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Mini review

Navigating the cellular landscape in tissue: Recent advances in defining the pathogenesis of human disease

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ABSTRACT

Over the past decade, our understanding of human diseases has rapidly grown from the rise of single-cell spatial biology. While conventional tissue imaging has focused on visualizing morphological features, the development of multiplex tissue imaging from fluorescence-based methods to DNA- and mass cytometry-based methods has allowed visualization of over 60 markers on a single tissue section. The advancement of spatial biology with a single-cell resolution has enabled the visualization of cell–cell interactions and the tissue microenvironment, a crucial part to understanding the mechanisms underlying pathogenesis. Alongside the development of extensive marker panels which can distinguish distinct cell phenotypes, multiplex tissue imaging has facilitated the analysis of high dimensional data to identify novel biomarkers and therapeutic targets, while considering the spatial context of the cellular environment. This mini-review provides an overview of the recent advancements in multiplex imaging technologies and examines how these methods have been used in exploring pathogenesis and biomarker discovery in cancer, autoimmune and infectious diseases.

Contents

Abbreviations: FFPE, Formalin-fixed paraffin-embedded; IHC, Immunohistochemistry; TSA, Tyramide signal amplification; HRP, Horseradish peroxidase; CODEX, Co-detection by indexing; DSP, Digital Spatial Profiler; FF, Fresh-frozen; ROI, Regions of interest; MCI, Mass Cytometry Imaging; IMC, Imaging Mass Cytometry; MIBI, Multiplexed Ion Beam Imaging; TOF, Time-of-flight; TME, Tumor microenvironments; HER2, Human epidermal growth factor receptor 2; PD-L1, Programmed death-ligand 1; IDO, Indoleamine 2,3-dioxygenase; PD1, Programmed cell death protein 1; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; FOXP3, Forkhead box P3; T1DM, Type 1 diabetes mellitus; MS, Multiple sclerosis; CNS, Central nervous system; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; IL-10, Interleukin-10; TNF-α, Tumor necrosis factor alpha.

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1. Introduction

Visualizing human tissue has become increasingly important in furthering our understanding of cellular mechanisms, pathogenesis, and treatment efficacy. In-depth analysis of the tissue landscape at a single-cell level is crucial in defining the pathogenesis of many diseases, where complex local cellular environments determine either the success or failure of the host response. Single-cell transcriptomics, using cell suspensions from dissociated tissue, has enabled the characterization of cellular processes and previously unidentified populations in both physiological and diseased states [1,2]. While able to capture the cell–cell variation present in tissue, single-cell transcriptomics is unable to provide the spatial context of cells [3,4]. Consequently, the scope of these studies may be limited as the native cellular environment of biological systems is not considered, omitting the functional roles from the spatial organization of tissue [5,6]. Recent advances in multiplex tissue imaging have tried to preserve the spatial context of tissues while retaining the depth of single-cell analysis, enabling the investigation of both cell–cell interactions and the spatial distribution of the cells in human tissues and organs [7,8]. However, as most clinical and archival samples are preserved as formalin-fixed paraffin-embedded (FFPE) tissue, this has limited the imaging technologies available that can overcome the inherent autofluorescence and damage to cell and tissue quality observed in FFPE tissue [9–11].

This mini-review will highlight how the development of major proteomic-based tissue imaging technologies over the past decade, ranging from multiplex immunofluorescence and mass cytometry to DNA barcoding methods, has deepened our understanding of human diseases.

