Research Techniques Made Simple: Use of Imaging Mass Cytometry for Dermatological Research and Clinical Applications

Jesse Veenstra
Peter Dimitrion
Yi Yao
Li Zhou
David M. Ozog

See next page for additional authors
Authors
Jesse Veenstra, Peter Dimitrion, Yi Yao, Li Zhou, David M. Ozog, and Qing-Sheng Mi
INTRODUCTION
Tissue architecture plays an essential role in both physiological and pathological processes necessary to understand normal homeostasis and the development of disease. Not surprisingly, histopathology remains among the most critical and widely used tools for dermatologic researchers and clinicians to investigate and diagnose cutaneous disease, where numerous structural elements and cellular types interact with one another. Despite the utility of routine histopathology, it has limited capability to define molecular features important to investigators to fully understand the tissue microenvironment. Although traditional immunohistochemistry (IHC) or immunofluorescence (IF) can quantify molecular markers to supplement H&E staining, it is inherently limited by its ability to analyze only several markers from a single section at a time, which hinders in-depth characterization and phenotyping of tissues. These limitations preclude IHC from simultaneously phenotyping multiple cell types, cellular interactions, and cellular states in parallel with tissue markers. Although techniques for high-dimensional histological imaging exist, such as flow cytometry or single-cell RNA sequencing. Resulting high-dimensional histological images permit spatially conserved analysis to identify unique cell populations, cellular interactions and avoidance s, and insight into activation and behavioral status based on tissue location. IMC can be performed on both frozen and formalin-fixed paraffin-embedded tissue, allowing for previously banked samples to be analyzed and correlated with known clinical outcomes. Expectedly, IMC will change the landscape of investigative pathology, particularly when used in coordination with multiomic platforms to combine transcriptomic and proteomic data at a single-cell resolution. Here, we aim to highlight the potential utility of IMC within dermatologic research and clinical applications.

Traditional immunohistochemistry (IHC) is inherently limited by its ability to analyze only several markers within a histological tissue section at a given time, which hinders in-depth characterization and phenotyping of tissues. Imaging mass cytometry (IMC), which combines IHC using metal-labeled antibodies with laser ablation and detection using mass cytometry by time-of-flight, overcomes this limitation with the capability to simultaneously analyze up to 40 protein markers to generate high-dimensional images from a single tissue section. IMC analysis preserves tissue architecture and spatial cellular relationships that would otherwise be lost or significantly altered in applications requiring tissue dissociation, such as flow cytometry or single-cell RNA sequencing. Resulting high-dimensional histological images permit spatially conserved analysis to identify unique cell populations, cellular interactions and avoidances, and insight into activation and behavioral status based on tissue location. IMC can be performed on both frozen and formalin-fixed paraffin-embedded tissue, allowing for previously banked samples to be analyzed and correlated with known clinical outcomes. Expectedly, IMC will change the landscape of investigative pathology, particularly when used in coordination with multiomic platforms to combine transcriptomic and proteomic data at a single-cell resolution. Here, we aim to highlight the potential utility of IMC within dermatologic research and clinical applications.

Abbreviations: cSCC, cutaneous squamous cell carcinoma; CyTOF, cytometry by time-of-flight; FFPE, formalin-fixed paraffin-embedded; HS, hidradenitis suppurativa; IF, immunofluorescence; IHC, immunohistochemistry; IMC, imaging mass cytometry; MIBI, multiplexed ion beam imaging; SCP, single-cell pathology; Treg, regulatory T cell; TSK, tumor-specific keratinocyte
IMC WORKFLOW

Before tissue imaging, the design phase is critical to ensure that appropriate metal-tagged antibodies are selected and optimized to tissues of interest with both positive and negative controls (Figure 1). It is recommended to optimize non-validated IMC antibodies with IF before metal conjugation, which serves as a surrogate for IMC detection performance. Antibodies to a wide array of targets, including extracellular, intracellular, and signal transduction pathways, have been successfully employed for IMC. The technical aspects of IMC and CyTOF are further reviewed in the complementary Research Techniques Made Simple article by Naderi-Azad et al. and prior similar articles, respectively (Doan et al., 2015; Matos et al., 2017). Once IMC is complete, raw images can be rendered to high-dimensional images with each marker pseudocolored per investigator preference. Single-cell segmentation from images is possible using a combination of different open-source software, such as Ilastik and CellProfiler. The resulting single-cell data is conducive to the same analysis pipelines used to analyze CyTOF data as previously reviewed (Matos et al., 2017), with the added dimensions of cell shape, size, and localization. Publicly available algorithms can integrate both cytometric and spatial data to enable a more detailed analysis of IMC data (Schapiro et al., 2017). Further integration with other data sets from relevant subjects and specimens enables a multiomics approach for improved sample classification and subsequent clinical correlation, which is further discussed hereafter.

