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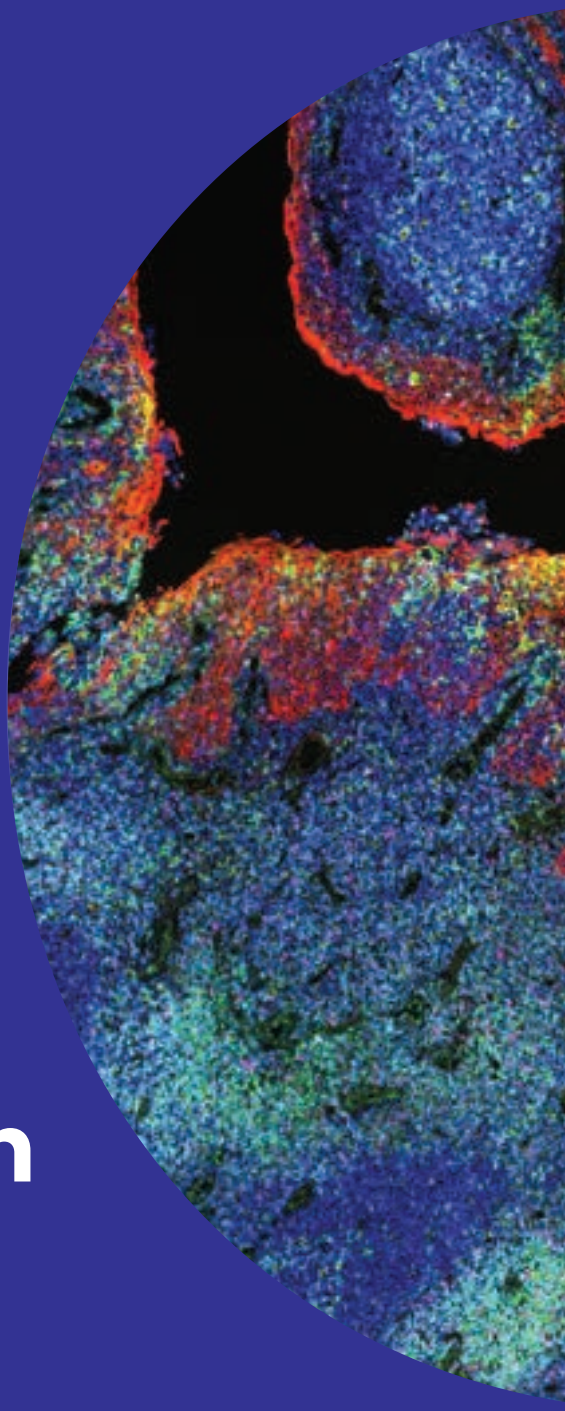

From Tissue Preparation Through Image Analysis: A Multiplex Cyclic Immunofluorescence Workflow

Multiplex immunohistochemistry (IHC) technologies are powerful tools to visualize multiple markers in the same tissue sample. These techniques, which include tyramide signal amplification and cyclic immunofluorescence, help scientists understand the cells that make up a tissue, what markers they express, their spatial distribution, and their potential interactions with one another. These methods depend on antibodies that are specific to the target, produce a clean signal, and have low background.

At Bethyl Laboratories (Bethyl), we provide end-to-end custom multiplex imaging and histology services to encompass the full spectrum of the imaging workflow. In this whitepaper, you will find an overview of our workflow for multiplex imaging using cyclic immunofluorescence.

Cyclic Immunofluorescence Overview

Cyclic immunofluorescence uses fluidics to flow multiple rounds of antibodies over the slide. This approach is divided into three steps (Figure 1), followed by image QC and analysis:

(1) **Staining:** After capturing the background autofluorescence of the sample, stain the slide for up to two targets and the nuclear stain. This involves flowing primary antibodies across the sample, followed by the addition of fluorescently labeled secondary antibodies and DAPI.

(2) **Imaging:** Fluorescent images can be acquired using an automated system such as the Lunaphore COMET™.

(3) **Elution:** Antibodies are eluted from the sample to prepare for the next round of staining and imaging.

This cycle can be repeated up to 20 times, to detect up to 40 antigens of interest. After all antigens have been detected, the images can be aligned, merged and analyzed.

