Mucosal immunology revisited through mass cytometry : from biology to bioinformatics and back

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CHAPTER 7

Summarising Discussion

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Definition of a distinct immune subset

It is increasingly recognized that in order to gain further insights into human disorders and develop new therapeutic strategies and diagnostic tools, it is critical to have a comprehensive overview of immune cell subsets resident in tissues under physiological and pathological conditions. Flow cytometry has been the golden standard for analyzing immune cell subsets, and with a typical experiment dozens of immune subsets can be discriminated. Due to the lack of spectrally-resolvable fluorochromes, mass cytometry utilizing metal-conjugated antibodies has shown to be a powerful tool for dissecting the immune landscape even further[1]. It currently allows for the simultaneous measurement of up to 40 markers, three times as many as flow cytometry. While most mass cytometry studies have so far focused on in-depth analysis of one immune lineage[1], we choose a broad coverage approach by analyzing across all immune lineages simultaneously. We applied this to intestinal samples obtained from controls and patients with intestinal diseases, celiac disease and inflammatory bowel disease (IBD) in particular. While the role of several immune subsets in driving intestinal pathology had been studied for various intestinal diseases[2, 3], this broad and system-wide approach revealed previously unappreciated heterogeneity in the mucosal immune system and provided evidence for tissue- and disease-specific immune signatures.

Indeed, mass cytometry and other high-dimensional, single-cell analysis techniques have greatly increased the number of phenotypically distinct cell subsets within the immune system. In our first study on gastrointestinal disorders (**Chapter 2**) we were able to distinguish 142 distinct immune subsets using a 28-antibody panel, and in our second study focusing on IBD (**Chapter 5**) we distinguished 309 distinct immune subsets in the intestine alone using a 36-antibody panel. These findings have raised obvious questions about the true distinctiveness and function of such cell subsets, and concerns on the definition of a "subset". Our view is that a subset is a set of similar cells displaying a distinct marker expression pattern based on the complete mass cytometry-antibody panel, where unsupervised computer-generated subset definition is manually checked for validity. Indeed, many of them could be defined as the same immune cell population displaying different levels of activation or maturation stages. To uncover potential relationships between immune subsets,

we exploited the ability of Cytosplore (**Chapter 3**) to visualize the evolution of the t-SNE map (**Chapter 6**), a pathway analysis. These results demonstrated several potential differentiation trajectories of innate lymphoid cell (ILC) subsets in the human fetal intestine, confirmed by *in vitro* experiments. This suggests that these subsets are indeed interconnected. Nonetheless, the biological significance of a large number of immune subsets we identified needs to be investigated in future studies.

Remaining challenges for data analysis

Up to recently, analysis of flow cytometry data was mainly performed with gating strategies based on primarily bimodal expression patterns. Mass cytometry data analysis, however, requires computational tools to distil this large body of data into interpretable forms. The high-dimensionality, large size, and non-linear structure of the data poses considerable challenges. Dimensionality reduction-based techniques like t-SNE offer single-cell resolution and is one of the leading techniques for data visualization and clustering. However, three major caveats to the t-SNE method were that it lacked interactivity, yields incomplete density-based clustering, and, most importantly, was limited by the number of cells that can be analyzed. Therefore, the existing dimension reduction techniques were not optimal for mass cytometry data. Through fruitful collaborations with computer scientists from Technical University Delft, we succeeded to solve these issues. We developed Cytosplore (**Chapter 3**), an interactive visual analysis system where we incorporated state-of-the-art clustering and t-SNE-based techniques, for the efficient data-driven specification of phenotypically distinct subsets in cytometry data. It provides a highly engaging user-friendly experience by providing direct feedback and linked views, and is coupled to clinical parameters allowing rapid identification and visualization of patient-specific features. In addition, the inclusion of the HSNE algorithm (**Chapter 4**) allows the exploration of millions of cells without the need for downsampling the data. What makes HSNE particularly useful is that it preserves local data structure while allowing examination of the full dataset at single-cell resolution. This application can be used not only for complex mass cytometry datasets, but also for standard 12-parameter flow cytometry datasets. Therefore, Cytosplore allows us to go beyond data sizes currently possible to handle with other tools, a useful development considering expected increases in acquisition rate and dimensionality in mass- and flow cytometry[4, 5].

