Immunofluorescent Multiplex (mIF) Assays

BETHYL LABORATORIES, INC

Step-by-Step Protocol for mIF Assays: Formalin-Fixed, Paraffin-embedded tissues and cell blocks. The use of 3-5µm paraffin sections on charged slides is recommended.

Reagents Required:

- Xylene
- 100% Reagent alcohol-histology grade
- Endogenous Peroxidase Block Solution: 0.9% Hydrogen Peroxide in Methanol
- Epitope Retrieval Solutions:
- Citrate Buffer pH 6.0. Measure 2.94 g Tris-sodium Citrate (dehydrate) volume to 1 Liter with distilled water and pH to 6.0.
- Tris Buffer pH 9.0. Measure 1.21 g Tris Base and 0.37 g EDTA volume to 1 Liter with distilled water and pH to 9.0.
- 1x Tris-Buffered Saline (TBS), 0.1% Tween® 20 (TBST)
- Blocking Solution: 20% Normal Goat Serum in PBS
- IHC Antibody Diluent: 50mM TBS, 1% BSA
- HRP-conjugated Secondary Antibodies: Anti-mouse A90-116P, Anti-rabbit A120-501P
- Fluorophore-conjugated Tyramide Signal Amplification (TSA®)
- DAPI nuclear counterstain
- Slide mounting media

Slide Preparation:

1. Xylene - 3 changes for 5 minutes each. Agitate 10 times up and down.
2. 100% Reagent Alcohol - 3 changes for 5 minutes each. Agitate 10 times up and down.
3. 0.9% Hydrogen Peroxide in Methanol 40 minutes. Agitate 10 times up and down.
4. Distilled water 2 X 5 minutes. Agitate 10 times up and down.
5. Epitope retrieval (Refer to Bethyl Antibody datasheet for recommended retrieval buffer). Heat epitope retrieval buffer to 92-96°C. Place slides in epitope retrieval buffer for 20 minutes. Cool on benchtop for 20 minutes. Drain epitope retrieval buffer and add distilled water to the container.
6. Circle tissue with hydrophobic barrier pen. Take care to prevent the samples from drying out.

Staining Cycles:

Cycle #1 (1st target)

7. Blocking: Incubate tissue in 20% Normal Goat Serum or serum (NS) matched to the host of the secondary antibody for 20 minutes at RT.
8. Primary Antibody: Remove Blocking Buffer and add primary antibody diluted in IHC antibody diluent (Refer to Bethyl data sheet for recommended dilution range. Optimal working dilutions should be determined experimentally by the investigator). Incubate for 20 minutes at RT.
9. Rinse with TBST - 3 changes for 5 minutes each. Agitate 10 times up and down.
10. HRP-conjugated Secondary: Add 1:400 diluted HRP-conjugated secondary (goat anti-rabbit: A120-501P or goat anti-mouse: A90-116P) and incubate for 20 minutes at RT.
11. Rinse with TBST - 3 changes for 5 minutes each. Agitate 10 times up and down.
12. TSA® Substrate: Dilute fluorophore-conjugated TSA® and incubate according to manufacturer's instructions.
13. Rinse with TBST- 3 changes for 5 minutes each. Agitate 10 times up and down.
14. Epitope retrieval: For the next cycle, repeat epitope retrieval with the buffer recommended on the datasheet of the primary antibody (refer to step #5).

Cycle #2 with 2nd target

15. Blocking: Incubate tissue in 20% Normal Goat Serum or serum (NS) matched to the host of the secondary antibody for 20 minutes at RT.
16. Primary Antibody: Remove Blocking Buffer and add primary antibody diluted in IHC antibody diluent (Refer to Bethyl data sheet for recommended dilution range. Optimal working dilutions should be determined experimentally by the investigator). Incubate for 20 minutes at RT.
17. Rinse with TBST - 3 changes for 5 minutes each. Agitate 10 times up and down.
18. HRP-conjugated Secondary: Add 1:400 diluted HRP-conjugated secondary (goat anti-rabbit: A120-501P or goat anti-mouse: A90-116P) and incubate for 20 minutes at RT.
19. Rinse with TBST - 3 changes for 5 minutes each. Agitate 10 times up and down.
20. TSA® Substrate: Dilute fluorophore-conjugated TSA® and incubate according to manufacturer's instructions.
21. Rinse with TBST- 3 changes for 5 minutes each. Agitate 10 times up and down.
22. Epitope retrieval: For the next cycle, repeat epitope retrieval with the buffer recommended on the datasheet of the primary antibody (refer to step #5).