2. Tissue imaging technologies

2.1. Sequential multiplex immunofluorescence

Multiplex immunofluorescence overcomes the limitations commonly associated with conventional immunohistochemistry (IHC), including the low label capacity and high inter-observer variability, by allowing simultaneous detection of multiple markers on a single tissue section and post-imaging digital analysis [12,13]. While several methods are available in multiplex immunofluorescence, multiplexing often presents challenges through the degradation of tissue architecture and epitopes after iterative stain cycles [14,15]. Tyramide signal amplification (TSA) is the core of many multiplex immunofluorescence technologies. As shown in Fig. 1, TSA methods consist of serial staining cycles where horseradish peroxidase (HRP) conjugated to secondary antibodies enzymatically catalyzes tyramide reactions, binding tyrosine-conjugated fluorophores near protein antigens of interest [8,15–18]. The subsequent covalent tyramide-tyrosine bond formed helps preserve fluorescent signals accumulating on the tissue while other non-covalently bound antibody pairs are removed by heat [15,16,19]. This allows the detection of markers with low expression and the use of primary antibodies from the same species, while still maintaining a high signal-to-noise ratio [16,17,19–21]. TSA is also more photostable compared to conventional immunofluorescence, which enables the long-term storage and re-imaging of the slides after the initial staining [22].

The most notable TSA method is the Opal Multiplex IHC assay (Akoya Biosciences), which can identify up to eight markers using FFPE tissue [15]. The Opal assay utilizes TSA-conjugated fluorophores which are covalently bound to tissue, and iterative rounds of antibody staining and stripping by heat-induced epitope retrieval, commonly performed in the microwave [21,23]. After the optimization of an Opal assay using single-colored controls and a spectral library, the resulting assay has the advantages of increased sensitivity, specificity, and compatibility with most fluorescent microscopes in research laboratories [16].

While the large-scale quantitative analysis and manual labor-intensive protocols are limiting in TSA-based technologies, the development of autostainers and automated protocols has allowed for consistent imaging of multiple whole tissue samples with quick turnover times [20]. However, these advancements in protocol automation do not address all the limitations. Tissue degradation from repeated heat-induced epitope retrievals required in TSA-based protocols and prolonged tissue exposure to chemicals required for multiplexing are particularly problematic when staining delicate samples or tissues with low cell density [8,14]. Furthermore, TSA does not recognize the intensity of antibody expression and is prone to false-positive staining from tyramide overreaction [12]. Fluorescence imaging is also limited by tissue autofluorescence and difficulties in assessing and recognizing tissue architecture, as opposed to imaging of conventionally stained tissue [21]. Ultimately, spectral overlap remains a barrier to marker capacity for panels from fluorescence-based multiplex imaging technologies.

2.2. DNA barcoding

To overcome the practical and spectral limitations of sequential immunofluorescence imaging, DNA-barcoded imaging probes have been developed. DNA barcoding utilizes antibodies conjugated with orthogonal DNA oligonucleotides (Fig. 1), which are detected by probes of complimentary single-stranded DNA often conjugated to a fluorescent dye [24]. Here we will focus on co-detection by indexing (CODEX), and nanoString Digital Spatial Profiler (DSP), two examples of DNA barcoding methods.

2.2.1. Co-detection by indexing (CODEX)

This technology incorporates DNA-conjugated antibodies with dye-labeled nucleotides for multiplex imaging [25]. In the first iteration of CODEX, cells are first stained with all tagged antibodies, where markers are iteratively revealed and imaged when exposed to a nucleotide mix, allowing the final multiplexed image to be reconstructed [25]. Notably, the recent commercialization of CODEX into the PhenoCycler by Akoya Biosciences has led to advancements in their workflow, which includes an automated microfluidic system for a single staining procedure capable of staining FFPE, fresh-frozen (FF) tissue, and single cells [26]. This advancement allows the quantification of up to 60 markers and streamlines the staining protocol in a single platform. The latest

