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HIGHLIGHTS

Basic

REVIEW

The end of omics? High dimensional single cell analysis in precision medicine

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High-dimensional single-cell (HDcyto) technologies, such as mass cytometry (CyTOF) and flow cytometry, are the key techniques that hold a great promise for deciphering complex biological processes. During the last decade, we witnessed an exponential increase of novel HDcyto technologies that are able to deliver an in-depth profiling in different settings, such as various autoimmune diseases and cancer. The concurrent advance of custom data-mining algorithms has provided a rich substrate for the development of novel tools in translational medicine research. HDcyto technologies have been successfully used to investigate cellular cues driving pathophysiological conditions, and to identify disease-specific signatures that may serve as diagnostic biomarkers or therapeutic targets. These technologies now also offer the possibility to describe a complete cellular environment, providing unanticipated insights into human biology. In this review, we present an update on the current cutting-edge HDcyto technologies and their applications, which are going to be fundamental in providing further insights into human immunology and pathophysiology of various diseases. Importantly, we further provide an overview of the main algorithms currently available for data mining, together with the conceptual workflow for high-dimensional cytometric data handling and analysis. Overall, this review aims to be a handy overview for immunologists on how to design, develop and read HDcyto data.

Keywords: bioinformatic tools · diagnostic biomarkers · flow cytometry · mass cytometry (CyTOF) · single cell analysis

In recent years, we witnessed an exponential growth in high-dimensional (HD) technologies, which resulted in a wide range of medical [1–11] and biological [12–14] applications. HD cytometers such as mass cytometry (Cytometry by time of flight, CyTOF) and flow cytometry (hereafter termed HDcyto) can detect more than 40 parameters (with the promise for detection of up to 100 parameters) with single-cell resolution. The data produced by HDcyto techniques together with appropriate computational methods of analysis have initiated a new era for translational medicine, enabling the unsupervised discovery of new cell populations, disease signatures as well as biomarkers of response to therapeutics in several pathologies, such as autoimmune diseases and cancer.

Single-cell RNA sequencing (scRNAseq) also provides a high-throughput platform for deep cell profiling, as reviewed elsewhere [15]. The number of transcripts detected, and the single-cell resolution are, however, burdened, today by high costs that strongly reduces the number of cells that can be analyzed (usually in the order of 10^2–10^3). In addition to this, pre-enrichment of cells samples is often required, therefore preventing the analysis

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of other potentially important cell subsets that might be present in the original sample. The analysis of cross-sectional cohorts of clinical samples is often exceedingly demanding, in terms of cost and time needed to acquire the samples. Conversely, well-designed HDcyto panels allow the measurement of fewer but more characterizing antigens on higher amounts of cell and most importantly, larger cohorts.

Compared to conventional "omics" approaches, such as bulk transcriptomics and proteomics, where high throughput performances are often hampered by a disadvantageous signal-to-noise ratio and lack of resolution at the cellular level [16], HDcyto technologies provide an opportunity to better describe the complex cellular population dynamics that regulate biological phenomena. The generation of scientific data from the integrated analysis of biological samples from different cohorts allows a detailed analysis of the different markers measured by HDcyto. The data obtained can then be integrated with clinical metadata such as age, sex, disease-scores, routine serology screens, and therapy plans, thus providing a valuable contribution for bridging cutting-edge technologies to tailored medicine [8, 17, 18].

New technologies in high-dimensional cytometry

In 1965, Fulwyler reported the discovery of an electronic device capable of separating erythrocytes from leukocytes based on their respective cell volume via their optical properties [19]. Since then, our ability to interrogate cellular heterogeneity through the means of tagged antibodies has dramatically expanded our understanding of biological processes, and revolutionized immunology research. To fulfill the demands for a higher dimensionality, conventional cytometer technologies have been implemented by expanding the range of emission and detection of wave light (e.g. PMT based FACS; Spectral Analyzer), the inclusion of means for cell morphology detection (e.g. Imaging flow cytometry, cyclic immunofluorescence), and novel strategies that combine features of flow-cytometry and mass-spectrometry (e.g. mass cytometry; Imaging mass cytometry) (Table 1).

Photomultiplier tube (PMT)-based flow cytometers

New generation photomultiplier tubes (PMTs)-based flow cytometers have currently integrated excitation lasers for newly designed fluorophores, therefore covering thus far untapped absorption spectra [20]. Moreover, up to 30 parameters can be detected with single cell resolution [21]. The complex spectra that derives from relatively broad emission of dyes requires, however, an adequate expertise in panel design as well as labor-intensive signal compensation of spectral overlap (Brummelmann et al, in revision). Conversely, spectral analyzer cytometers utilize an elegant approach to circumvent these hurdles by dispersing the entire emitted fluorescence signal according to the respective wavelength using either prisms or gratings, granting a higher photon throughput, and a better resolution respectively [22]. Consequently, the entire fluorescence spectrum is captured by a linear array of charge-coupled device or PMT detectors, and spectral unmixing algorithms deconvolute the signal [22]. Without the need for further compensation, algorithms are able to discriminate antibody-mediated fluorescence signals from autofluorescence, making spectral cytometry especially suitable for cell suspensions derived from tissues like heart, brain or intestines that have to deal with high autofluorescence [23].