Cycles #3-8

23. Repeat steps 15-22 as needed for the desired number of targets in multiplex, up to 8

Counterstain and Mount

24. Rinse slides in dH2O
25. Rinse slides in TBST
26. Incubate slides in DAPI solution according to manufacturer's protocol
27. Rinse slides with TBST for 5 minutes
28. Rinse slides in dH2O for 5 minutes
29. Mount with preferred mounting media

Imaging Recommendations:

Multiplex IF can be imaged on multiple systems. We recommend using fluorescent slide scanners. While standard epi-fluorescent and confocal microscopes can be adapted to image multiplex experiments, the ability to image entire tissue sections and automated controls make slide scanners the preferred instruments. If fluorophores exhibit overlapping spectra that do not allow for complete isolation by filters, some form of multispectral imaging will be needed.
The type of imaging instrument is dependent on the number of targets and whether your set of fluorophores exhibit spectral overlap. The list below includes some instrumentation for multiplex (not an exhaustive list).

- 3-color or less (2 antibodies + DAPI):
  - Standard Research grade fluorescent microscopes
  - Confocal microscopes
  - Mantra
  - Aperio Versa slide scanners
  - Zeiss Axioscan
  - 3DHisTech Panoramic slide scanners
  - Vector, Vector 3, and Vector Polaris
- 4-color or more (3 or more antibodies + DAPI):
  - Confocal microscopes
  - Mantra
  - Aperio Versa slide scanners
  - Zeiss Axioscan
  - 3DHisTech Panoramic 250 slide scanners
  - Vector, Vector 3, and Vector Polaris
- Fluorophores whose spectra overlap:
  - Advanced confocal systems
  - Mantra
  - Vector, Vector 3, and Vector Polaris

Troubleshooting:

- Low signal
  - Ensure antibody is suitable for IHC by DAB detection
  - Titer primary antibody using TSA substrate
  - Increase secondary concentration
  - Increase TSA substrate concentration
  - Increase TSA incubation times
  - Test different HIER methods
  - Test primary on a known positive tissue
  - Reoptimize order of antibodies
- High signal
  - Titer primary antibody using TSA substrate
  - Decrease secondary concentration
  - Decrease TSA substrate concentration
  - Decrease TSA incubation times
- High background
  - Perform Peroxidase block prior to staining
  - Titer antibody components
  - Increase rinse times and number of rinse exchange
  - Filter all buffers to ensure no particulates contact the tissue
  - Switch blocking buffers
- Excess TSA signal (fluorescent speckles or particulates on tissue)
  - Increase rinse times
  - Rinse slides with more vigorous motion
  - Dilute TSA substrate
  - Dilute HRP-conjugated secondary
- Signal for one antigen overlaps with another antigen
  - Ensure HIER was used to strip antibodies before beginning next cycle of staining
  - Image using the proper filters for each fluorophore
  - If fluorophore spectra overlap, use multispectral imaging
- Tissue autofluorescence
  - Use multispectral imaging
  - Shift fluorophores from the green to red portion of the spectrum

mIF Assays FAQs

What do I need to get started with mIF?
mIF requires high quality IHC validated primary antibodies, HRP-conjugated secondary antibodies, tyramide detection fluorophores, and an imaging system capable of separating the fluorophores being used in the assay.

Can I use any primary?
Primary antibodies of any host species can be used in mIF, even if the antibodies are from the same host species. It is advisable to begin developing mIF with highly validated IHC antibodies. Bethyl offers and extensive portfolio of IHC validated antibodies. Antibodies from other companies are compatible with Bethyl antibodies but will need to be validated by the researcher.

What types of samples do I need?
The most common mIF sample is formalin fixed paraffin embedded tissue (FFPE). Since mIF with tyramide detection uses multiple rounds of heat induced epitope retrieval, non-FFPE tissue tends to degrade during the assay.

Where do I purchase tyramide detection fluorophores?
Bethyl antibodies are validated for mIF with the Opal detection system from Akoya Biosciences. Use of other tyramide reagents will require independent validation by the researcher.

Do I have to perform any optimization steps?
The mIF protocol outlines the basic optimization parameters. The recommendations given for mIF applications are starting points for assay development. Further optimization of antibodies will be needed using your own tissue. Differences in tissue fixation, epitope retrieval buffer, instruments used for epitope retrieval, and technical skill can impact the mIF assay. The mIF protocol outlines the basic optimization parameters.

Why do I observe signal in singleplex stains but not during multiplex stains?
Order of staining can impact the detection of antigens. Multiple rounds of epitope retrieval can destroy some epitopes. In this case, antibodies recognizing these markers need to be used early in the staining order. Bethyl validated mIF panels will have established an initial staining order. This order should be confirmed on your own tissue before proceeding mIF studies.

Do you have any recommendations for antigens that are localized to the same cell type within the tissue?
Fluorophore pairings should be carefully considered when staining for markers that occur in the same cell type, especially if those markers are localized to same subcellular location. In these cases, it is advised to use fluorophores whose spectra are non-overlapping.

What do I need to perform heat induced epitope retrieval (HIER)?
Bethyl uses steamers for HIER because this method is widely used. Other methods, such as microwave treatment, may be used but will require independent validation by the researcher.

How many targets can I detect in one tissue sample?
The number of targets detectable is dependent on available tryramide fluorphores and the imaging system. Several slide scanners such as the Vectra Polaris offer the capablilty to perform a 9-color scan (8 immunostained markers + DAPI).