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

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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.

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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 serial IF, their utility is limited by the precision of target detection with fluorophores prone to spectral overlap and the time-intensive nature, making them less clinically useful (Gerdes et al., 2013). Therefore, a need exists for a highly multiplexed histology platform that is compatible with routinely collected formalin-fixed paraffin-embedded (FFPE) tissues, enables quantification of many markers simultaneously, provides single-cell resolution and high-throughput analysis capability, allows for fully customizable panels, and can be readily analyzed.

Recent advances using cytometry by time-of-flight (CyTOF) has led to the advent of imaging mass cytometry (IMC), which combines IHC using metal-labeled antibodies with laser ablation and detection by mass spectrometry to generate multiplexed images. IMC overcomes the limitations of traditional IHC and IF with the current capacity to analyze up to 40 targets from a single scan with 135 available detection channels, significantly augmenting the ability to evaluate complex cellular systems and processes. IMC can also be performed on FFPE or frozen tissues, allowing for previously banked samples with known clinical outcomes to be analyzed. Although IMC remains a relatively new technology and has not been widely used in dermatology thus far, its use

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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

SUMMARY POINTS

- Imaging mass cytometry (IMC) of tissues, which utilizes mass cytometry by time-of-flight (CyTOF) technology, enables highly multiplexed histological imaging with single-cell resolution using up to 40 markers from a single scan.
- Resulting histological images permit spatially conserved single-cell analysis to identify and phenotype unique cell populations and associated cellular interactions and provide insight into activation and behavioral status and signal transduction cascades.
- Previously banked formalin-fixed paraffin-embedded or frozen tissues can be used and correlated to known clinical outcomes.
- Tissue microarrays from multiple samples can be constructed on a single slide to enable high-throughput analysis.

Advantages

- In a single scan, IMC enables quantification of up to 40 markers, with 135 available detection channels.
- There is minimal to no spectral overlap or background signal with the use of metal-tagged antibodies in contrast to fluorophores used in immunofluorescence.
- Antibody panels are fully customizable with metal conjugation reagents.
- Multiple regions of interest can be selected for scanning from a single slide, which can be visualized in real time and remotely monitored.

Limitations

- Initial CyTOF equipment cost is substantial.
- There is limited availability of institutions offering IMC Core services to outside investigators.
- Metal-labeled antibodies are more costly than fluorophore-conjugated antibodies.
- The entire slide is not imaged, only the region of interest.
- Region of interest size is limited based on the time required to perform ablation.
- Imaged tissue is laser ablated and cannot be reused for subsequent applications.
- High-dimensional imaging requires advanced analysis tools to utilize all data to the greatest extent.

should rapidly increase given myriad advantages over tissue analysis methods (Table 1) and the ability to integrate within multiomics analysis pipelines. In this review, we describe the potential applications of IMC for dermatologic research and practice.

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

Table 1. Differences between IMC and IF

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

CyTOF, cytometry by time-of-flight; IF, immunofluorescence; IMC, imaging mass cytometry.
[a]Serial 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).

