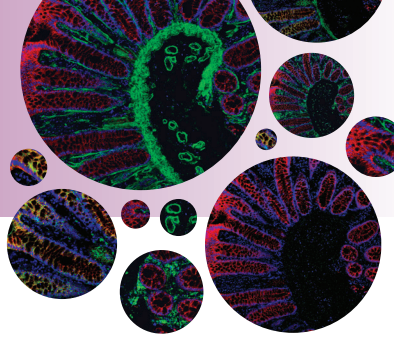
FLUIDIGM® | Technical Note



Imaging Mass Cytometry Antibody Verification Process

Introduction

Imaging Mass Cytometry™ (IMC™) provides unprecedented visualization of complex cellular phenotypes and their relationships in tissue and tumor microenvironments in the context of cancer, immuno-oncology and immune-mediated diseases. Bringing together trusted CyTOF® technology with imaging capability to uncover pathology insights, new biomarker correlations and cell interactions, IMC enables deep profiling of tissue samples in a simple workflow. The production and pathologist verification process outlined in this technical note helps ensure optimal sensitivity and performance of each Maxpar® metal-conjugated antibody.

Objectives

- To introduce the IMC workflow and advantages of the technology.
- To provide an in-depth description of the Fluidigm Maxpar antibody verification and quality control (QC) processes for IMC.
- To provide examples of staining on formalin-fixed, paraffin-embedded (FFPE) human tissue.

Workflow

Using the Hyperion™ Imaging System, IMC is easily applied after a single staining step to paraffin-embedded tissue sections using a familiar immunohistochemistry workflow. This enables highly multiplexed cellular phenotyping in tissue and tumor microenvironments, all from a single scan.

1

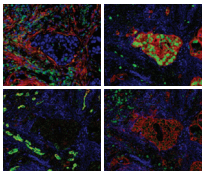
DESIGN
panels using pathologist-verified Maxpar antibodies conjugated to metal tags.

2

STAIN
tissues (FFPE or frozen) or fixed cells using a simple process.

3

IMAGE
protein markers at subcellular resolution using the Hyperion Imaging System.



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ANALYZE
images in minutes using the MCD Viewer software and easily export for publication or secondary analysis.

Figure 1. The IMC workflow is a simple 4-step process.

Advantages of IMC

The Hyperion Imaging System combines a precisely directed laser beam focused to 1 µm to collect biological samples stained with metal-tagged Maxpar antibodies and direct these tags for analysis by CyTOF technology. The system provides spatial resolution and quantitation of up to 37 distinct markers on a single tissue section.

- Trusted CyTOF technology essentially eliminates autofluorescence, spectral overlap and other related issues.
- Easy IMC protocol is similar to traditional immunofluorescence (IF) and immunohistochemistry (IHC).
- Automated image acquisition allows unsupervised batch collection of many regions of interest (ROIs).
- Readily visualize, review and export IMC data with MCD™ Viewer post-acquisition data processing software.

Verification Process

All the pathologist-verified IMC antibodies have undergone the following:

1. Novel target identification

A new marker of interest is identified based on customer feedback and research trends.

✓ **Ensures biological relevance to research interests.**

2. Clone selection

Clones are selected based on vendor data and existing literature.

✓ **Ensures high clone fidelity.**

3. Clone screening

Selected clones are tested by IMC and IF to assess method comparability and the compatibility with the staining protocol.

✓ **Ensures compatibility with the single-step staining protocol.**

4. Verification testing

Positive and negative control tissues are stained with an IMC panel that includes antibodies targeted to proteins that co-localize and counter-localize with the antibody of interest.

✓ **Ensures specificity and staining quality.**

5. Pathologist review and approval

A verification report is produced for each antibody and staining is evaluated by an independent pathologist.

✓ **Ensures product quality.**

Figure 2. Overview of production and pathologist verification process for IMC antibodies.

1. Novel Target Identification

Candidate antibodies are identified from the literature and through customer needs. If the antibody is available in a metal-tagged format, it is tested directly by IMC. If it is unavailable in the catalog, we investigate whether it is available in a format that is compatible with metal conjugation and select the best clone for the target.

