CD3. FOXP3, however, becomes
502 brighter with more microwaving steps and is barely visible if placed at the first or second round
503 of multiplex[13,14].
504
505 The staining process starts with FFPE tissue cut onto charged slides. The first problem that may
506 be encountered is the tissue falling off of the slide after microwaving. When charged slides are
507 not used, adherence of tissue to the glass surface is limited and the tissue will have either
508 excessive folding or may slide off entirely. To further ensure the tissue adheres to the slides
509 during the staining process, several techniques are performed. First while cutting the blocks onto
510 slides, the purest form of water works best. For some laboratories, deionized water may be
511 sufficient but distilled water has worked best. The slides should be dried flat immediately after
512 cutting at 37 °C overnight. To further ensure adherence, the slides are then placed in a
513 hybridization oven and baked lying flat at 60 °C for one hour just prior to use. Although formalin
514 fixation has already occurred during the initial tissue preparation process, placing the slides in
515 neutral buffered formalin for 30 min after the deparaffinization and rehydration process
516 enhances the structural integrity of the mounted tissue[1,13].
517
518 The manual staining process can take up to three, 8-hour days depending on the number of
519 antibodies used. Avoiding pitfalls is essential in saving time and sparing reagents. The first
520 common mistake often occurs in reagent preparation. Water differs among laboratories and can
521 be the one single difference in results from one lab to the next. Using the same as well as a clean
522 water source for all reactions and reagents ensures a consistent result. The antibodies, blocking
523 solution, and fluorophores work best at room temperature. To avoid pitfalls in reagent and
524 working solution preparation, use the same water for all reagents. Also use all of the working
525 solutions and reagents at room temperature.
526
527 Troubleshooting of mfIHC is important and adherence to strict laboratory guidelines crucial to
528 limit contamination of components and/or operator error. Production of suboptimal images will

529 skew results and lead to false positive and negative cell populations which can negatively impact
530 data. Because the ultimate analysis typically includes computer generated phenotypes based on
531 machine learning, small errors in staining can be magnified to affect the entire data set. For
532 example, although all the stains may work perfectly, an error or omission of DAPI will prevent
533 future cell segmentation and counting rendering the experiment useless. Analysis software uses
534 DAPI staining not only to identify cells but assigns x and y coordinates to each cell and if the
535 multiplex has dim, poorly circumscribed, or diffuse DAPI staining, the image cannot be further
536 used and the multiplex must be redone or attempted rescue and re-staining of DAPI. Use of
537 software components such as the pathology and brightfield views will enhance confidence in
538 sensitivity and specificity early in the optimization process and prevent mistakes early in the
539 multiplex process.
540
541 The modifying aspects of the imaging process can also aid in image optimization. For example,
542 taking an image using DAPI as the visual aid to focus helps to avoid blurry images. Adding the
543 newly made fluorophore library to the software will ensure a clean image and less spectral
544 overlap of each fluorophore during the analysis phase and using the blank slide to extract the
545 autofluorescence intensifies the markers and enhances the image quality.
546
547 The use of tissue specimens has and will remain as an essential tool in investigating
548 pathophysiology of disease. Emerging technology has increased our understanding of cell-to-cell
549 interactions and mfIHC is a promising new player. Optimization and accuracy during the staining
550 process in this technique is paramount for proper utilization and further analysis of cell-to-cell
551 interactions.
552