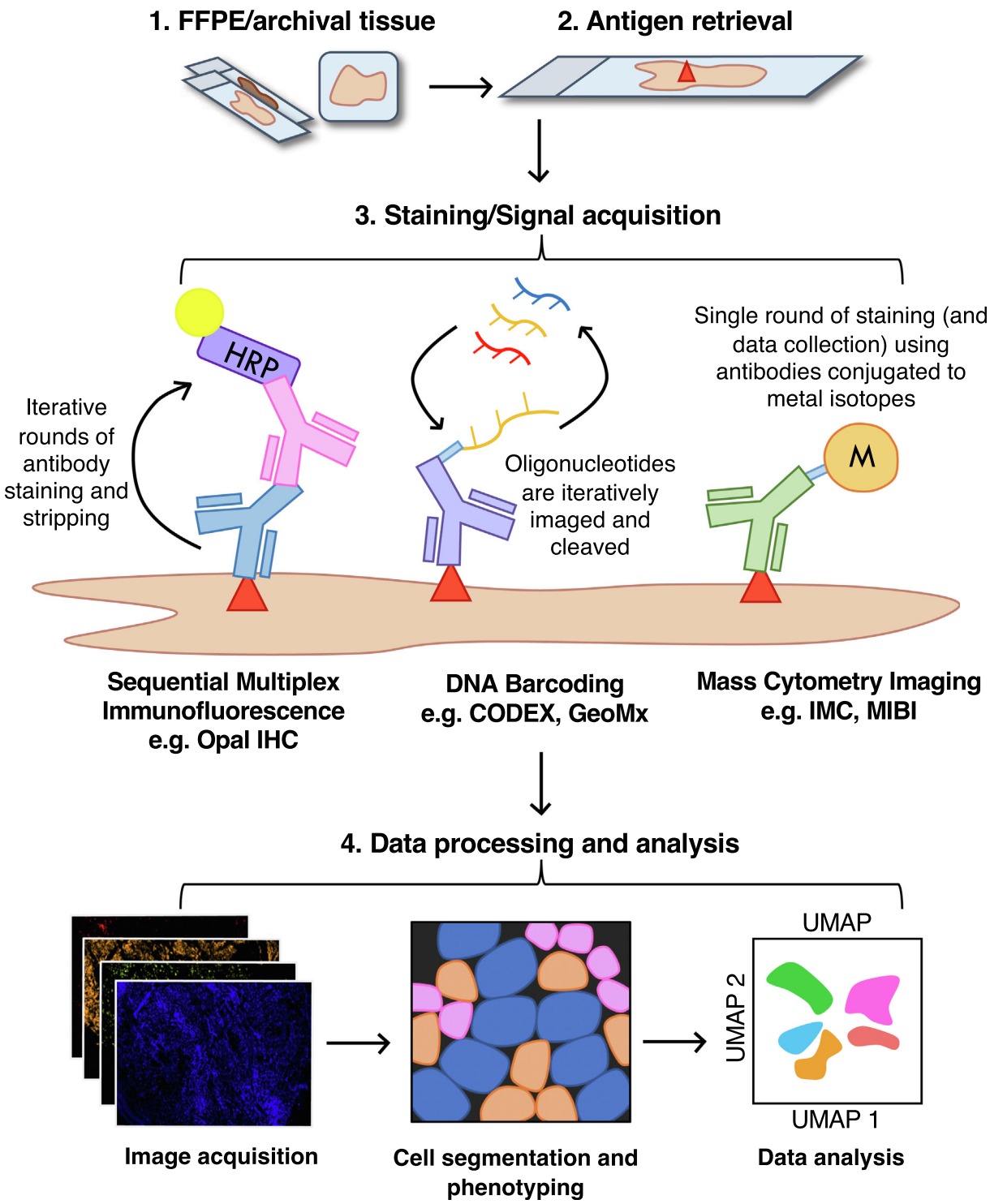

Fig. 1. Workflow for multiplex imaging technologies. Formalin-fixed paraffin-embedded (FFPE) tissue samples first undergo a round of antigen retrieval to prepare the tissue for staining. In sequential multiplex immunofluorescence, each marker is stained separately, where the fluorescent signal is amplified from the horseradish peroxidase (HRP)-tyramide reaction (left). DNA barcoding instead consists of a single staining step with antibodies conjugated to oligonucleotides. These oligonucleotides are detected, imaged, and then quantified to create the final multiplexed image (middle). Mass cytometry imaging also utilizes a single staining step with metal-conjugated antibodies. These metal isotopes (M) are ionized and detected by mass spectrometry, where the signal detected can be used to reconstruct the image (right). After staining, the images acquired from all imaging technologies follow a similar analytics pipeline including cell segmentation and phenotyping to further data analysis and visualization.

system, PhenoCycler-Fusion, has an integrated imaging platform for faster single-cell and tissue analysis, while increasing sample capacity [27]. This system is also compatible with RNAscope [28] to enable RNA detection alongside protein markers.