2. Clone Selection

Prior to metal tagging, each antibody clone is selected based on both vendor references and use in the literature as a reliable and robust histology reagent. Figure 3 shows IHC staining of FFPE human tonsil (Newcomer Supply 3770A) with anti-human CD3.

Once the clone has been chosen, the antibody is metal-tagged. Conjugation involves a simple chemical reaction between a partially reduced antibody and a metal-chelating polymer. Maxpar metal labeling kits are available in the Fluidigm catalog to give our customers the flexibility to include novel markers or tailor panels for their research questions.

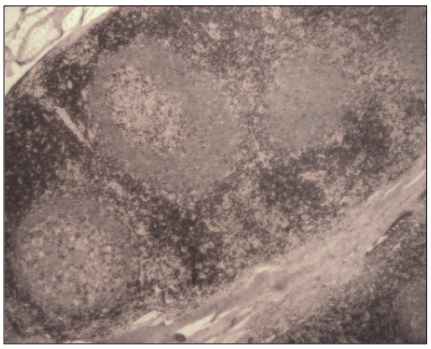


Figure 3. Anti-human CD3 IHC staining on FFPE human tonsil (DAB) from Newcomer Supply. CD3 localized around the follicle in dark brown.

3. Clone Screening

IMC vs. IF

Each metal-tagged clone is tested by IF to confirm staining patterns produced by IMC. In the images below, anti-human CD3 (polyclonal) was tested by IMC (left) and IF (right) (Figure 4). Once method reproducibility has been established, we continue to screen the clone for specificity.

IMC vs. IF: FFPE human tonsil

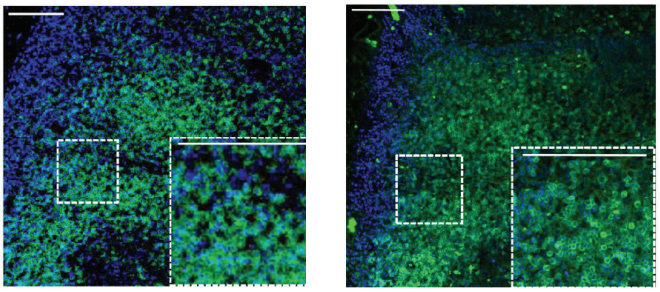


Figure 4. Comparison of anti-human CD3 by IMC (left) and IF (right) on FFPE human tonsil. Images show CD3 staining in green and DNA counterstain in blue. Scale bars = 100 µm.

Specificity

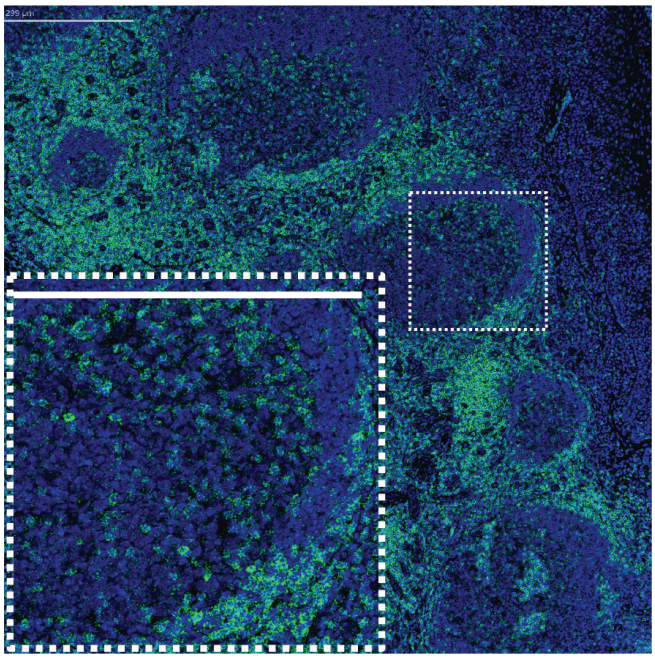
To assess antibody specificity, we test staining patterns in two different target tissues:

- Positive control: tissue positive for target
- Negative control: tissue negative for target

Following the example of anti-human CD3 (polyclonal), we demonstrated antibody specificity using human tonsil as a positive control, where CD3 expression is known to be found in follicles, and a negative control of human testis.

