Research Techniques Made Simple: Experimental Methodology for Imaging Mass Cytometry

Sheida Naderi-Azad1,6, David Croitoru2,6, Saeed Khalili2, Lihi Eder3,4 and Vincent Piguet2,5

Technological advances in flow cytometry and the development of mass cytometry by time-of-flight (CyTOF) have led to progressive increases in the number of proteins and biochemical processes that can be simultaneously measured. The most recent development of these platforms, imaging mass cytometry (IMC), allows for the visualization of up to 40 unique cellular markers and also employs rare metal isotopes conjugated to antibodies. However, IMC also adds the important benefit of preserving two-dimensional (2D) tissue architecture; this is accomplished by staining in situ and direct tissue vaporization followed by generation of a 2D spectral reconstruction using CyTOF-captured events. We review the experimental methodology for IMC that enables high-resolution multilayer images depicting protein expression, cellular localization, and interaction in situ in dermatology research.

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Introduction of imaging mass cytometry

Applying principles from mass spectrometry (MS) to flow cytometry (FC) protocols has catalyzed a shift toward multidimensional analysis with cytometry by time-of-flight (CyTOF) (Table 1). A 2017 Research Techniques Made Simple (RTMS) article by Matos et al. (2017) reviews the techniques underpinning CyTOF. Unlike conventional FC, the immunogenic staining in mass cytometry (MC) involves metal-conjugated antibodies (MCAs) that differ by atomic weight rather than by fluorophores. This confers improved accuracy and precision, bypassing the overlap in fluorophore emission spectra that leads to reduced resolution with multiple parameters in FC. Imaging mass cytometry (IMC) offers an additional, substantial benefit of resolving cellular spatial orientation within tissues. In IMC, a laser vaporizes tissues to generate plumes of aerosolized, ionized molecules, which are atomized by the CyTOF mass spectrometer, quantified, and reconstructed to a two-dimensional (2D) representation.

Overview of experimental design

The steps involved in the IMC workflow are illustrated (Figure 1). Tissue samples in IMC can be prepared in fresh-frozen or formalin-fixed, paraffin-embedded (FFPE) blocks (Chang et al., 2017). Cells are incubated with affinity MCA probes, which are commercially available or conjugated, following existing protocols, in situ using standard immunohistochemistry (IHC) protocols (step 1). The next step involves passing the stained histology slide through an inductively coupled plasma (ICP) ion source with a high-energy laser, which breaks covalent bonds to produce a cloud of charged heavy-metal-tagged ions (step 2). Tagged ions are filtered by their mass-to-charge (m/z) ratios and quantified by a time-of-flight (TOF) mass spectrometer (step 3). Quantification with CyTOF enables the generation of a matrix-relating localization and concentration of each native and non-native (antibody-conjugated) metal isotope during signal analysis (step 4). Given that the generated ion cloud for any MCA is correlated to the specific location of the laser-ablated tissue sample, the TOF data can then be used to recreate a multidimensional image (Giesen et al., 2014). Notable modifications to the overall IMC workflow have included the use of classical IHC to validate results and identify potential regions of interest of varying inflammatory stages before IMC interrogation. Ramaglia et al. (2019) have demonstrated this in affected and unaffected brain tissue to differentiate inflammatory cell subsets at various stages of the pathogenesis of multiple sclerosis, which has powerful application to inflammatory immune disorders of other organ systems such as the skin, with early work demonstrating promising results.

Metal-antibody panel design and conjugation

Rare earth metal isotopes are coupled with selected antibodies to generate a panel of probes with a distinct m/z signal, typically using lanthanide series (Han et al., 2018).
There are multiple commercially available panels, which can be modified considering design factors such as isotope sensitivity, high concentration (>1 mg/ml), cross-talk, and the ratio of antigen abundance to the environmental background (signal-to-noise ratio). Design tools can help to predict signal overlap per panel using algorithms that rely on preloaded signal and tolerance to optimize panel performance for metal-antibody conjugation (Fluidigm, 2020; Guo et al., 2019). After conjugation, the optimal antibody concentration can be titrated with serial antibody dilutions, and the dilution with the highest signal-to-noise ratio is selected. In cases with considerable spillover to the adjacent channels, lower concentrations are used. All MCAs should be validated using fluorescent antibodies in FC or other IHC assays with relevant controls to ensure assay congruence (described in detail below) (Guo et al., 2019).