CODEX is advantageous due to its ease of use while minimizing spectral overlap and batch effects from the single antibody staining step [29]. The commercialized version of CODEX is compatible with many existing inverted fluorescent microscopes and does

not cause significant damage to the tissue sample. While CODEX panels have been validated for up to 56 antibody markers in lymphoma and colorectal cancer samples [30,31], over 100-plex panels have been developed for use with the PhenoCycler-Fusion system, enabling the spatial characterization of clinical samples with a single-cell resolution. CODEX has the capacity to increase the number of markers in antibody panels, only limited by the development of new, unique oligonucleotide sequences and long imaging acquisition times [26]. While CODEX overcomes many technological barriers in multiplex tissue imaging, the cost of CODEX-specific reagents is one of the primary limiting factors. Many of the antibodies validated and compatible with CODEX are more expensive than antibodies available for staining in FF tissue [26]. As CODEX increases the capacity for more markers in antibody panels, minimizing the speed of each imaging cycle to visualize the entire barcoded tissue remains an issue to be addressed [25].

2.2.2. GeoMx Digital Spatial Profiler

GeoMx Digital Spatial Profiler (DSP), a commercial system developed by nanoString, is an alternative method that allows the visualization of both mRNA and protein using oligonucleotide probes conjugated to either antibodies or RNA probes with a photocleavable UV linker [32,33]. The DSP platform also integrates fluorescent staining to identify morphological features and help select regions of interest (ROI). After the oligonucleotide incubation, the slides are scanned into the automated DSP system to select for ROI, where for each region the oligonucleotides are photoreleased and collected into a microtiter plate for quantification [32,33]. ROI selection and shape can be customized by the user with the DSP platform capable of imaging regions at sizes of 5 μm by 5 μm to 660 μm by 785 μm [34]. Currently, DSP has been validated for use with up to 44 proteins and 96 genes in lymphoid, colorectal tumor, and inflammatory bowel disease tissue and is compatible with both FFPE and FF tissue [33,34].

One of the primary advantages of DSP is the capability to profile both RNA and protein using targeted panels in the same tissue section multiple times for in-depth sequencing of only targets of interest with minimal damage to the tissue section [33,35]. Currently, over 300 antibodies have been validated for use with the DSP platform in addition to pre-designed reagent panels and custom-designed antibody or RNA probes [34]. Finally, the DSP platform is simple to use, automated, and does not require additional instrumentation to image, quantify and undertake spatial profiling [33,36]. However, DSP is restricted to analyzing information within each region of interest and cannot profile the complete slide, unlike CODEX and sequential immunofluorescence imaging [33]. While DSP can achieve a near single-cell resolution for some proteins, this cannot be achieved for all markers and relies on the sensitivity of each antibody or probe used [33,37]. Further development of DSP needs to improve processing capacity and reduce the current turn-around time of up to four days, which is usually lengthened by increased ROI size and complex profiling strategies [32,34]. Another platform recently developed by nanoString, the CosMx Spatial Molecular Imager, aims to overcome the issues with resolution and speed in DSP, enabling detection of both RNA and protein at a subcellular resolution [38].

2.3. Mass cytometry imaging technologies

Mass cytometry imaging (MCI) applies the principles of mass cytometry used for analyzing cells in suspensions to visualize FFPE and FF tissue samples using metal-conjugated antibodies (Fig. 1) [39,40]. Mass cytometry-based imaging technologies overcome many of the limitations in fluorescence-based methods, allowing the visualization of up to 40 markers [39,41,42]. Imaging Mass Cytometry (IMC) [42] and Multiplexed Ion Beam Imaging (MIBI) [41] are the two primary methods, only differing in how they ionize the metal isotopes for detection by the time-of-flight (TOF) mass spectrometer.