PRACTICAL USES IN DERMATOLOGIC RESEARCH

With the advent of single-cell omics, researchers have begun to appreciate the importance of individual cellular subsets and states in the pathogenesis of numerous diseases. IMC can capture this heterogeneity at single-cell resolution while preserving the spatial orientation of different cell types, subclasses, and activation states (e.g., inflammatory or anti-inflammatory). The flexibility in assessing the localization of specific cell types and states lies in the development of a question-driven IMC antibody panel. Questions may be broad and exploratory, such as, “Is there a spatially regulated cellular or tissue biomarker for a certain disease state or stage?” or “What is the immune cell landscape in lesional versus perilesional skin or epidermis versus dermis?” Indeed, one recent publication utilized IMC to assess the infiltration and spatial localization of various immune cell subsets within lesions from patients with hidradenitis suppurativa (HS) (Gudjonsson et al., 2020). The investigators used an IMC panel of 12 markers targeting different immune subsets, such as plasma cells, B cells, monocytes and macrophages, CD8 T cells, and neutrophils, which found substantially increased leukocyte infiltration in HS lesional tissue relative to normal skin. In contrast, questions can also be more targeted, such as, “What is the ratio of activated and exhausted CD8 T cells infiltrating tumor versus stroma?” or “What is the landscape of T-cell phenotypes in a cutaneous malignancy?” Defining regions of interest based on tissue architecture or lesional or tumor–stroma boundaries can further provide meaningful biological context to spatial data.

As previously noted, cell segmentation can be performed on IMC images to permit single-cell phenotypic-based...
clustering to define cell clusters and populations within the sample, which can then be linked back to the original histological image to gain a deeper understanding of where cellular subsets are localized; this process has been coined round-trip analysis (Schapiro et al., 2017). In other words, instead of identifying specific cellular markers (i.e., CD3, CD4, Histone 3) in tissue sections, round-trip analysis condenses these markers and allows the visualization of a cellular phenotype (i.e., memory, proliferating, activated, and exhausted T-cell phenotypes).

It is well known that immune cells utilize cell surface contacts to mediate cellular crosstalk and coordinate function. From localizing cell types, one can also determine cell-cell interactions or groups of cells interacting from IMC data, so-called neighborhood analysis. This analysis relies on the number and frequency of interactions and avoidances.

Table 1. Differences between IMC and IF

<table>
<thead>
<tr>
<th>Imaging Attributes</th>
<th>IMC</th>
<th>IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly multiplexed imaging ≥40 markers</td>
<td>Yes</td>
<td>Only with serial IFa</td>
</tr>
<tr>
<td>Resolution</td>
<td>Subcellular (1 μm)</td>
<td>Subcellular (dependent on microscope)</td>
</tr>
<tr>
<td>Simultaneous target readout</td>
<td>40</td>
<td>1-5</td>
</tr>
<tr>
<td>Scanning automation with real-time visualization</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Background signal and spectral overlap</td>
<td>Minimal to none</td>
<td>Yes (further increases with number of cycles)</td>
</tr>
<tr>
<td>Tissue ablated</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Area imaged</td>
<td>Regions of interest</td>
<td>Objective field of view</td>
</tr>
<tr>
<td>Throughput</td>
<td>~2 hrs/mm²</td>
<td>Hours per cycle</td>
</tr>
<tr>
<td>Marker coloring</td>
<td>User-selected (pseudocoloration)</td>
<td>Fluorophore dependent</td>
</tr>
<tr>
<td>Equipment required</td>
<td>CyTOF mass cytometer</td>
<td>Confocal or super-resolution microscopes</td>
</tr>
</tbody>
</table>

CyTOF, cytometry by time-of-flight; IF, immunofluorescence; IMC, imaging mass cytometry.

*aSerial immunofluorescence is an IF technique with the capability to generate highly multiplexed images using multiple cycles of staining and fluorophore bleaching (Gerdes et al., 2013).