**Cell staining**
The antibody targets for the immunohistochemical staining are diverse. Extracellular molecules (e.g., drugs), cell surface markers (e.g., cluster of differentiation, chemokine receptors, collagen), intercellular molecules (e.g., within the tumor microenvironment), intracellular targets (e.g., histone proteins, DNA), and signal transduction (e.g., phosphorylation) have all been used. One report by Chang et al. (2016) provides a compelling example of IMC utility in cutaneous drug distribution by detecting cisplatin accumulation in the skin of treated mice bearing pancreatic tumor xenografts. In the study of structures of healthy skin in murine models, full-thickness IMC has been shown to effectively stain epidermal cadherins and intracellular markers, dermal collagen, and vessels using smooth muscle actin as well as others (Chang et al., 2017). In terms of endogenous human skin disease, Guo et al. (2019) identified mechanistically relevant lymphoid cell subsets (lymphoid tissue–inducer [Lti] cells and regulatory T cells [Tregs]) in patients with psoriasis compared with those in controls.

Cell staining of FFPE samples requires dewaxing with xylene, rehydration, and antigen retrieval, whereas these steps are not required with fresh-frozen samples. From here onward, the workflow for both FFPE and frozen tissue samples involves blocking with BSA or other commercially available reagents, followed by MCA incubation. For the dewaxing step, it is typically recommended that fresh-cut tissue sections be used as opposed to precut because the latter would result in tissue degradation over time. Various methods can be used for antigen retrieval depending on the antibody kit and cellular targets, including heat-induced epitope retrieval (HIER) and proteolytic-induced epitope retrieval (PIER). PIER uses various enzymes such as proteinase K, trypsin, and pepsin and has not been validated to the same degree as HIER. Various parameters such as antigen retrieval solution, duration, and temperature can be optimized as appropriate. Because the optimization of the staining protocol often requires numerous efforts, it is recommended that smaller panels of 3–5 markers be initially used on positive and negative control tissue sections. Many of the antibody kits include information on recommended dilution ranges on the technical datasheets.

**Laser ablation**
A duplicate slide with the region of interest can be identified with the help of immunofluorescence staining and microscopy, with standard IHC to plan the localized target of laser ablation (Ramaglia et al., 2019) (Figure 2). Ramaglia et al. (2019) demonstrate congruence of localization between immunofluorescence microscopy (IFM) and IMC using CD3+ lymphocytes, CD68+ myeloid cells, and antiprotoechistoprotein–stained myelin (Figure 2a). There are various lasers that can be coupled to the ICP mass spectrometer or a comparable prototype for sample detection (Chang et al., 2016; Guo et al., 2019).
### Table 1. Summary of Advantages and Disadvantages with IMC, CyTOF, and FC Platforms

<table>
<thead>
<tr>
<th>Overview</th>
<th>IMC</th>
<th>CyTOF</th>
<th>FC</th>
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<tbody>
<tr>
<td><strong>Distinguishing mechanistic feature</strong></td>
<td>Uses same metal-conjugated probe and image capture principles as CyTOF with in situ staining and direct tissue vaporization</td>
<td>Uses rarely occurring MCAs that differ by atomic weight</td>
<td>Uses fluorophores probes to differentiate antigen targets</td>
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<tr>
<td><strong>Advantages</strong></td>
<td>Can analyze over 45 cellular event parameters</td>
<td>Provides molecular weights of proteins and peptides with high accuracy</td>
<td>Relatively fast and cost-effective</td>
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<tr>
<td>Calibration for probe detection often not required</td>
<td>Improved accuracy and precision by bypassing overlap in fluorophore emission spectra</td>
<td>Can be used to study a large cell population</td>
<td></td>
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<tr>
<td>Resolved cellular spatial orientation within tissue through direct laser ablation and postacquisition image reconstruction</td>
<td></td>
<td>Established standard for cell population expression and cycling</td>
<td></td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>Time-consuming</td>
<td>Time-consuming</td>
<td>Fluorophore overlap leads to background noise</td>
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<tr>
<td>Costly</td>
<td>Costly</td>
<td>Limited channels for parametric analysis (15–20)</td>
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<tr>
<td>Requires careful validation with IF and/or FC standards and complex data analysis</td>
<td>No cellular spatial orientation within 2D reconstruction</td>
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Abbreviations: 2D, two-dimensional; CyTOF, cytometry by time-of-flight; FC, flow cytometry; IF, immunofluorescence; IMC, imaging mass cytometry; MCA, metal-conjugated antibody.

![Image of IMC experimental design](image-url)

**Figure 1. Schematic representation of IMC experimental design.** The IMC workflow involves antibody target selection, antibody staining in situ, capturing of ablated tissue, postacquisition data processing, and five-channel IMC overlaying. DC, dendritic cell; GC, germinal center; IMC, imaging mass cytometry; PMN, polymorphonuclear; ROI, region of interest; TFH, T follicular helper.
The tissue samples are ablated with a pulsed deep UV 213–219 nm wavelength laser with a diameter spot size of 1 μm, frequency of 20–200 Hz, and a translation speed of 20 μm/s (Fluidigm, 2020). The laser can be calibrated daily with a tuning slide spiked with known metal elements to ensure consistency. The vaporized plumes of tissue are ionized and passed through the TOF spectrometer where isotope abundance is captured.