Further analysis improvements are still needed to exploit the full potential of mass cytometry. Methods that can quantify cellular heterogeneity, identify critical cell subset features and assign biological identity to computer-identified subsets

will be particularly useful. For the latter two, a recent report has taken the first steps into this direction. Diggins *et al.* presented marker enrichment modeling[6], an algorithm that objectively describes cell subsets by quantifying contextual marker enrichment. This provides a standardized language to annotate the key distinguishing features of immune subsets identified in cytometry data.

Moreover, despite the vast amounts of data generated by the mass cytometry community, increasing exponentially each year (**Figure 1**), there has hardly been any comparisons of datasets as of yet. This is a serious shortcoming as much more can be learned from these experiments. To accomplish this, a public mass cytometry data repository needs to be established through international efforts, with the aim of data integration. How to shape this? One of the challenges is the antibody panel composition designed specifically for each study. Different datasets can only be matched based on the presence of overlapping markers, where the accuracy of matching is proportional to the number of shared markers in the panels. Although thousands of immune-system-wide markers are available, generally the core immune markers providing phenotypic information are confined to a much smaller number. Also, recently Fluidigm started providing standardized antibody panel kits, such as the 29-antibody cocktail specifically designed for human immune monitoring. Therefore, I anticipate that many independent mass cytometry studies (will) share a sufficient number of markers for comparative dataset analysis. Machine-learning and classification methods need to be developed to impute marker expression profiles and assess cell subset similarities between mass cytometry studies. This will enable meta-analyses across multiple mass cytometry experiments, revealing differences and commonalities in cellular profiles between different types of immune-mediated diseases.

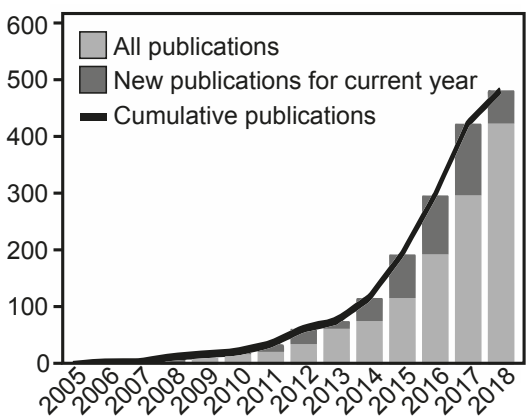

Figure 1. Number of cumulative mass cytometry publications. Quantification of number of papers as of June 2018. Adapted from https://www.fluidigm.com/publications

Unique individual fingerprint of the immune system

In our first study (**Chapter 2**), the peripheral blood and intestinal samples from six patients that were biopsied twice, with a 3- to 6-months time interval, clustered tightly together in the data analysis. When comparing the immune cell composition of intestinal biopsies from the same individual but at different intestinal location or inflammatory state, the large interindividual variation as compared to intraindividual variation was striking (**Chapter 5**). In several cases, samples derived from the same individual clustered together. Indeed, cell compositions and other immune markers have been described to be different across individuals by several reports investigating peripheral blood[7, 8, 9, 10]. These studies demonstrate that the heterogeneity in the immune system increases with age, largely driven by non-inheritable factors. In addition, it was observed that collective sets of immune cell frequencies could predict diverse functional responses[7, 8], suggesting that the composition of an individual's immune system reflects a functional network of cell subsets and that the balance between these cell subsets determines the overall responsiveness of the immune system. In agreement, our results confirm the presence of a unique individual 'fingerprint' in immune cell composition in the periphery. In addition, we have provided evidence that the collective immune cell composition in the intestine is also individual-specific, emphasizing the need for personalized care.

Mass cytometry as an application in clinical medicine

The heterogeneous nature and suboptimal clinical response to treatment observed in gastrointestinal and other immune-mediated disorders highlight the need for improved strategies and personalized patient care. Mass cytometry has shown great promise in identifying immune profiles that associate with disease. So how far is mass cytometry as an application in clinical medicine? Recently, clinical studies implementing mass cytometry have been performed to investigate immunological aspects that underlie clinical outcomes[11, 12], help monitor disease progression[13], and to predict responses to therapy[14, 15]. As a direct diagnostic tool, mass cytometry might be rendered unsuitable due to the currently relative low sample throughput and high cost. Rather, mass cytometry is more often applied as a discovery tool. In this thesis, we have mainly investigated diagnostic specimens from untreated patients with gastrointestinal diseases, mapping immune compositions in health and disease (**Chapter 2** and **Chapter 5**). An important next step would be to design a longitudinal study where the effect of treatment (such as anti-TNF) in IBD could be examined, to identify immunological parameters that correlate with therapeutic responses.