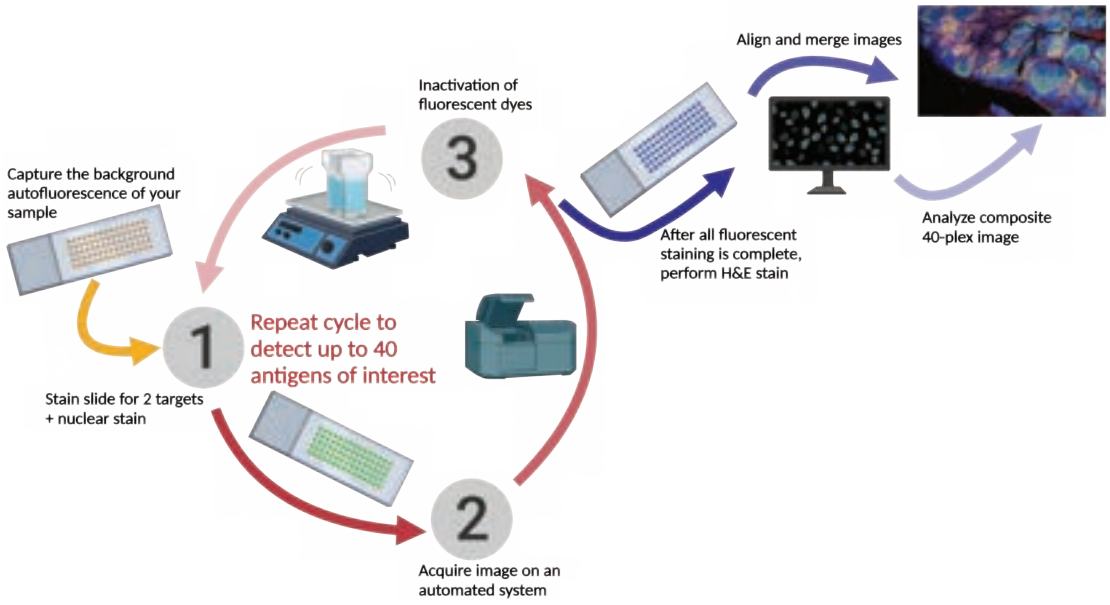

Figure 1: Cyclic immunofluorescence staining and imaging.

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The Multiplex IHC Workflow

Step 1: Tissue and Slide Preparation

Prior to imaging, tissue samples must be paraffin embedded. This might be a tissue block of a single sample or a microarray representing dozens of tissue samples from an individual biopsy or experiment or from multiple biopsies or experiments. These tissue blocks are then sectioned onto slides. At Bethyl, we provide services to encompass all steps of sample preparation.

Step 2: Antibody Selection and Acquisition

The first step in selecting antibodies for a multiplex panel involves choosing the markers necessary for imaging. Marker selection should be related to the overall goals for the experiment (ex: monitor changes in expression, localization of specific proteins, or differentiate between different cell types). Antibody selection is also done in the context of the instrument used for analysis as each instrument has a different maximum number of markers it can evaluate and its own requirements for antibody host species conjugation. Other considerations for antibody selection include specificity of antibody for the target to minimize cross-reactivity with other proteins and affinity to ensure a strong signal.

An advantage of cyclic immunofluorescence is the ability to use label-free primary antibodies. This expands the possible antibodies and targets that can be detected because antibodies from unique host species can be built into a panel and are not dependent upon the availability of numerous non-overlapping fluorophores. However, this also has the potential to be a limiting factor in panel size. Each round of staining in cyclic immunofluorescence requires that all the antibodies being used are raised in a different host species. For example, a rabbit and a mouse antibody can be used together in a single round of staining, but two rabbit antibodies will need to be used in sequential rounds. This is because cyclic immunofluorescence uses indirect immunofluorescence, relying on the addition of secondary antibodies recognizing each primary antibody species during each round of staining.

At Bethyl, we begin with our in-house validated antibodies as a starting point for antibody acquisition. The advantage to using these antibodies is that they have already been validated for our internal multiplex IHC workflows. If an external antibody is needed, we can help to identify options that are specific for the target and validated for multiplex IHC. A careful review of the antibody's data sheet can help assess whether the antibody is likely to be successful in multiplex IHC.