IMC Workflow

Figure 1. IMC Workflow. (a) After experimental planning and selection of appropriate samples and markers, staining of tissues of interest and appropriate controls are optimized. (b) FFPE or frozen tissues are prepared on glass slides and can be in the form of custom-built tissue microarrays composed of numerous specimens to aid in high-throughput analysis. (c) Slides are stained with metal-tagged antibodies and then IMC is performed by laser ablation in conjunction with CyTOF. (d) Multidimensional images are generated and pseudocolored per user specifications for visualization. (e) Preliminary cell phenotyping is possible based on targets analyzed, and single-cell segmentation is performed from raw image files to enable downstream (f) single-cell analysis with phenotype-based clustering algorithms (i.e., Phenograph, FlowSOM). (g) If available, IMC data can be incorporated with other relevant data sets, such as spatially defined transcriptomics, for a multiomics approach. (h) Each sample can be classified based on the composition of cell types and cellular interactions (e.g., single-cell pathology subgroups). (i) Newly classified samples can be correlated with known clinical outcomes from imaged tissues. CyTOF, cytometry by time-of-flight; FFPE, formalin-fixed paraffin-embedded; IMC, imaging mass cytometry.
between defined populations of cells. Such analyses can help generate cell–cell interaction networks that go awry in disease to identify key pathways and potential targets for immunotherapy or define disease-specific cellular communities with prognostic value. Additionally, neighborhood analysis can determine whether certain cell types have a predilection or aversion for clustering in a tissue, which may indicate a disease-driving process. Defining interacting communities of cells and neighborhoods adds another level of depth to IMC analysis. This opens a new avenue of investigation into defining the etiology and pathogenesis of skin disease by expanding the scope of questions from a cell type to a community type.

Recently, a multiomics investigation of human cutaneous squamous cell carcinoma (cSCC) utilizing multiplexed ion beam imaging (MIBI) with a panel of 38 markers to perform high-dimensional imaging, spatial transcriptomics, and single-cell RNA sequencing identified a previously uncharacterized population of tumor-specific keratinocytes (TSKs) in tumor, but not matched normal, skin (Ji et al., 2020). MIBI is similar to IMC with the use of metal-labeled antibodies but, instead of a laser, uses an ion beam to raster over the tissue to generate secondary ions for detection by a sector field mass spectrometer (Figure 2c) (Bodenmiller, 2016); serial immunofluorescence and IMC methods are illustrated respectively in Figure 2a and b. Specimen imaging with MIBI (Figure 3a) uncovered prominent inter- and intratumor microenvironment heterogeneity across cSCC clinical specimens (Figure 3b and c). The integration of data across all platforms revealed that TSKs serve as the principal hub for intercellular communication within cSCC that influences tumor progression, immunosuppression, and heterogeneity. Despite considerable tumor heterogeneity, a more in-depth analysis of spatially resolved immune infiltrates revealed a strong correlation between CD8 T cells, CD4 T cells, regulatory T cells (Tregs), and macrophages (Figure 3d). Fibroblasts, macrophages, and Tregs were predominantly at the tumor–stroma border, whereas CD8 T cells and neutrophils were largely excluded from the tumor, indicating that Treg and macrophage positioning may limit effector lymphocyte access to the tumor (Figure 3e and f). B cells, which may mediate either antitumor or suppressive
immunity, were the only cell type found to infiltrate the tumor compartment preferentially.

Ji et al. (2020) also demonstrate the integration of high-dimensional imaging with other single-cell technologies, such as single-cell transcriptomics and spatial transcriptomics. Although IMC requires targets to be defined a priori, single-cell transcriptomics and spatial transcriptomics have no such requirement, which allows for remarkable de novo discovery of pathogenic cell states, phenotypes, behaviors, and communities. Notably, single-cell transcriptomic technologies, which simultaneously classify cells based on epitope expression and transcriptomic profiles, integrate seamlessly with IMC because the user can match IMC targets with the epitope index (Stoeckius et al., 2017). This allows deeper characterization of IMC-defined cellular subclusters and states and spatially regulated gene expression networks and together provides more confidence in newly discovered cell populations, as transcriptionally defined subsets are not always readily distinguished at the protein level. An integrative omics approach that includes IMC provides an unprecedented breadth and depth of analysis that evaluates tissue architecture and spatial relationships between cellular subsets and communities, which will undoubtedly be valuable to defining the etiology of skin diseases and improving our understanding of cutaneous biology.

**CLINICAL UTILITY OF IMC**

Many complex cellular phenotypes and cellular relationships can be identified within the spatial context of intact tissues using highly multiplexed imaging via IMC, which enables superior histopathological classification of clinical samples and potential for improved prognostication (Giesen et al., 2014). The feasibility and power of this concept were recently demonstrated in breast cancer, where a panel of known and putative prognostic biomarkers was used to characterize novel single-cell pathology (SCP) subgroups.