Depending on the marker, it is also possible to assess signal and background within a single sample. For example, tonsil follicles contain CD3+ regions, whereas tonsil stroma should be CD3–. Figures 4 and 5 shows expected patterns of CD3+ staining in the follicles and lack of staining in stromal borders, indicating high specificity.

FFPE human tonsil: positive control

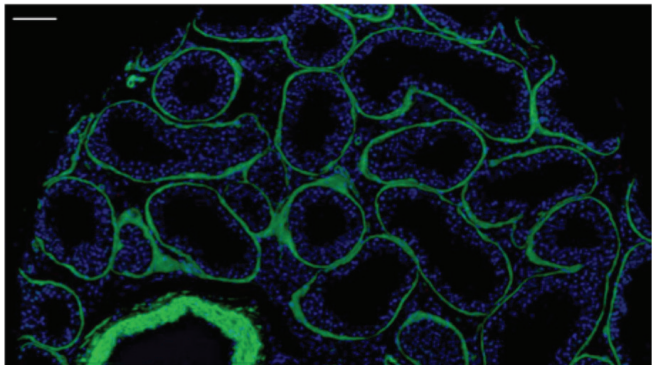


CD3 DNA

Figure 5. FFPE human tonsil sample stained with anti-human CD3. Pattern from interfollicle and intrafollicle CD3 staining (green) is comparable to IHC pattern shown in Figure 4. Scale bar = 300 µm.

In Figure 6, human testis samples were stained with anti-human alpha-smooth muscle actin and anti-human CD3. As expected, there is a lack of CD3 staining in the negative control. We were also able to assess nonspecific staining with the negative control.

FFPE human testis: negative control



CD3 aSMA DNA

Figure 6. FFPE human testis tissue showing lack of anti-human CD3 staining. Scale bar = 100 µm.

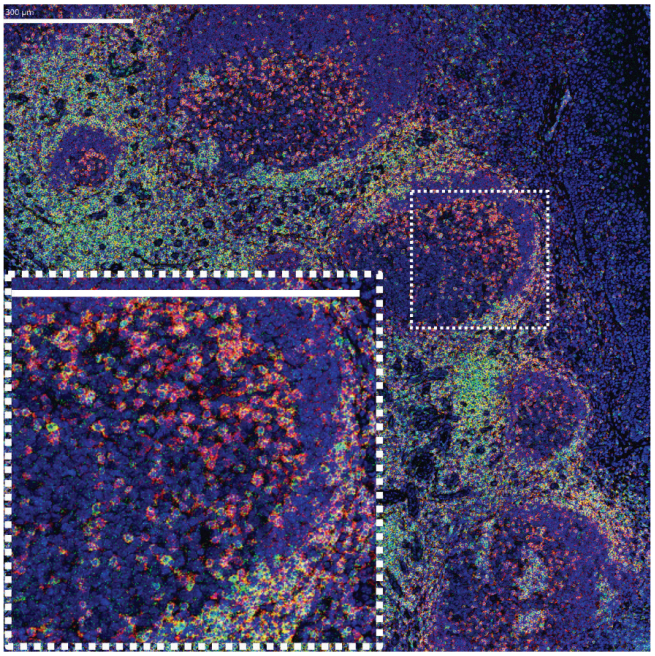
Each clone is also tested in a cocktail of other antibodies to further assess its specificity by:

- Co-localization
- Counter-localization

In the following image, human tonsil samples were stained with both anti-human CD45 and anti-human CD3. Since CD45 is expressed on the majority of immune cells and CD3 is expressed only on T cells, we expect all CD3+ cells to be CD45+. Figure 7 shows CD45+/CD3+ cells at two different scales.