Step 3: Internal Validation of Primary Antibodies

Given the critical role of antibody quality in data accuracy, in-house validation of primary antibodies is essential, even for those already validated by the manufacturer for the same application. For IHC validation, this means selecting tissues that should be positive for the antibody target and tissues that should be negative for the target, and comparing how they perform in imaging. Tissues that express the marker should produce a signal while tissues that do not contain the marker should not produce a signal.

To internally validate an antibody for IHC, we use DAB (3,3'-Diaminobenzidine) chromogenic testing at the recommended dilution provided by the antibody manufacturer. Our standard in-house protocol involves:

1. Antigen retrieval using tris-EDTA pH9 - 20 min
2. Blocking serum - 20 min
3. Primary incubation - 1 hr
4. Secondary incubation - 1 hr
5. DAB chromogen incubation - 5 min
6. Whole slide imaging with the 3DHistech PANNORAMIC® 250 high-capacity slide scanner

In the example below, tonsil cells were used as a positive control and liver squamous cell carcinoma (SCC) was used as the negative control for CD20 (Figure 2). This antibody is considered validated for IHC as it produces a signal in the positive control and not the negative control tissue.

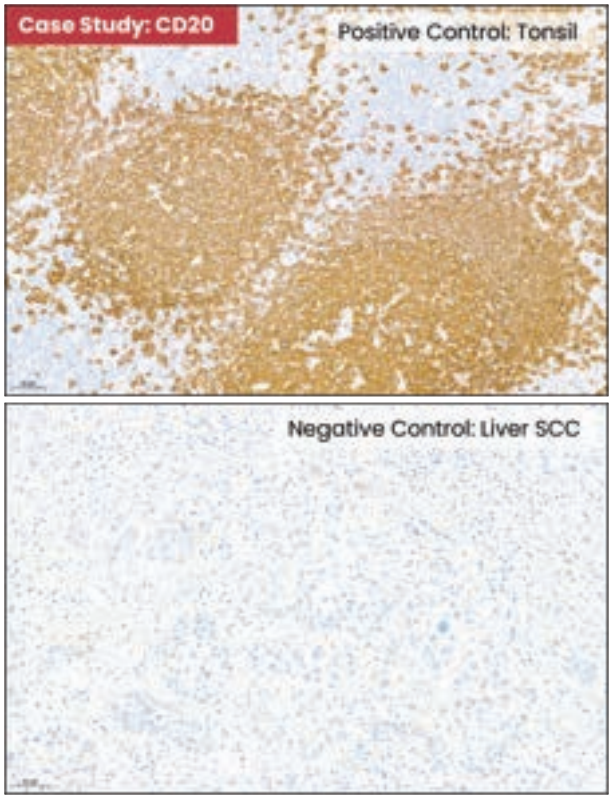

Figure 2: Comparison of CD20 staining in tonsil tissue (positive control) and liver SCC tissue (negative control).

Step 4: Primary Antibody Dilution Series on Instrument

Once the antibody has been internally validated with DAB chromogenic testing, the next step is to test the antibody in fluorescence microscopy. This stage also evaluates the best antibody concentration for the panel by using a dilution series. This dilution series incorporates the concentration used during DAB chromogenic testing and two other concentrations: one below the original concentration and one above the original concentration.

In this example, we tested the original concentration of the CD20 antibody (5 µg/ml) and two additional concentrations: 1 µg/ml and 8 µg/ml. The same area of tissue was visualized with the same exposure settings at each concentration (Figure 3). We ultimately decided that the original concentration (5 µg/ml) is strong enough to produce adequate signal, but still allows room for intensity adjustments during the image analysis step.