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

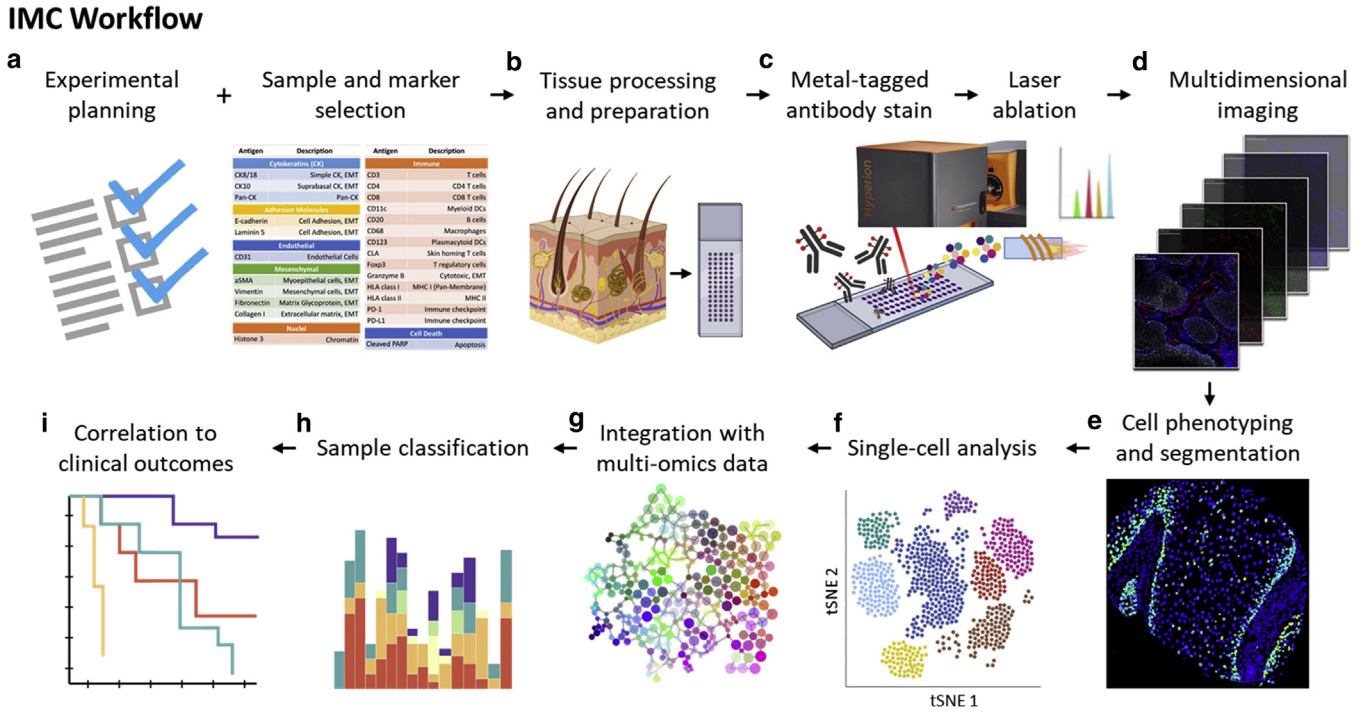

Figure 1. IMC Workflow. (**a**) After experimental planning and selection of appropriate samples and markers, staining of tissues of interest and appropriate controls are optimized. (**b**) FFPE or frozen tissues are prepared on glass slides and can be in the form of custom-built tissue microarrays composed 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.