IMC detects molecular targets using metal-conjugated antibodies which are ionized in an ablation chamber by a UV laser, then measured and indexed by TOF mass spectrometry [40,42,43]. The speed and size of the laser ablation spots determine the resolution of the final image acquired, where a rastering speed of 200 pixels per second using a 1 μm laser spot size can image 1 mm^2 of tissue in under two hours [43].

Meanwhile, MIBI follows a similar principle but uses an alternative to a pulsed laser to vaporize the metal isotopes bound to the tissue [43]. Instead, a tunable oxygen primary ion beam is used to rasterize the tissue, ionizing metal isotopes while only ablating a thin layer of tissue [39,41,43]. This enables the tissue to be rescanned, unlike IMC [8]. Like IMC, MIBI is capable of imaging tissue sections of up to 1 mm^2, with the resolution of the final image reliant on the acquisition time [44]. However, in MIBI, the ion beam can be adjusted, allowing a higher resolution of as low as 260 nm to be scanned [39,44]. Detailed comparisons between IMC and MIBI have been previously reviewed [8,39].

MCI is advantageous over fluorescence-based imaging as it utilizes atomic mass in generating the tissue image, reducing endogenous autofluorescence and the need for amplification [42,45]. The subsequent low signal-to-noise ratio allows for high sensitivity with detection limits of approximately 50 copies of an epitope per pixel [46]. The use of heavy-metal isotopes ensures that each isotope has a unique atomic mass that can be easily identified and quantified, resolving issues related to spectral overlap common in fluorophore-based imaging technologies [46]. Currently, the largest panels validated for imaging diseased tissue using MCI consist of 30–40 markers [42,44,47–49]. However, MCI has the potential to expand and detect up to 100 markers depending on the discovery of new rare metal isotopes [39]. IMC can also be expanded to detect mRNA expression by conjugating RNAscope probes to heavy metal isotopes [28,40,50]. Currently, *in situ* detection of up to three mRNAs can be obtained simultaneously, allowing visualization of the relationships seen in and between mRNA and protein signaling networks [51].

Both IMC and MIBI rely on high-quality, purified antibodies for use with a TOF mass spectrometer to minimize non-specific signals from impurities, where most of these antibodies are only optimized for use in single-cell suspensions. Consequently, developing a high parameter panel is both expensive and time-consuming, requiring antibody validation and optimization [7,8,43,46]. In addition, the time-consuming nature of image acquisition limits the use of MCI for whole-slide imaging. Instead, MCI is usually reserved to analyze smaller ROI to ensure high spatial resolution while balancing the speed of image acquisition [43]. Manually selected ROI are vulnerable to visual and cognitive bias and may ignore spatial and cell–cell interactions that would otherwise be identified during whole-slide imaging [52]. Furthermore, the 1 μm^2 resolution used in IMC is still unable to accurately resolve and image subcellular organelles, limiting the ability to assign cellular phenotypes based on marker expression [40]. However, the recent adaptation of a positively charged cesium primary ion beam to MIBI has enabled the imaging of subcellular structures, achieving approximately 30 nm in the lateral resolution and 5 nm in the axial resolution [53].

3. Application of tissue imaging in human disease

3.1. Cancer

The demands for better biomarkers and a deeper understanding of mechanisms underlying successful immunotherapy have played

a pivotal role in spearheading the development of cutting-edge multiplex tissue imaging technologies [41,42]. These approaches have characterized tumor microenvironments (TME) with single-cell resolution and defined the local cellular response to immunotherapy [31,44,54,55]. In this regard, breast cancer tumor samples were explored in two original papers, which first described the utility of IMC and MIBI [41,42] in in-depth cell phenotyping of tissue, allowing the visualization of the cell–cell interactions, tissue architecture, and tumor heterogeneity.