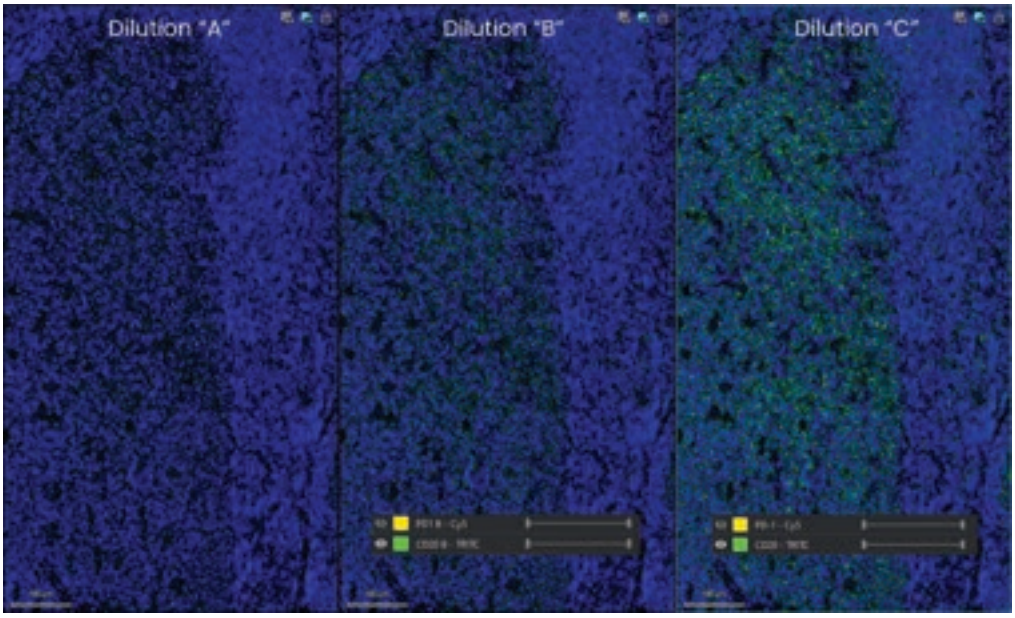

Figure 3: Fluorescence imaging of the same tissue section at different dilutions. Dilution "B" (5 µg/ml) was chosen for the full panel.

Step 5: Full Panel Run Incorporating All Antibodies

Once all antibodies have been validated and concentrations determined, imaging with the full panel can begin. During imaging, each channel should be assessed individually for background, signal intensity, and specificity (Figure 4).

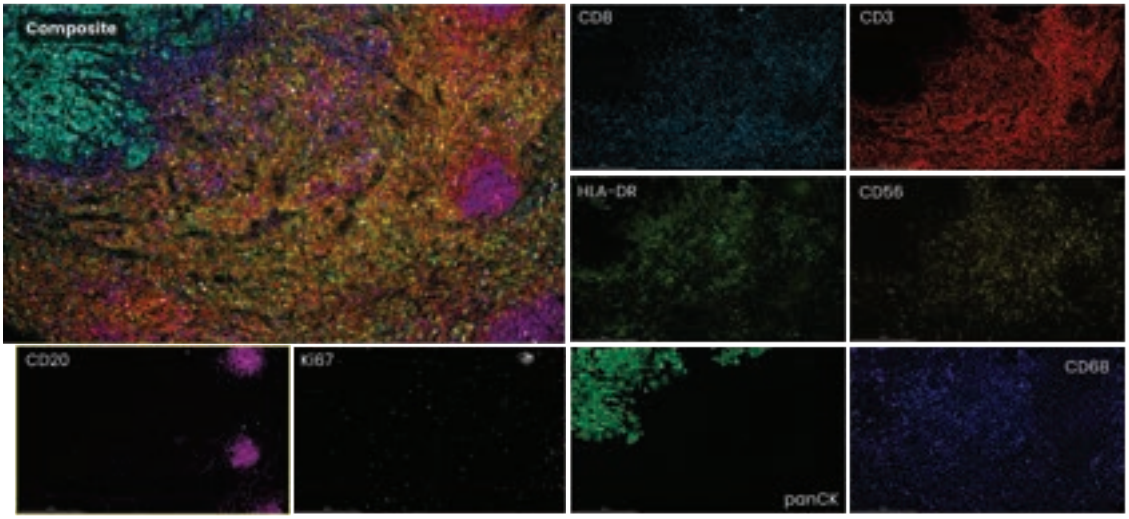

Figure 4: Composite image after staining with 8 antibodies and images of each channel.

Step 6: Image Quality Control and Evaluation

Image QC involves assessing the images for potential issues such as concerns with the intensity or localization of the antibody signal, inefficient elution of an antibody from a previous cycle, tissue damage or loss, and imaging quality issues such as improper focus or bubble formation.