A population of cells is expected to be CD45+/CD3– because CD3 expression is restricted to T cells. These can be seen within the same ROI, providing further evidence for clone specificity.

FFPE human tonsil: positive control



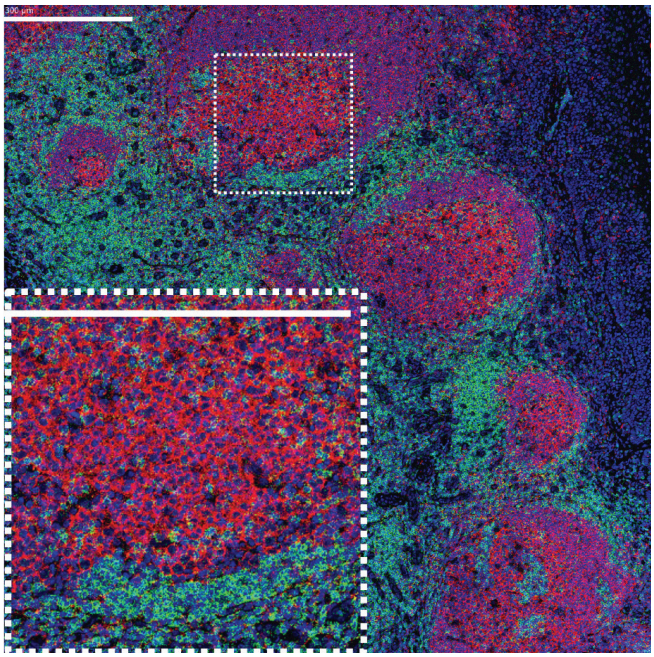
CD3 CD45 DNA

Figure 7. Co-localization of CD3 and CD45 on human tonsil samples. CD45 (red) and CD3 (green) staining patterns overlap (yellow), indicating the expected pattern of localization. Scale bar = 300 µm.

Counter-localization is also assessed to establish antibody specificity. To test anti-human CD3, we included anti-human CD20 as a co-stain (Figure 8). Since CD20 is a marker found only on B cells, all CD3+ cells should be CD20–.

Furthermore, B cells in tonsil tissue are known to aggregate in the center of each follicle, forming germinal centers. Figure 8 shows appropriate clustering of CD3+ cells around CD20+ germinal centers. Coexpression cannot be observed, even in the enlarged image.

FFPE human tonsil: counter-localization



CD3 CD20 DNA

Figure 8. Counter-localization of anti-human CD3 (green) and anti-human CD20 (red) on FFPE human tonsil at two scales.
Scale bars = 300 µm.

4. Verification Testing

Positive and negative control tissues are stained with an IMC panel that includes antibodies targeted to proteins that co-localize and counter-localize with the antibody of interest.

5. Pathologist Review and Approval

Once specificity and reproducibility have been established, a report summarizing the data is submitted to an external pathologist for review and approval. All Maxpar metal-conjugated antibodies for IMC have been verified by an independent pathologist. The antibody is approved only if the following conditions have been released:

- Positive control
- Negative control
- Co-localization
- Counter-localization

Following the case of anti-human CD3, the verification conditions are summarized below.

Positive control	Co-localization
Tonsil	CD45
Negative control	**Counter-localization**
Testis	CD20

Figure 9. Conditions required for pathologist-verified status of anti-human CD3.

Resources

To help assess antibody staining in the tissue of interest, we recommend consulting the Human Protein Atlas (proteinatlas.org) in conjunction with GeneCards® (genecards.org) for reference.

Conclusion

Imaging Mass Cytometry is a powerful and dynamic tool that can be used to simultaneously analyze up to 37 different markers in a single experiment. The information provided will prove invaluable in gaining insight into complex cell-to-cell relationships, spatial distributions and activation status in many different disease states.

Tips for Success

We employ these best practices as part of our verification process and we recommend the same to our customers.

Sample integrity

To ensure best results, we recommend using FFPE samples that are freshly cut, not stored long-term.