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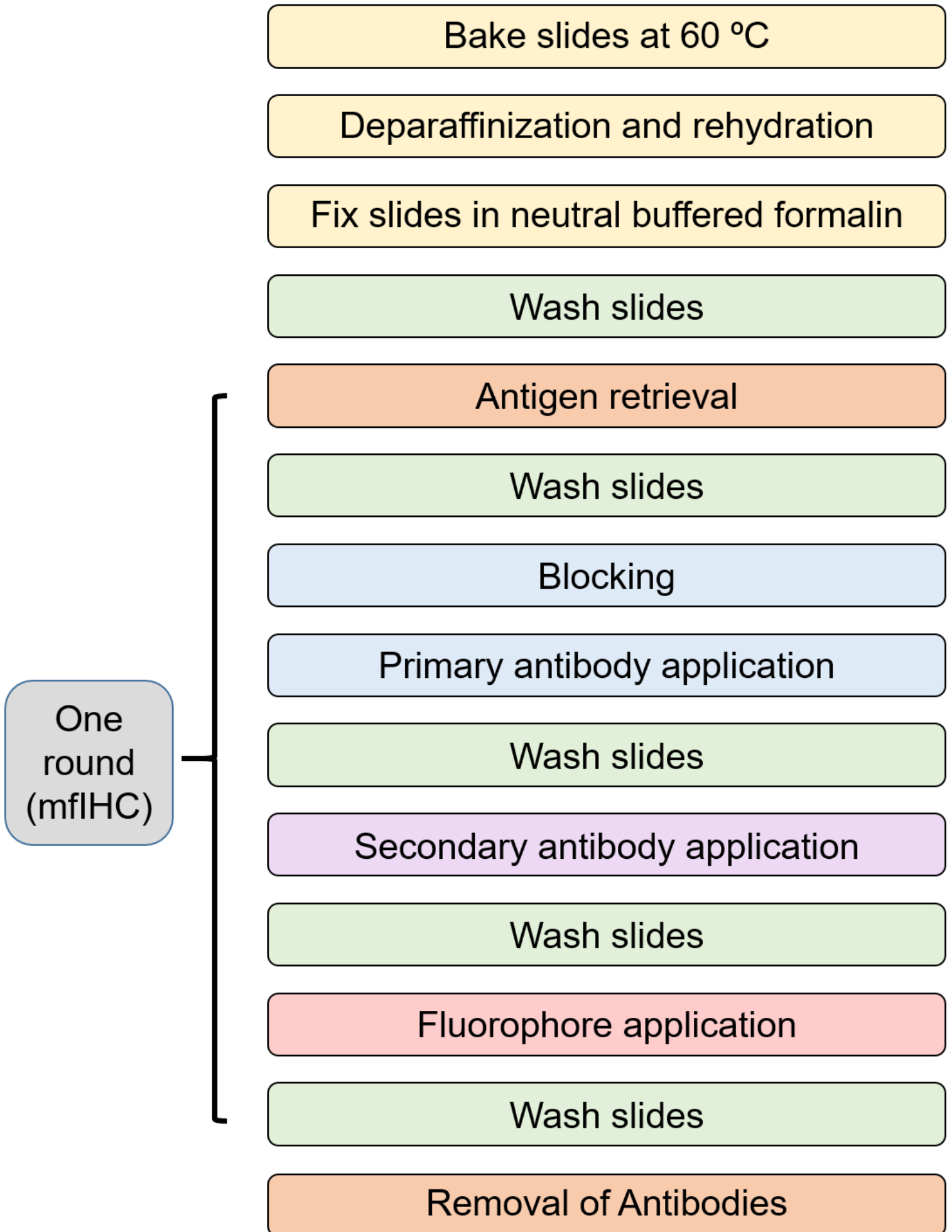600   for Reed-Sternberg cells in Hodgkin lymphoma. *Blood.* **130** (22), 2420-2430, (2017).
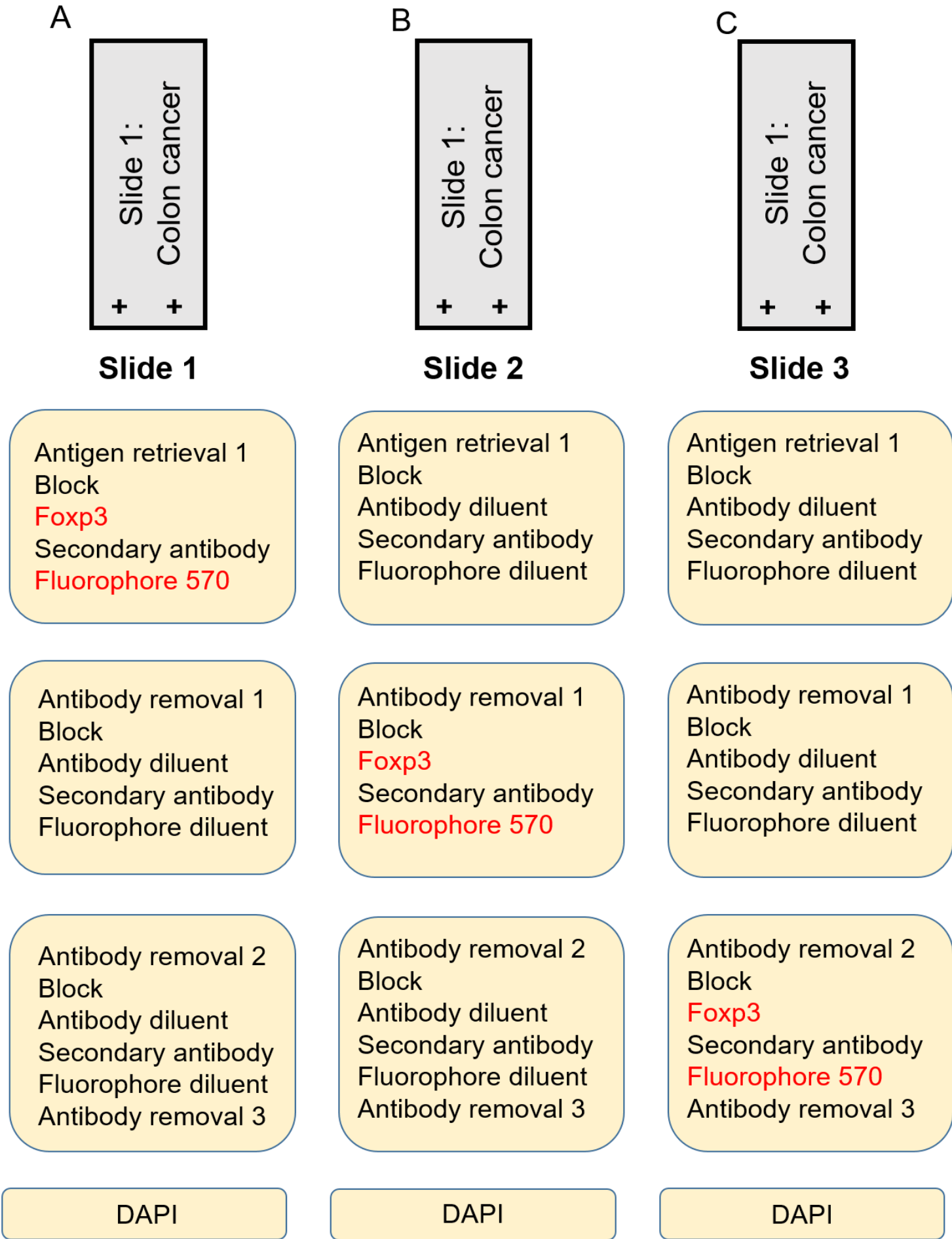
601