---

**Figure 3. Spatial architecture of lymphocyte subsets in cSCC.** (A) Select MIBI FOVs for patient samples with expression of highlighted features. (b) Heatmap of feature expression across cell types identified by MIBI. (c) Top, bar plots of proportion of nontumor cell types across all FOVs. Bottom, bar plots of total numbers of nontumor cells identified in each FOV. (d) Correlation heatmap of nontumor cell types across all FOVs. (e) Nontumor cells flagged by location relative to tumor and stromal compartments. (f) Heatmap of relative abundance of cell types in each compartment. Values represent proportion of total nontumor cells in compartment contributed by each cell type. cSCC, cutaneous squamous cell carcinoma; dsDNA, double-stranded DNA; FOV, field of view; KC, keratinocyte; MIBI, multiplexed ion beam imaging; Treg, regulatory T cell. Adapted from (Ji et al., 2020) under a CC-BY license.
based on cellular composition, cellular interactions, and tissue organization among a large cohort of banked FFPE breast cancer specimens with known clinical outcomes (Jackson et al., 2020). The newly identified SCP subgroups were found to better predict and segregate clinical outcomes when compared with current standard clinical grading criteria using hormone receptor and HER-2 status (Figure 4a–h). They found that almost no solitary marker was independently associated with clinical outcomes, whereas the composition of cellular communities that comprised SCP subgroups did (Figure 4i). Similar methodology will likely be able to be utilized for other diseases, such as melanoma, to better classify and correlate tissue pathology with patient survival, disease progression, and likelihood of response to targeted therapies or immune checkpoint blockade.

It bears mentioning that although other single-omics platforms, such as single-cell RNA sequencing and suspension-based CyTOF, require fresh tissue for analysis, IMC can utilize previously banked FFPE tissue with known clinical outcomes. This is especially helpful in dermatology given the sheer number of cutaneous biopsies from a multitude of different conditions that are available. This facilitates
MULTIPLE CHOICE QUESTIONS

1. What is the detection method for imaging mass cytometry (IMC)?
   A. Immunofluorescence
   B. Substrate—chromogen reaction
   C. Phase-contrast microscopy
   D. Cytometry by time-of-flight (CyTOF)

2. Which of the following is the primary consideration when planning an IMC experiment?
   A. Availability of fresh tissue
   B. Appropriate antibodies are selected and optimized to tissues of interest with both positive and negative controls
   C. What commercial IMC kits are available
   D. Selection of your top 25 markers of interest

3. Which of the following is an advantage of IMC over immunofluorescence techniques?
   A. Highly multiplexed imaging with a single scan
   B. Minimal to no background signal and spectral overlap
   C. User-defined marker coloring (pseudocoloration)
   D. All of the above

4. Which of the following cannot be analyzed by IMC from human skin samples?
   A. Localization of cellular markers (i.e., CD4, IFNγ) in different layers of the epidermis
   B. Whole transcriptomes from keratinocyte subsets within a melanoma section
   C. Perifollicular immune cell populations from patients with moderate to severe psoriasis
   D. Extracellular matrix proteins in the dermis from a perilesional tissue sample from a patient with lichen planus

5. Which of the following is a potential application of IMC in the clinic?
   A. Determination of tumor heterogeneity and immune infiltrates within preserved tissue architecture
   B. Identification of novel single-cell pathology subgroups to better predict clinical outcomes
   C. Characterization of single-cell interactions and relationships
   D. Integration with transcriptomic data
   E. All of the above
REFERENCES


1. What is the detection method for imaging mass cytometry (IMC)?

**CORRECT ANSWER:** D. Cytometry by time-of-flight (CyTOF)

IMC combines metal-labeled antibodies with laser ablation and detection using mass CyTOF. The other choices are not used as a method of detection in IMC.

2. Which of the following is the primary consideration when planning an IMC experiment?

**CORRECT ANSWER:** B. Appropriate antibodies are selected and optimized to tissues of interest with both positive and negative controls

Before tissue staining and imaging, the design phase is critical to ensure that appropriate metal-tagged antibodies are selected and optimized to tissues of interest with both positive and negative controls. Fresh tissue is not required for IMC; both formalin-fixed paraffin-embedded and frozen tissues can be used. Users can create fully customized antibody panels performing metal conjugation to selected antibodies. IMC currently has the capacity to detect up to 40 markers in a single scan.

3. Which of the following is an advantage of IMC over immunofluorescence techniques?

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

All of the choices listed are advantages of IMC over immunofluorescence.

4. Which of the following cannot be analyzed by IMC from human skin samples?

**CORRECT ANSWER:** B. Whole transcriptomes from keratinocyte subsets within a melanoma section

All of the options except for B assess proteins from different skin samples. Although antibodies do exist to analyze some noncoding RNAs and studies have been able to measure mRNA using oligonucleotide antibodies, IMC cannot be used to efficiently acquire the entire transcriptome from any kind of cells. This is where the integration with other omics technologies complement IMC.

5. Which of the following is a potential application of IMC in the clinic?

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

All of the choices listed are potential applications of IMC.