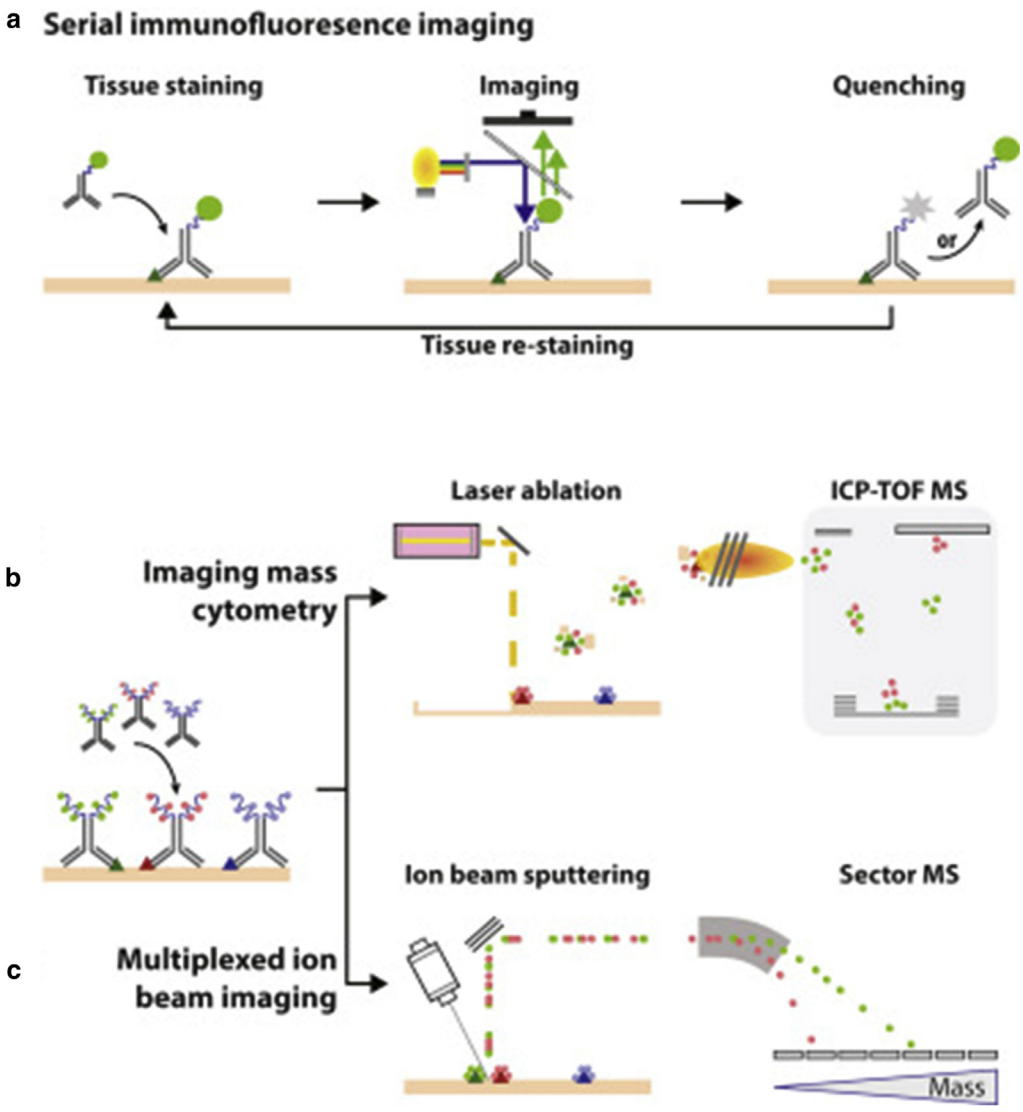

Figure 2. Immunofluorescence versus mass cytometry for highly multiplexed imaging. (**a**) Serial immunofluorescence imaging uses cycles of tissue staining and imaging with one or a few fluorescently labeled antibodies, quenching of the fluorescence by fluorochrome destruction or antibody removal, and restaining with additional antibodies. (**b**) In CyTOF imaging mass cytometry, a tissue is stained with dozens of metal-labeled antibodies simultaneously. A high-resolution laser ablation system is then used to transfer the tissue spot-by-spot into the CyTOF mass cytometer to determine metal isotope content and, therefore, epitope expression. (**c**) In multiplexed ion beam imaging, the tissue is stained with 10 metal-labeled antibodies simultaneously. A primary ion beam is used to raster over the tissue to generate secondary ions, among them the metal isotopes that were bound to the antibodies. A sector field mass spectrometer is then used to determine the metal isotope content and, therefore, epitope expression in each rastered area. CyTOF, cytometry by time-of-flight; ICP-TOF MS, inductively coupled plasma time-of-flight mass spectrometer; MS, mass spectrometer. Reprinted from (Bodenmiller, 2016) with permission from Elsevier.