The success of these studies helped establish the prognostic value of studying tumor heterogeneity and how characterizing TME can predict treatment and patient outcomes. One of the landmark studies [44] used MIBI with a 36-plex panel to identify TME in triple-negative breast cancer, a form of breast cancer with increased tumor-infiltrating lymphocytes and a lack of therapeutic breast cancer targets (estrogen receptor, progesterone receptor, and HER2). This study found that the spatial organization of immune infiltrates and immunoregulatory proteins were differentially expressed across patients and that PD-L1 and IDO were hallmarks of tumor compartmentalization, which could be associated with increased overall survival. This seminal study was important in forming a framework for visualizing and analyzing high parameter data, linking the cellular composition of tumor microenvironments to clinical cohorts with improved survival. Similarly, a recent CODEX study developed a 56-plex panel to analyze clinical cutaneous T cell lymphoma samples [30]. Expansion to a 56-marker panel with CODEX allows more extensive immunophenotyping by classifying cell types using multiple markers, especially important for cells with similar lineages, and visualization of cells with spatial context. An extensive panel is also important in enabling the detection of novel biomarkers and immunomodulatory proteins. This identifies the intra-tumor and patient heterogeneity present and allows a more accurate understanding of the cellular basis underlying the host responses to immunotherapy. Together, these studies establish a framework and validated panel for studying cell–cell interactions in TME and immunotherapy responsiveness in FFPE tumor samples [30,44].

Imaging technologies have also been employed clinically to characterize the changes in immune cells and checkpoints during immunotherapy [54,56]. Due to easier reagent and instrument availability, smaller 4- and 5-plex Opal panels have been used to perform immune profiling on melanoma patients treated with anti-PD1 monotherapy and combination therapy of anti-PD-1 and anti-CTLA-4 [56,57]. Responders to monotherapy and combined therapy had higher initial and final infiltrates of immune cells, consistent with previous studies describing the relationship between the TME and immunotherapy [30,56,58–60]. Additionally, while PD-1 and PD-L1 increased in responders, non-responders had significantly fewer infiltrates of activated T cells and regulatory FOXP3$^+$ T cells in tumors [56]. This has direct translational relevance to clinical oncology, as it allows the prediction of non-responders, which have low tumor-infiltrating lymphocytes, and enables early selection of combination therapy to maximize treatment efficacy.

Alternatively, larger IMC panels have been used to assess the changes in immune cell infiltration and immunomodulatory proteins in tumors after immunotherapy. An 18-plex panel showed that breast cancer tumors, expressing the extracellular domain of HER2, responded well to treatment with trastuzumab and were spatially associated with increased CD8$^+$ T cell infiltration [54]. Similarly, a 25-plex panel profiled melanoma tumors treated with immune checkpoint inhibitors, pembrolizumab, nivolumab, or a combination of ipilimumab and nivolumab [61]. The study observed that patients with increased survival had higher levels of CD8$^+$ T cell infiltration. In addition, higher beta2-microglobulin expression in tumors was associated with a better response to immunotherapy and may be a potential biomarker indicating immunotherapy responsiveness. While these IMC studies only profile ROI, they highlight how broad marker panels and subsequent spatial analysis can capture biomarkers indicative of successful immunotherapy, with potential for future routine use in predicting patient immunotherapy responsiveness.

3.2. Autoimmune diseases

3.2.1. Type 1 Diabetes

Imaging beta-cells, the main source of insulin, in pancreatic islets has been of interest in studying disease progression and the immunopathogenesis of type 1 diabetes mellitus (T1DM) [62]. Two IMC studies sought to characterize disease progression and immunopathogenesis of T1DM by using archival pancreas samples [47,49]. A 35-marker panel strived to elucidate the mechanisms behind beta-cell destruction and examined if proximity to immune cells and blood vessels correlated with increased destruction [49]. Surprisingly, they discovered that while cytotoxic and helper T cells are recruited to pancreatic islets before beta-cell destruction, the spatial analysis suggested that they were not directly involved as previously thought, exemplifying the importance of spatial information in understanding pathogenesis. In a separate study, a 33-marker panel quantified the structural and cellular changes in islets during T1DM progression [47]. They found that while cytotoxic and helper T cells were also found in proximity to beta-cell-containing islets, there was heterogeneity in the spatial distribution and proportions of immune cell infiltration within each sample and across patient samples. Together, both these studies help identify the changes in pancreatic tissue and islet cells for early detection of diabetes, uncovering the mechanisms behind pathogenesis.