Figure 1

Figure 1

Bake slides at 60 ºC

Deparaffinization and rehydration

Fix slides in neutral buffered formalin

Wash slides

Antigen retrieval

Wash slides

Blocking

Primary antibody application

Wash slides

Secondary antibody application

Wash slides

Fluorophore application

Wash slides

Removal of Antibodies

One round (mfIHC)

Figure 2

Figure 2

Figure 3

Figure 3

A

Fluorphore 570:
signal intensity 14.5

50 µm

B

50 µm

C

Fluorphore 570:
signal intensity 0.83

50 µm

D

50 µm

E

25 µm

F

25 µm

Figure 4

Figure 4

A

Multiplex

+ +

Antigen retrieval 1
Block
CD8
Secondary antibody
Fluorophore 570

Antibody removal 1
Block
CD3
Secondary antibody
Fluorophore 520

Antibody removal 2
Block
CD163
Secondary antibody
Fluorophore 650

Antibody removal 3
Block
Pancytokeratin
Secondary antibody
Fluorophore 690

Antibody removal 4
Block
PD-L1
Secondary antibody
Fluorophore 540

Antibody removal 5
Block
Foxp3
Secondary antibody
Fluorophore 570
Antibody removal 6

DAPI

B

Blank

+ +

Antigen retrieval 1
Block
Antibody diluent
Secondary antibody
Fluorophore diluent