Image analysis

After image capturing, single-cell segmentation can be achieved in various ways. First, it can be done by detecting cell boundaries by cell membrane proteins as well as nuclei by localized proteins such as histones or intercalated iridium (Giesen et al., 2014). This may be enhanced by watershed transformation, which extrapolates cell boundaries (cytoplasm) and attempts to minimize noise by Gaussian blurring (Giesen et al., 2014). Other adjunct algorithms are established with the help of pathologist-defined rulesets to distinguish cell types such as stromal and epithelial cells according to cell morphology, size, proteins, and location (Chang et al., 2016).

Several algorithms can be used for data analysis that aid in the spectral reconstruction of the ablated tissue in 2D cross-sections, depending on the goals of the project. Dimensionality reduction channels (e.g., visual scholastic neighbor embedding [viSNE] channels) are helpful in identifying clusters of cell infiltrates while maintaining the single-cell resolution (Chester and Maecher, 2015). In contrast, spanning-tree progression analysis of density-normalized events (SPADEs) can visualize fold-change data over a population at the cost of single-cell resolution. SPADE clusters events according to a shared expression of proteins to produce a minimum spanning tree (Giesen et al., 2014; Guo et al., 2019). Guo et al. (2019) used the SPADE technique to evaluate seven cellular signaling pathways common to lymphoid cells with suspected roles in psoriasis pathogenesis (Figure 3b). Using clustered changes in protein expression within these pathways, as visualized by SPADE, and a subsequent visualization

Figure 2. Comparison of IMC with IF in MS lesions. (a) Ramaglia et al. (2019) assess two serial sections by IF (a′ and a′1) and IMC (a′′ and a′′1). (b) They use CD3+FITC and CD3+170ER-metal tag by IF and IMC, respectively, to demonstrate lymphocyte localization by both methods. (c) Their IMC workflow to detect regions of interest and capture with IMC is also shown. IF, immunofluorescence; IMC, imaging mass cytometry; MS, mass spectrometry; NAGM, normal appearing grey matter; no., number; PLP, proteolipid protein; WML, white matter lesion.

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Figure 3. Visual analysis techniques employ multiple algorithms to display large datasets of IMC. Guo et al. (2019) demonstrate two techniques for mapping and 2D visualization of immense IMC datasets from their study on lymphoid subsets in psoriatic plaques compared with those in normal skin. (a) Heatmap demonstrating lymphoid subpopulation. (b) They utilize SPADE analysis to hierarchically map T-cell subsets and compare known signaling transduction activity. (c) They also employed viSNE to incorporate expression signatures into density maps that demonstrate cellular colocalization. 2D, two-dimensional; HC, healthy control; IMC, imaging mass cytometry; SPADE, spanning-tree progression analysis of density-normalized event; STAT, signal transducer and activator of transcription; PS, psoriasis; viSNE, visual scholastic neighbor embedding.
MULTIPLE CHOICE QUESTIONS

1. What is the aim of serial antibody dilutions in imaging mass cytometry (IMC)?
   A. To ascertain the optimal antibody concentration
   B. To select the correct antibody for cellular targeting
   C. To select the sample with the lowest signal-to-noise ratio
   D. To select higher titrations in cases with noteworthy spillover to the adjacent channels

2. What are the advantages of spanning-tree progression analysis of density-normalized event (SPADE) compared with other algorithms (e.g., FlowSOM, Citrus, etc.) in IMC?
   A. Identifying clusters while keeping the single cell
   B. Visualizing fold-change data with hierarchical clustering
   C. Identifying rare cell types without density-based subsampling
   D. Identifying all cells with an experimental end point

3. Which option does not help to achieve single-cell measurement?
   A. Detecting cell boundaries by cell membrane proteins as well as cell centers by molecules such as histone proteins
   B. Watershed transformation (by defining the maximum cell boundaries and minimizing noise)
   C. Using the platforms with the help of pathologist-trained rulesets
   D. Using SPADE analysis

4. What are the potential contributors to channel cross-talk?
   A. Isotope impurity
   B. Metal oxidation
   C. Errors in ion detection
   D. All of the above

5. Which aspects of investigative dermatology can be studied using IMC?
   A. Skin inflammation
   B. Skin cancer
   C. Stem cells and tissue repair
   D. Normal skin physiology
   E. Psoriasis, eczema, and hidradenitis suppurativa
   F. All of the above

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Normalization and error propagation

Antibody testing can be done both before and after metal conjugation, with each step requiring both positive and negative validation. IMC data are often validated with other single-cell measurement techniques such as MS, MC, FC, and IFM (Guo et al., 2019). Using the initial validation, only antibodies with expression patterns consistent with the literature are used. Testing after metal conjugation ensures that antibody specificity is not affected by conjugation. For instance, in the recent IMC study by Guo et al. (2019) that identified Lti and Treg populations in psoriasis, FC was used to validate MCAs.