More recently, another study has incorporated whole-slide imaging with multiplex tissue imaging, allowing analysis of the cellular heterogeneity of T1DM pancreas at an organ level [63]. Here, Opal fluorophores detected immune and endocrine cells for infiltration and spatial analysis of islets [63]. While this study does not utilize an extensive panel of markers, the incorporation of whole-slide imaging with QuPath [64], a machine learning-based image analysis platform, allows accurate and objective quantification of the structural and cellular changes in T1DM pancreas.

3.2.2. Multiple Sclerosis

While our knowledge of the pathogenesis and treatments for multiple sclerosis (MS) have advanced over the past decade, the immunopathology behind the initiation of disease and triggers of central nervous system (CNS) injury remains unknown [65]. The limited availability of high-quality samples of early and active disease has led to difficulties in characterizing this stage of disease [65]. Multiplex imaging technologies have enabled the study of immune and CNS cell interactions while simultaneously generating high parameter data from rare archival samples [66].

A 13-plex IMC panel of myeloid and glial activation markers mapped macrophage and astrocyte phenotypes in various lesional regions [67]. By comparing myeloid cell and astrocyte phenotypes in early and late MS lesions, the authors observed that in later stages of disease, spatial interactions between these cellular populations significantly increased alongside increased activated macrophages throughout perivascular spaces. This study uncovers the changes in the cellular spatial organization of pro-inflammatory phenotypes in the lesion rim and center throughout disease progression, highlighting the capability of IMC in collecting high-throughput data from limited tissue samples.

Another recent study [68] used a 15-plex IMC panel to characterize different lesional stages of MS brain tissue after treatment with natalizumab. This panel enabled the authors to pinpoint the

anatomic localization of multiple T and B cell populations, identifying immune cells with demyelinating activity in both normal-appearing white matter and peaking in the core of active lesion. The identification of cell–cell interactions with demyelinating macrophage phenotypes in white matter before obvious signs of demyelination, and near blood vessels and the border of active-inactive lesions is instrumental to improving our understanding of MS tissue injury and inflammation, an area still poorly understood.

Both these studies feature the analysis of high dimensional IMC data, highlighting the potential of multiplex tissue imaging in addressing the current knowledge gaps in the initiation and progression of MS, improving diagnosis and identification of therapeutic targets, especially with limited sample availability.

3.3. Infectious diseases

3.3.1. Tuberculosis

More recently, multiplex imaging technologies have been used to address the limited knowledge surrounding the cellular architecture of tuberculosis granulomas. It has been previously established that there is high lesion heterogeneity in mycobacterial granulomas, where lesion pathology reflects bacterial persistence, quality of the local immune response, and clinical outcomes [69,70]. A 37-plex MIBI panel consisting of immune cells, non-immune structural cells, and functional immunoregulatory markers imaged granulomas to identify hallmarks of active tuberculosis [71]. They identified a unique spatial relationship between PD-L1 and IDO-1, where elevated co-expression of these markers was associated with an immunosuppressive phenotype with a high bacterial burden. Hence, the application of MCI to profile tuberculosis granulomas has revealed new immunoregulatory pathways and can help direct the future design of host-directed therapies to improve outcomes for tuberculosis patients.

3.3.2. COVID-19

Multiplex tissue imaging has also been applied to analyze tissue response in COVID-19. The collection of high-parameter data has helped the in-depth characterization of tissue pathology in the novel disease through the analysis of the changing tissue structures and immune infiltrates [72]. Multiple studies using IMC, DSP and Opal IHC have characterized the immune response and spatial landscape of COVID-19 organ pathology [73–79].