Antigen retrieval

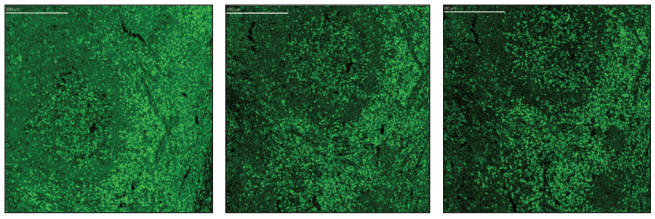
For consistency and to reduce the need for end-user protocol optimization, all of our antibodies are tested on FFPE tissue using a heat-induced epitope retrieval method with Tris-EDTA (pH 9) solution at 96 °C.

Antibody titration

Maxpar metal-conjugated antibody staining and signal intensity can vary greatly between different tissues (for example, spleen vs. lung), tissue types (for example, FFPE vs. fresh frozen) and method of antigen retrieval. Each antibody should be titrated on the tissue of interest to determine optimal staining concentration. A recommended range is provided on each antibody technical data sheet (TDS) to guide the user in determining an initial test dilution.

An example of an antibody titration is shown (Figure 10). Anti-human CD45 RO UCHL1 was tested on serial sections of FFPE human tonsil samples at three different dilutions. The 1:150 concentration yields the best signal with the least amount of background.

FFPE human tonsil: CD45 RO UCHL1 titration



1:50 **1:100** **1:150**

Figure 10. Titration of anti-human CD45 RO UCHL1 on human tonsil FFPE samples. Scale bars = 300 µm.

Storage

Once staining has been done, keep samples in plastic slide holders inside a sealed bag to minimize humidity. Desiccant can be added for long-term storage if desired. High humidity can damage tissue integrity and decrease shelf life of stained sample slides.

Available IMC Antibodies

More than 100 pathologist-verified Maxpar antibodies are commercially available in our IMC catalog, including markers for:

- Immunophenotyping
- Immune activation and immunosuppression
- Primary cancer and metastasis
- Stromal and vascular architecture
- Cell cycle state
- Phosphorylation state

Services

We provide an additional service of custom antibody conjugations upon request to ensure that our researchers have the freedom and flexibility of targeting the markers of interest to answer relevant biological questions. Please contact your local sales manager for a quote.

Panel kits

Specific antibody panel kits that target defined subpopulations are also available for purchase:

- Maxpar® Human Tumor-Infiltrating Lymphocytes IMC™ Panel Kit (Cat. No. 201502)
- Maxpar Human Immune Activation IMC Panel Kit (Cat. No. 201503)
- Maxpar Human Tissue Architecture IMC Panel Kit (Cat. No. 201504)
- Maxpar Human Immuno-Oncology IMC Panel Kit (Cat. No. 201505)

To provide better service to our customers, these panels have been pre-optimized on positive control tonsil tissue.

Appendix: Materials and Method

Materials

For the full list of Maxpar antibodies available for IMC, please go to **fluidigm.com**.

Vendor	Name	Catalog/ Part Number
Fluidigm	Maxpar PBS	201058
	Cell-ID Intercalator-Ir	201192A
	Maxpar® Water	201069
	Maxpar antibodies	Multiple
Third-Party Reagents		
Sigma-Aldrich®	M-xylene ReagentPlus	185566-1L
Sigma-Aldrich	Anhydrous ethyl alcohol	676829
Agilent®	Antigen Retrieval Solution pH 9 (10x)	S236784-2
Thermo Scientific™	Triton™ X-100	85111
Sigma-Aldrich	Bovine Serum Albumin (BSA)	A3059
Newcomer Supply	FFPE human tonsil sections	3770A

Protocol

The Imaging Mass Cytometry Staining for FFPE Protocol (PN 400322) can be found on our website. For detailed instructions on instrument operation, see the Hyperion User Guide (PN 400311). Acquisition was performed using CyTOF Software v7.0, and pseudocolor images were generated using MCD Viewer v1.6.

Learn more at **fluidigm.com**

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