An important consideration in the design of a longitudinal study employing mass cytometry is the need for standardizing experimental procedures thoroughly. This is especially important because of the required unbiased data analysis. Currently, mass cytometry cell acquisitions are accompanied with calibration beads for data normalization between experiments, enabling correction of signal fluctuations[16]. Further standardization might include multiplexing clinical samples into one processing tube using (live-cell) barcoding[17, 18], stained with a pre-aliqouted antibody cocktail (stable at -80 °C), combined with a consistent internal control sample for normalization of staining variability between experiments.

Results of clinical studies utilizing mass cytometry might help establish minimal sets of measurements critical in immune monitoring and personalized patient care. These could either be translated to high-throughput and cost-effective technologies, such as flow cytometry. Alternatively, if mass cytometry continues to develop further, the identification of disease-associated changes in immune composition may potentially lead to the development of unbiased diagnostic procedures based on a single mass cytometric analysis.

Rare cell subsets matter

The study of rare cell populations is of growing importance in diagnostics and therapeutics. Specifically, the detection of circulating tumor cells[19], tumor stem cells[20], endothelial cells[21], hematopoietic stem cells[22], HIV-infected cells[23], invariant NK-T cells[24], fetal cells[25], ILCs[26], antigen-specific T cells[27], and the monitoring of minimal residual disease[28] provides valuable clinical information. The term "rare" typically refers to cell counts with a frequency of 0.01% or less[29]. It is challenging to accurately identify rare cells due to the requirement of many phenotypic markers, large data sizes and limitations of pre-existing computational tools. Often, rare cells had been mistaken for noise by clustering algorithms or lost in the downstream analysis due to the necessity of downsampling the data. In fact, we have demonstrated that downsampling introduces a potential bias in observed heterogeneity in the immune system, affecting mainly rare cells (**Chapter 4**). We have shown that the HSNE method (**Chapter 4**) Is superior to other single-cell analysis methods in identifying rare cell populations in mass cytometry data. Two features make HSNE particularly suitable for this: (1) The preservation of nonlinearity of the data, key for separating distinct rare cells from abundant cell populations. (2) Allowing the examination of the full dataset at single-cell resolution, providing increased detail and confidence in establishing the phenotypes of rare cells.

Our analysis on rare cells in the human fetal intestine resulted in the identification of a previously unidentified innate cell population, termed int-ILCs (**Chapter 6**). These cells have the capacity to differentiate into NK cells and ILC3-like cells *in vitro*. We speculate that this cell population provides plasticity in the intestine in response to external stimuli. Interestingly, we identified the same cells in intestinal ileum biopsies of children and adults associated with an unaffected-IBD and control profile (**Chapter 5**), while effector NK cells and ILC3s were more readily detectable in affected-IBD specimens. It is tempting to speculate that under inflammatory conditions the int-ILC may differentiate into these effector cell types in the intestine. This also illustrates that research on the physiological and early-life development of the mucosal immune system improves our understanding of factors that may contribute to derailment of disease-associated immune responses.

Moreover, our results have indicated that disease-specific immune subsets reside mainly in the affected organ and are much less readily detectable in peripheral blood (**Chapter 2**). However, upon reanalyzing the dataset using HSNE (**Chapter 4**), we were able to identify a rare population of $CD28^-$ effector memory $CD4^+$ T cells specifically in blood of some Crohn's disease patients, which was missed in the original analysis (**Chapter 2**) due to downsampling. This suggests that rare intestinal disease-associated $CD4^+$ T cells can be detected in peripheral blood. In addition, we identified a $CD4^+$ T cell effector memory population distinguished by the expression of HLA-DR and CD38 while lacking CD27 and CD127 specifically in the intestine of 17 out of 29 IBD patients (**Chapter 5**). Together with previous observations[30, 31], there is strong evidence that in a subgroup of IBD patients the $CD4^+$ T cell population is altered during inflammation, and possibly detectable in blood. This might present an opportunity to circumvent invasive gastrointestinal endoscopy for disease monitoring, but requires further investigations. Therefore, the identification of circulating, rare immune subsets offers possibilities to determine cellular parameters that correlate with disease.