To examine the multi-organ alterations caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a 27-marker IMC panel imaged the lungs, intestine, spleen, liver, and kidneys [75]. Analysis established that the lungs and intestine displayed strong immune responses, where increased infiltrate of $CD11b^+$ macrophages and $CD11c^+$ dendritic cells were observed. Intriguingly, elevated production of both the anti-inflammatory cytokine, IL-10, and pro-inflammatory cytokine, TNF-α, was observed in the two organs. This study lays the foundation for mapping COVID-related organ damage throughout the body and identifies immune signatures that need to be addressed when developing therapeutics.

A later study [73] undertook a more extensive 36-plex IMC study to define and compare the immune landscape of COVID-19 lung pathology to other respiratory infections, using a panel containing structural, immune cell, and functional markers. In addition to reporting increased macrophages and dendritic cells as disease progressed, the study revealed an increase in interactions between macrophages and fibroblasts, contributing to alveolar wall thickening observed in COVID-19 pathology. To further validate the findings, the study compared the changes in cellular composition from the IMC data to gene set signatures obtained from DSP, where similar increases in fibroblast and macrophage cell populations from the progression of early to late COVID-19 were observed. Here, this study outlines the immune landscape of COVID-19 relative to other respiratory diseases and highlights how a combination of imaging technologies can be used to validate trends observed.

Imaging technologies have also been used to elucidate the host determinants driving the increased severity of COVID-19 lung pathology. A 6-plex Opal panel profiled immune cells in COVID-19 lungs in comparison to healthy, uninfected lungs [79]. A significant increase in the frequency and density of $CD163^+$ monocytes and macrophages were observed in the alveolar spaces of infected lungs, while there was little change in lymphocyte and neutrophil populations. Importantly, a similar spatial arrangement was also found to be present in individuals with severe influenza, implicating that increased monocyte recruitment at alveolar spaces is associated with alveolar damage and COVID-19 severity and represents a common feature of acute respiratory viral infection. With further analysis in larger cohort sizes, future clinical investigations may direct effective therapeutic development to reduce monocyte recruitment and thus reduce disease severity.

These studies all highlight the power of multiplex imaging in identifying the cellular mechanisms controlling the pathophysiology and immunopathology of human diseases, and in guiding the development of future therapeutics.

4. Summary & outlook

While the high dimensional data obtained from multiplex tissue imaging enables phenotyping and analysis of tissue with a single-cell resolution, this leads to an inevitable computational challenge. A robust and objective workflow for imaging data analysis and visualization still needs to be established, especially if these methods are developed for clinical use [18,80,81]. Robust and standardized workflows from panel design and imaging to data acquisition are fundamental in ensuring these results are valid and are reproducible across different tissue samples and studies [7,22]. As summarized in Fig. 1, most laboratories have adopted a similar analytics pipeline consisting of single-cell segmentation, cell phenotyping, dimensionality reduction, spatial analysis, and data visualization [26,41,44,74,82]. However, both analysis and reporting of tissue imaging studies have yet to be standardized. The development of more user-friendly, open-source analysis software, such as QuPath [64], is required to support and streamline this process [13,83]. Finally, as many of these imaging studies form part of early-stage discoveries of biomarkers and therapeutic targets, it is unknown how well these findings translate clinically.

The power of the multiplex imaging technologies has only just been uncovered, expanding from the well-established use of studying TME in cancer to studying the pathogenesis of autoimmune and infectious diseases. Our knowledge of the mechanisms behind immunotherapy and local cellular environments in disease is evolving alongside the technological advances in multiplex imaging technologies, which are increasing their marker capacity, speed, and resolution. While multiplex imaging technologies are still limited in panel size compared to spatial transcriptomics, this is rapidly being addressed by the continuing development of MCI and DNA-barcoding methods to feasibly increase the number of molecular targets in panels [82]. Nevertheless, multiplex tissue imaging will certainly have clinical utility in the future, cementing the importance of spatial biology in our understanding of human diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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