Conclusions

IMC, although a very recent development of CyTOF and FC, is a remarkable investigative tool for dermatology and
translational researchers in immunology that has, so far, been used in three primarily cutaneous studies to elucidate T-cell subsets in psoriasis and drug biodistribution in the skin as well as in healthy murine models. Because IMC is a recently adapted technique, it remains to be limited currently owing to the relatively small number of studies that have validated this technique with more established techniques such as FC and MS. Nevertheless, the experimental methodology from IMC studies in organ systems demonstrates the potential to produce high-resolution multilayer images in translational dermatology. Early reports highlight the immediate application of this platform to examine subcellular populations, detect single-cell protein expression, and elucidate cell-cycle changes, providing profound implications for disease pathogenesis and pharmacodynamic effects in the skin.

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**CONFLICT OF INTEREST**
Fluidigm (Markham, Canada) funding was provided to VP and LE. VP undertakes advisory work for Pfizer, AbbVie, Janssen, UCB, Novartis, Almirall, and Celgene and has received departmental support from AbbVie, Bausch Health, Celgene, Janssen, LEO Pharma, Eli Lilly, NAOS, Novartis, Pfizer, Pierre-Fabre, and Sanoﬁ. We have, however, made efforts throughout the article to refrain from using any commercial names for devices and have tried to adhere to generic names to avoid commercialization of imaging mass cytometry equipment and products.

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**AUTHOR CONTRIBUTIONS**
Conceptualization: VP, DC; Supervision: DC, VP; Visualization: SNA; Writing – Original Draft Preparation: SNA, DC; Writing - Review and Editing: DC, SNA, SK, LE, VP

**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

**REFERENCES**
1. What is the aim of serial antibody dilutions in imaging mass cytometry (IMC)?

**CORRECT ANSWER:** A. To ascertain the optimal antibody concentration

The aim of the serial antibody dilutions is to titrate the optimal antibody concentration after conjugation (A is correct, B is incorrect). Specifically, the dilution with the highest signal-to-noise ratio is selected (C is incorrect). Furthermore, lower concentrations are used in cases where there is considerable spillover to adjacent channels (D is incorrect).

2. What are the advantages of spanning-tree progression analysis of density-normalized event (SPADE) compared with other algorithms (e.g., FlowSOM, Citrus, etc.) in IMC?

**CORRECT ANSWER:** B. Visualizing fold-change data with hierarchical clustering

SPADE can visualize fold-change data over a population at the cost of single-cell resolution (B is correct). SPADE clusters events according to a shared expression of proteins to produce a minimum spanning tree and consequently does not help with single-cell segmentation (A is incorrect). FlowSOM is a self-organizing map (or neural network algorithm) that can be used to identify rare cell types without density-based subsampling and additional time investment (C is incorrect). Citrus can identify all cells with an experimental end point (D is incorrect).

3. Which option does not help to achieve single-cell segmentation?

**CORRECT ANSWER:** D. Using SPADE analysis

Single-cell segmentation can be achieved by detecting cell boundaries by cell membrane proteins as well as nuclei by localized proteins such as histones or intercalated iridium (A is incorrect). Watershed transformation extrapolates cell boundaries (cytoplasm) and attempts to minimize noise by Gaussian blurring (B is incorrect); pathologist-defined rulesets further help to distinguish cell types such as stromal and epithelial cells according to cell morphology, size, proteins, and location (C is incorrect). SPADE analysis is helpful for hierarchical mapping, such as cellular subtyping by expression, but not for unicell segmentation (D is correct).

4. What are the potential contributors to channel cross-talk?

**CORRECT ANSWER:** D. All of the above

Channel cross-talk can occur as a result of all, from isotopic impurity leading to signal leakage, metal oxidation, and errors in ion detection (D is correct).

5. Which aspects of investigative dermatology can be studied using IMC?

**CORRECT ANSWER:** F. All of the above

IMC is a remarkable investigative tool for dermatology with potential for use in cutaneous studies pertaining to inflammation, cancer, stem cell and tissue repair, various dermatologic conditions, and normal skin physiology (F is correct).