Assessing antigen-specificity

Celiac disease is an intestinal autoimmune disease driven by dietary gluten and gluten-specific $CD4^+$ T cell responses. We have identified a celiac disease-associated immune composition in the small intestine, mainly characterized by the presence of distinctive TCR$\gamma\delta$ subsets and $CD8^+$ T cell subsets while lacking certain innate lymphocyte subsets (**Chapter 2**). Following oral gluten challenge, concomitant with gluten-specific $CD4^+$ T cells, the appearance of activated, gut-homing $CD8^+$ T cells

and TCRγδ cells in peripheral blood of celiac patients was detected in a recent study[32]. However, it is currently unclear where the gluten-specific $CD4^+$ T cells reside in the high-dimensional immune landscape in the intestine we described. Possibly within the $PD\text{-}1^+CD161^+$ $CD4^+$ T cell effector memory subset as this subset was increased in numbers in celiac biopsies compared with control (**Chapter 2**). As mentioned above, the $CD4^+$ T cell compartment also appears to be implicated in IBD (**Chapter 5**), but unlike celiac disease the potential causative antigens in IBD are yet to be identified.

Newell *et al.* developed an elegant method that allows the simultaneous identification of many antigen-specific T cells, by combining mass cytometry with combinatorial peptide-MHC tetramers[33]. Using this method, they succeeded to screen for 109 different tetramers while retaining 23 metal channels to analyze other phenotypic or functional markers in a single sample. In addition, such an approach revealed that virus-specific $CD8^+$ T cells occupied distinct niches of phenotypic and functional diversity[34]. However, these analyses focused on $CD8^+$ T cells which require the more stable MHC class I tetramer reagents, while the development of MHC class II tetramers required for analyzing antigen-specific $CD4^+$ T cells is more complicated. Combining mass cytometry with tetramers has a great potential in providing a comprehensive analysis of specific T cell responses, distinguishing antigen-specific T cells from bystander T cells, and might be a useful application to study inflammatory intestinal diseases.

Functionality of immune subsets

Functionality of immune cells relates to their gene and protein expression profiles. As mentioned above, studies have shown that cellular immune compositions could predict functional responses[7, 8]. In contrast, a study on acute myeloid leukemia revealed that although surface and signaling phenotypes of immune cells displayed tight coregulation in healthy samples, this was not the case in leukemia[35]. This raises the question to what extent the surface phenotypes of immune subsets serve as proxies of cellular state and function in intestinal disorders. We have mainly focused on characterizing the immune cell landscape in health and disease (**Chapter 2** and **Chapter 5**), not its functionality. There are several ways how mass cytometry can be utilized for assessing immune cell function. For example, once immune subsets of interest have been discovered, a minimal marker gating strategy can be devised, either supervised or automated, to specifically identify these subsets using flow cytometry. This allows for the purification of viable cells of these subsets for further *in vitro* analysis. We have applied this approach to study the functionality of various ILC subsets in the human fetal intestine (**Chapter 6**), assessing their

cytokine production profiles and differentiation potential. Alternatively, mass cytometry can also be used directly for the analysis of functional states of the immune system[36, 37, 38, 39]. Due to the availability of many channels, mass cytometry permits the combination of multiple phenotypic surface markers with multiple functional intracellular markers, under unstimulated and stimulated conditions.

An elegant study by Bodenmiller *et al.* demonstrated the power of applying multiplexed mass cytometry for the profiling of cellular states perturbed by small molecule regulators[39]. By dedicating 7 metals to cellular barcoding they could multiplex an entire 96-well plate, characterizing immune cell signaling dynamics and the effects of 27 inhibitors on this system. For each inhibitor, they could measure 14 phosphorylation sites in 14 cell types at 96 conditions, resulting in 18,816 quantified phosphorylation levels from each multiplexed sample. Therefore, this analysis allows the high-throughput characterization of cell type selectivity and responsiveness to a given stimulation or drug for dozens of cell types simultaneously. The cellular responsiveness can either be measured by analyzing the major signaling pathways or production of effector molecules, such as cytokines. Now that we have profiled the immune system with a 36-surface antibody panel in IBD **(Chapter 5**), it would be interesting to determine the minimal set of markers required to preserve most of the disease-associated immune heterogeneity. This would make space in the panel for building in several intracellular signaling or cytokine markers to investigate the functionality of these disease-associated subsets. A major challenge would be the determination of the minimal number of cells required per condition for a reliable read-out using this assay. With the Helios-upgraded CyTOF system, approximately 50,000 live immune cells can be detected from 2 intestinal biopsies in total, while in the original study about 200,000 cells per well was used[39]. If feasible, this assay may provide a unique opportunity to screen for the functionality of the immune subsets implicated in IBD with a high-dimensional approach. It might also be an attractive method for drug screening of immune cells from clinical biopsies, to categorize drug effects or drug combinations, to eventually guide personalized therapeutic strategies in IBD.