Antibody removal 1
Block
Antibody diluent
Secondary antibody
Fluorophore diluent

Antibody removal 2
Block
Antibody diluent
Secondary antibody
Fluorophore diluent

Antibody removal 3
Block
Antibody diluent
Secondary antibody
Fluorophore diluent

Antibody removal 4
Block
Antibody diluent
Secondary antibody
Fluorophore diluent

Antibody removal 5
Block
Antibody diluent
Secondary antibody
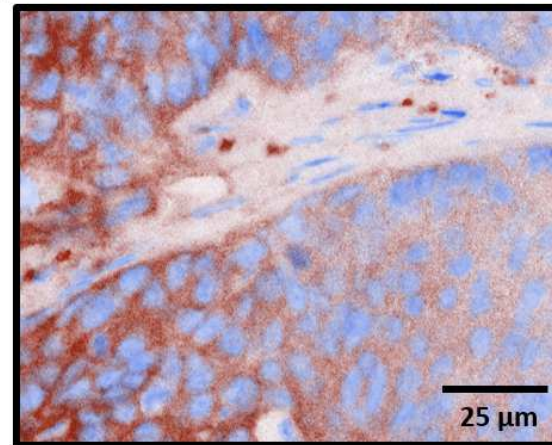Fluorophore diluent
Antibody removal 6

DAPI

Figure 5

Figure 5

Name of Material/Equipment	Company	Catalog Number	Comments/Description
100% ethanol	Fisher	HC8001GAL	
70% ethanol	Fisher	HC10001GL	
95% ethanol	Fisher	HC11001GL	
Analysis software	Akoya Biosciences	CLS135783	inForm version 2.3.0
Antifade mountant	ThermoFisher	P36961	ProLong Diamond
Blocking solution	Vector	SP-6000	Bloxall
Bovine Serum Albumin (BSA)	Sigma Life Sciences	A9647-100G	
Cover Slips	Fisher	12-548-5E	
Delicate task wipe	Kimberly-Clark	34120	
Fluorescent diluent	Akoya Biosciences	ARD1A01EA	Opal TSA diluent
Fluorophore 520	Akoya Biosciences	FP1487001KT	1:100
Fluorophore 540	Akoya Biosciences	FP1494001KT	1:100
Fluorophore 570	Akoya Biosciences	FP1488001KT	1:100
Fluorophore 620	Akoya Biosciences	FP1495001KT	1:100
Fluorophore 650	Akoya Biosciences	FP1496001KT	1:100
Fluorophore 690	Akoya Biosciences	FP1497001KT	1:100
Fluorophore DAPI	Akoya Biosciences	FP1490	3 drops in TBST or PBS
Heat resistant box	Tissue-Tek	25608-904	Plastic slide box-green
Humidified Chamber	Ibi Scientific	AT-12	
Hybridization oven	FisherBiotech		
Hydrophobic barrier pen	Vector	H-4000	ImmEdge
Microscope	Perkin Elmer	CLS140089	Mantra quantitative pathology workstation
Microwave	Panasonic	NN-A661S	with inverter technology
Neutral buffered formalin	Fisher Scientific	SF100-4	10% neutral buffered formalin
pH 6 antigen retrieval buffer	Akoya Biosciences	AR600	AR6
pH 9 antigen retrieval buffer	Akoya Biosciences	AR900	AR9
Phosphate buffered saline	Fisher	BP3994	PBS
Plastic slide box	Tissue-Tek	25608-906	
Plastic wrap	Fisher	NC9070936	
Polysorbate 20	Fisher	BP337-800	Tween 20

Primary antibody CD163	Lecia	NCL-LCD163	1:400
Primary antibody CD3	Dako	A0452	1:400
Primary antibody CD8	Spring Bio	M5390	1:400
Primary antibody FOXP3	Dako	M3515	1:400
Primary antibody pancytokeratin	Cell Signaling	12653	1:500
Primary antibody PD-L1	Cell Signaling	13684	1:200
Secondary antibody	Akoya Biosciences	ARH1001EA	Opal polymer
Slide stain set	Electron Microscopy Sciences	6254001	
Tris buffered saline	Corning	46-012-CM	TBS
Vertical slide rack	Electron Microscopy Sciences	50-294-72	
Xylene	Fisher	X3P1GAL	

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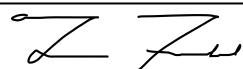## CORRESPONDING AUTHOR

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