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

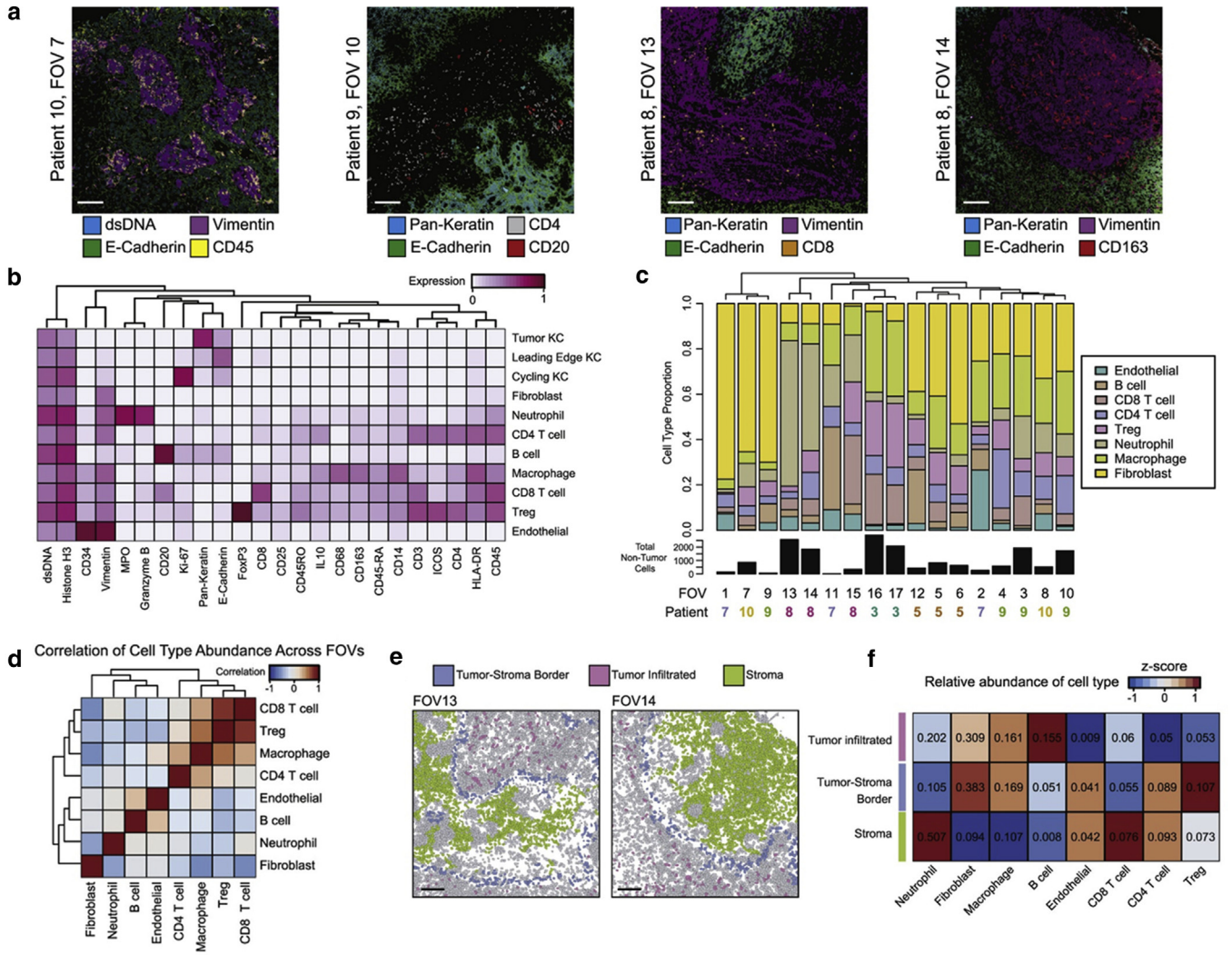

Figure 3. Spatial architecture of lymphocyte subsets in cSCC. (A) Select MIBI FOVs for patient samples with expression of highlighted features. (**b**) Heatmap of feature expression across cell types identified by MIBI. (**c**) Top, bar plots of proportion of nontumor cell types across all FOVs. Bottom, 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.