The architecture of the tissue microenvironment in health and diseases

Many types of cells must cooperate in tissues in order to mount an inflammatory immune response. Therefore, we choose a broad coverage approach by analyzing across all immune lineages simultaneously. Results of the gastrointestinal studies we employed (**Chapter 2** and **Chapter 5**) elucidated that underlying the identified disease-associated profiles, the differentiating subsets were often a combination of

both the innate and the adaptive immune compartment. This raises the question whether these disease-specific cells are interacting with each other in the tissue. However, with conventional mass cytometry we analyze single-cell suspensions and, therefore, lose the spatial context. Many studies of intestinal immunology have not taken into account that the intestine comprises several anatomically defined segments that each have distinct physiological roles and immunological components[40]. A promising technological development is imaging-mass cytometry, a combination of mass cytometry with laser ablation that allows the simultaneous analysis of over 30 markers on tissue sections with subcellular resolution[41, 42].

We performed preliminary experiments using imaging-mass cytometry with a 15-antibody panel on sections from the fetal intestine (**Figure 2**). Prior to ablation, the tissue section can be examined by a camera allowing the identification of regions of interest, such as the intestinal villi (**Figure 2A**). After data acquisition, the expression of a few individual markers can be visualized with classic coloring overlays (**Figure 2B**). The resolution of imaging-mass cytometry is comparable with standard immunofluorescence, but unhampered by autofluorescence. Coloring overlays of individual markers, however, is not capable of visualizing over 4 markers simultaneously. To comprehensively visualize all markers simultaneously at 1 μM pixel resolution, we exploit the ability of HSNE (**Chapter 4**) of allowing the high-dimensional analysis of millions of data points, followed by clustering of similar pixels. Subsequently, the pixels are projected back onto the original image, but now color-coded according to the HSNE clustering (**Figure 2C**). Using this approach we obtain an immediate, reconstructed overview of the architecture of the intestine in a complete data-driven fashion. This analysis demonstrates the presence of a tissue organization between T cells, ILCs and myeloid cells in the fetal intestine, and this will be investigated further in the future. Furthermore, we are currently working on integrating the imaging-mass cytometry analysis over several layers, from the pixel level to the cellular level to the tissue level, each interacting with another.

This provides a unique opportunity to comprehensively determine the cellular neighborhood of disease-associated immune subsets in the tissue-specific microenvironment. Ultimately, a three-dimensional architecture of the intestine could be reconstructed where the microenvironment of immune cells composed of structural proteins and stromal cells could be visualized and analyzed interactively, providing a more integrated view of disease-specific changes.