If changes are needed, our team will make the requisite adjustments and rerun the full panel on test samples. Once the test sample produces a satisfactory image, we then run the full panel on the customer samples.

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Step 7: Image Analysis

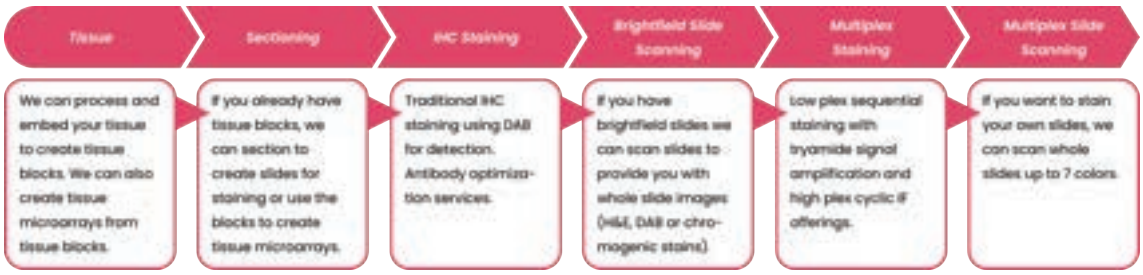
To analyze multiplex IHC images, you first need to stack the individual images to create a composite image. This composite image can then be analyzed to identify where the cells are, what markers they express, and what structures they are near. This is done using the following steps:

1. **Cell segmentation:** Cell segmentation is the digital isolation of cells within the image. This is commonly done using a technique called "watershed segmentation" where the image is treated as a topographic surface and simulates how water would fill the area to outline cells. AI-based methods use large datasets to train segmentations that can better handle dense areas, overlapping cells, and irregularly shaped cells.
2. **Cell phenotyping:** This step identifies what markers are expressed by each cell. Several methods are available for analyzing images including supervised classification, which are guided by a pathologist to determine the threshold for a positive population, and unsupervised approaches, which use algorithm-guided clustering of expression patterns. The output from this stage is a list of markers expressed by each cell and can help identify major cell types or cellular subsets of interest defined by the presence of multiple markers (ex: CD4+ FoxP3+ regulatory T cells).
3. **Area segmentation:** Area segmentation methods allow scientists to understand the structural elements of the tissue so that cells can be analyzed in context of their surroundings. Area segmentation relies on AI models to recognize designated structures across all images within an experimental dataset.
4. **Spatial feature augmentation:** With structures and cellular phenotypes identified, this information can be combined to understand the proximity between cells (nearest neighbor analysis) and structural elements or between different cell types. For example, it could be useful to know the distance between a tumor and the nearest blood vessel.

By simultaneously visualizing multiple markers to distinguish cell populations and identifying the structural elements they are near, spatial biology increases our understanding of biology by allowing scientists to make observations on numerous cell types in context of the tissue microenvironment.

Custom CRO Histology and Multiplex Imaging Supporting the Entire Imaging Workflow

At Bethyl, we provide end-to-end solutions that support the entire imaging workflow from tissue preparation through multiplex slide scanning, image analysis, and all steps in between. Since we have a catalog containing over 100 internally validated antibodies to choose from and have already built core panels using these antibodies, we can expedite the imaging workflow by eliminating the need to always acquire and validate external antibodies.



Our custom histology and multiplex imaging service offerings include:

- **Tissue preparation:** Tissues are processed and embedded in paraffin to create tissue blocks.
- **Tissue sectioning:** Sections are cut from tissue blocks to create slides or tissue microarrays.
- **IHC staining:** Traditional IHC staining is conducted using DAB for detection, with antibody optimization included in the process.
- **Brightfield slide scanning:** H&E, DAB, or chromogenically stained slides, whether created in-house or provided by the customer, are scanned to produce whole slide images.
- **Multiplex staining and imaging:** Service offerings include low plex staining up to 7-plex using tyramide signal amplification (Akoya PhenoImager™ HT) and high plex (up to 40-plex) cyclic immunofluorescence (Lunaphore COMET™).
- **Multiplex slide scanning:** Customers can provide slides up to 7 colors for scanning at our facility.

Every project is unique. Visit fortislife.com/custom-multiplex-imaging to see how our tailored histology and imaging solutions can help enhance your research.