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

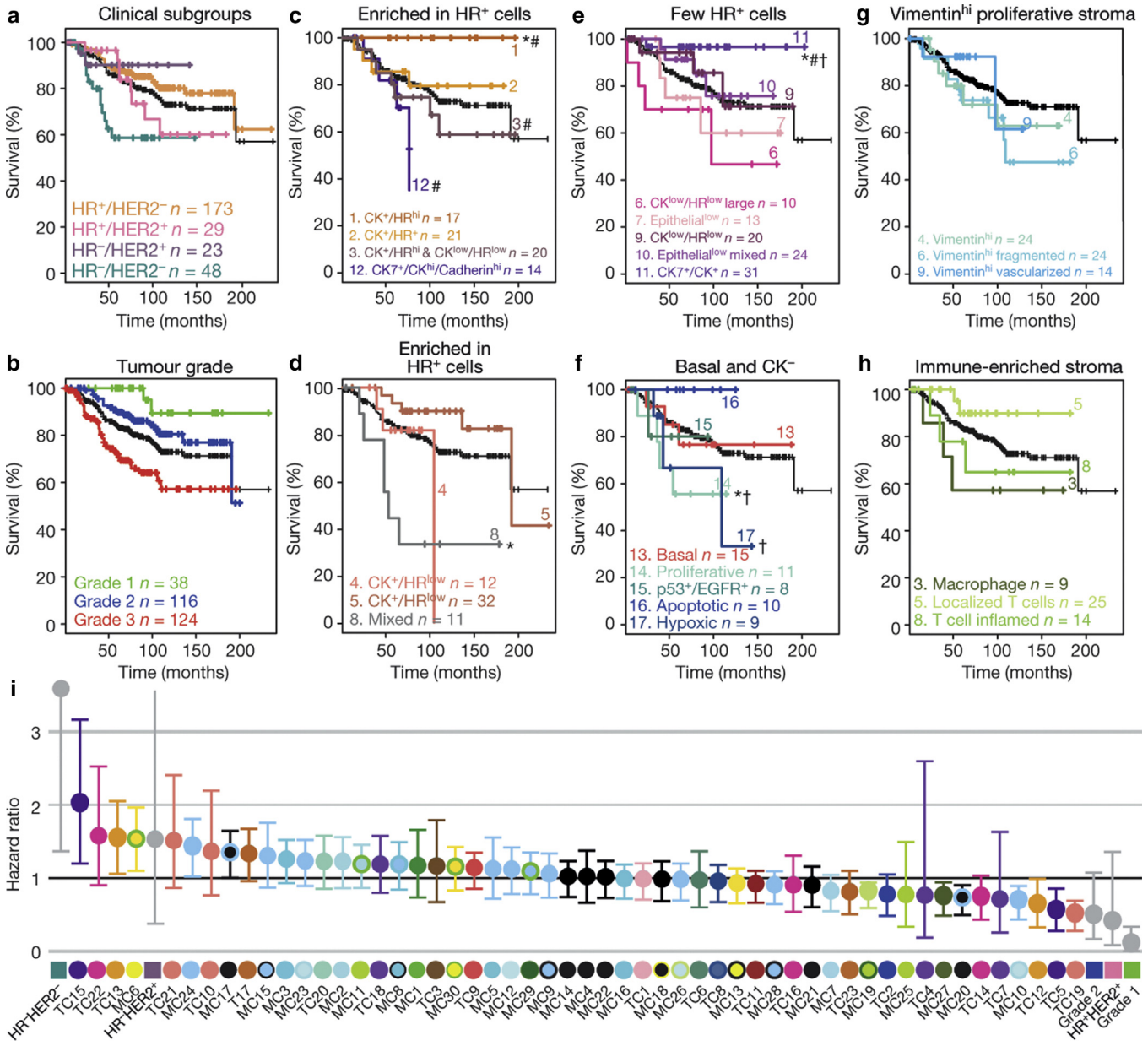

Figure 4. SCP subgroups have distinct clinical outcomes. (**a**–**h**) Kaplan–Meier curves of overall survival for each patient group (n = 278 patients in total) on the basis of (**a**) clinical subtype, (**b**) clinical grade, (**c**–**f**) SCP subgroup, or (**g, h**) stromal environment. $^{*}P < 0.05$ compared with all other samples, $^{\#}P < 0.05$ compared with similar subgroups, $^{\dagger}P < 0.05$ compared with other $HR^{+}HER2^{-}$ patients (log-rank test). (**i**) Relative hazard ratios and 95% confidence intervals of disease-specific overall survival for densities of tumor communities and microenvironment communities and clinical categories (molecular subtype and grade) estimated using a Cox proportional hazards model (n = 266 patients, reference group $HR^{+}HER2^{-}$ grade 3 for molecular subtypes and grades, n = 15 were excluded as only communities with fewer than 10 cells were identified in these patients). SCP, single-cell pathology. Reprinted from (Jackson et al., 2020) with permission from Springer Nature.

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

investigators and clinicians alike to pose a research question, select relevant tissues, perform analysis, and obtain meaningful results that can be correlated to patient-specific outcomes within a relatively brief time frame in comparison to lengthy prospective studies that would otherwise be required. Implementing IMC in this fashion will likely aid in expanding precision medicine throughout dermatology.

CONCLUSION

Although, to date, there have not been many studies utilizing IMC in investigative dermatology, its use will undoubtedly expand given its powerful capability to provide highly multiplexed spatially resolved imaging with single-cell resolution of banked clinical specimens. Furthermore, recent work has shown how IMC integrates with transcriptomics to enable unparalleled multiomics analysis that has begun to characterize previously unknown cell populations and interactions to shed light on disease pathogenesis. This approach facilitates the ability to capture the full potential of multiplexed imaging to investigate biologic complexities at both the tissue and cellular level. Thus, it is clear that spatially preserved, single-cell analysis via IMC is an exciting new histological imaging platform that can characterize tissue, cellular, and cellular community heterogeneity in a previously unfeasible manner, which will prove invaluable to deeply investigate cutaneous pathology. Resulting data have the potential to improve on predictive value for disease diagnosis, prognosis, or relapse and better inform patient-specific clinical decisions relative to current standards.

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CONFLICT OF INTEREST

The authors state no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: QSM, JV, LZ; Funding Acquisition: QSM, LZ, JV; Investigation: JV, PD, YY, DO, LZ, QSM; Methodology: JV, PD, YY, DO, LZ, QSM; Project Administration: JV, LZ, QSM; Visualization: JV, PD, LZ, QSM; Writing - Original Draft Preparation: JV, PD, QSM; Writing - Review and Editing: JV, PD, YY, DO, LZ, QSM.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.12.008.

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DETAILED ANSWERS

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.