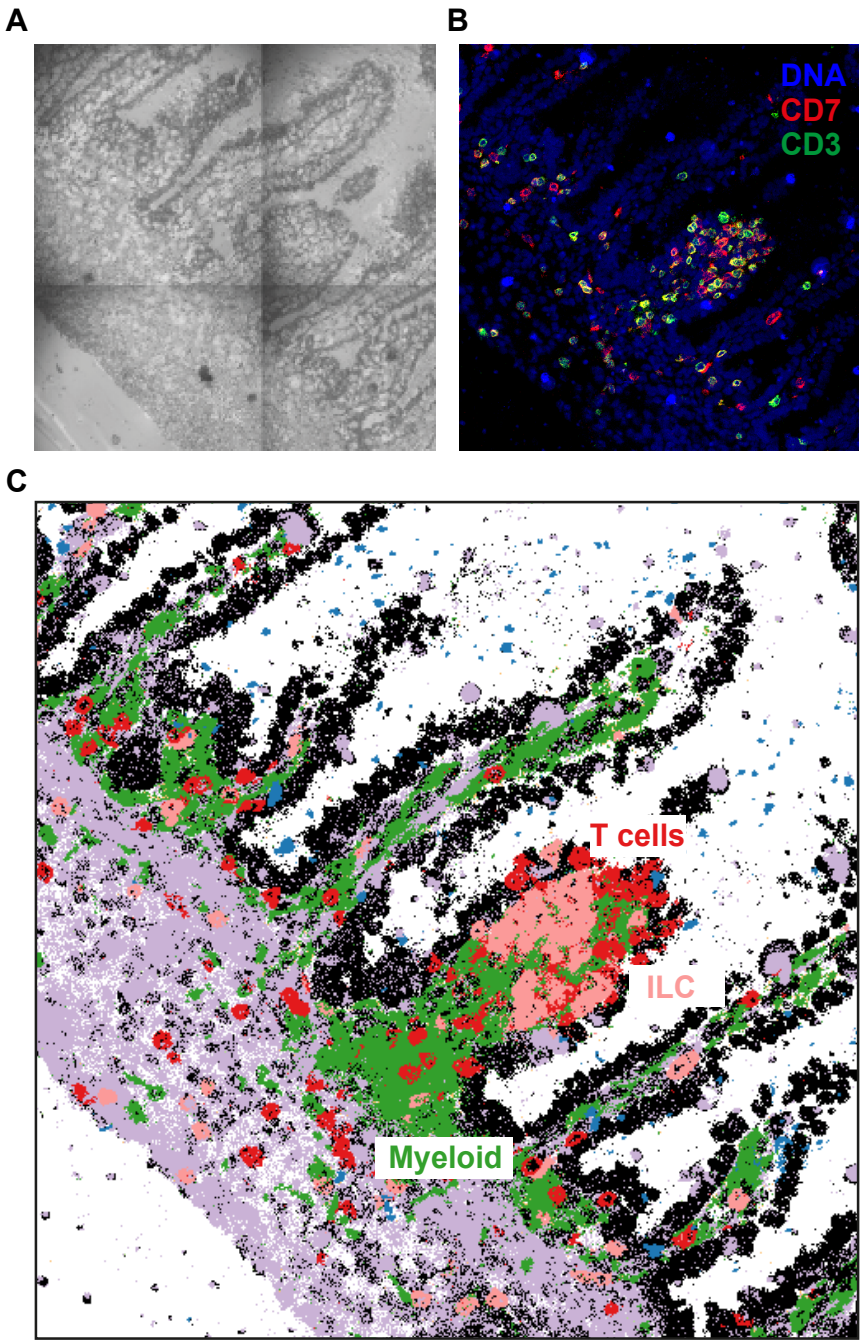

Figure 2. Imaging-mass cytometry on the human fetal intestine. (**A**) A camera showing regions of the tissue section prior to ablation. (**B**) Color overlays for the markers DNA (blue), CD7 (red), and CD3 (green). (**C**) HSNE analysis of the single-pixel data projected back onto the image. Color represents HSNE pixel clusters, indicating epitelium (black), nerves (purple), IgM^+ cells (blue), T cells (red), ILCs (pink) and myeloid cells (green)

Systems Immunology

Moving forward, it will be important to investigate the immune system by integrating mass cytometry with other technologies, such as single-cell transcriptomics, proteomics, metabolomics, T cell and antibody repertoire sequencing, multi-parameter imaging and cytokine bead-based multiplex assays. Such an approach, also known as 'Systems Immunology'[43], will enable us to gain insight into how different components of the immune system function and interact in the tissue context in health and disease. Additionally, It would be of great value to correlate this with the composition of the microbiota, since multiple profiling studies have described characteristic shifts in the microbiota composition associated with the pathogenesis of IBD[44]. Moreover, single-cell RNA sequencing of samples prior to CyTOF antibody panel design might aid in the identification of unanticipated candidate markers in an unbiased manner. A great barrier to implementing systems immunology successfully, however, is the requirement of a highly-skilled multi-disciplinary team. Collaborations between clinicians, technology experts, bioinformaticians and immunologists are key for such an undertaking. Combining these technologies will offer new opportunities to define a comprehensive landscape of the immune system and provide a framework for understanding the essential features of an immune response that may correlate with useful clinical outcomes.

Concluding remarks

Inflammatory intestinal diseases can only be understood by studying specialized cell types within the tissue niche itself. Mass cytometry and data-driven, automated analysis approaches have proven to be a powerful approach to investigate complex compositions of heterogeneous cell subsets, such as those encountered in intestinal biopsies. Future improvements on interactions between datasets and integrations with other –omics technologies, such as imaging, are required to exploit the full potential of mass cytometry.These types of data have the potential to greatly improve our understanding on human disease. In concert with clinical data, mass cytometry could enable a finer classification of patients, and might aid in the development of improved diagnostics, prognostics and personalized therapeutic regimens.

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