Imaging cytometry

Other technologies aimed to implement dimensionality by recording expression data in even subcellular resolution and morphological features. For instance, imaging flow cytometry allows the simultaneous imaging in the bright field, dark field, and up to ten fluorescence channels, thereby offering morphological and spatial information of each individual cell [24]. This principle makes imaging flow cytometry a rewarding approach for diagnostic settings that hold cellular morphological changes, and might be used for the clinical diagnosis of hematological malignancies [25]. On the other hand, slide-based cytometry (Chip cytometry or Cyclic immunofluorescence) immobilizes histologic sections or cell suspension on a chip instead of passing them through a flow. The iterative loops of staining-acquisition-bleaching of the employed fluorophores enable a theoretically unlimited number of markers that can be detected [26]. The applicability of highly multiplexed assays, the possibility to store cryopreserved samples for up to 20 months, and the opportunity to re-stain for subsequent analysis has advantages in clinical settings when invasive procedure are needed to collect samples and material is limited (e.g. analysis of cerebrospinal fluid, or tissue biopsies) [27]. The caveats of such technologies include: (i) problems to detect antigens requiring incompatible antigen retrieval strategies, (ii) decreasing antigen stability after multiple bleaching rounds, and (iii) the time-consuming acquisition process.

CyTOF

Fusing the fundamental principles of mass spectrometry and flow cells led to the mass cytometry platform, also termed cytometry by time-of-flight (CyTOF). Antibodies labeled with stable metal isotopes prevents spectral overlap issues (albeit some level of compensation might be still required [28]). Limitations in labeling chemistry restrict today the amount of parameters measured per cell to up to 50 to date [29, 30], which is still sufficient to apply barcoding strategies (crucial in mass cytometry for the exclusion of doublets and reducing inter-experimental variability) while preserving high-sensitivity detection channels [31–33]. Importantly, once combined, all samples are stained and acquired simultaneously, virtually eliminating technical sample to sample variations and batch effects. These characteristics are particularly suitable for automated high-dimensional analysis of large sample groups, in which even rare immune subsets can be identified [32]. Further development of this platform aimed to embed the

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Table 1. Overview of described technologies methods and their coverage of the typical cytometry data analysis workflow

Feature	Flow cytometry	Spectral analyzers	Imaging flow cytometry	Cyclic immuno fluorescence	Mass cytometry	Imaging mass cytometry
Theoretical number of detectable parameters	50	64	10	Unlimited	135	135
Actual number of parameters	30 [21]	32 [64]	10 [65]	60 [66]	50 [30]	32 [67]
Morphological information	Detection of cell size and granularity	Detection of cell size and granularity	Includes subcellular information	Includes subcellular information	None	Includes subcellular information
Acquisition speed	10 000 cells/second	10 000 cells/second	5000 cells/second	Time intensive iterative approach	500 cells/second	14h/mm^2
Need for compensation	Yes	Spectral unmixing algorithms	Pixel by pixel compensation	No	Ongoing debate [28]	Ongoing debate [28]
Key advantages	Fast, reproducible, established	No autofluorescence and spillover	Valuable morphological and subcellular information	Highly multiplexed, sensitive, long-term storage of samples	High-dimensionality, barcoding excludes technical variations	High-dimensional informative of tissue samples
Key drawbacks	Spectral overlap, cumbersome panel design	Not compatible with every fluorophores, Difficult panel design	Low dimensionality, difficult compensation	Low throughput, time-intensive staining	Low acquisition speed, destructive approach	Challenging analysis, low acquisition speed
Best suited application	Routine assays, clinical diagnostics	Highly autofluorescent tissue [23]	Detection of hematological malignancies [25]	Precious clinical samples with low cell numbers [27]	Unbiased biomarker and disease signature discovery [7]	Interrogate spatial intercellular interactions in cancer tissue [4]

dimensionality of CyTOF with spatial resolution of imaging technologies. By staining, laser ablation and acquisition of tissue section, imaging mass cytometry currently allows the detection of more than 30 specificities [4, 34].

Unambiguously, more than 50 years of continuous development since the first flow cytometer led to a versatile platform for the detailed investigation of cellular phenotypes. The responsibility of modern cytometry is to define computational workflows that allow the translation of high-dimensional phenotypic single-cell profiles into clinically meaningful outcomes. In the following sections we will outline some of the most successful study designs and how basic research and clinical medicine can benefit from the embracement of data science and cutting-edge cytometry technologies.

Identification of molecular signatures and disease-associated phenotypes

One of the most prominent application of HDcyto technology is the investigation of human pathologies, as it allows a deep understanding of inter-cellular perturbations and the identification of disease-specific signatures that may serve as diagnostic biomarkers or, eventually, therapeutic targets. HDcyto has already proved to be a powerful platform for the investigation of idiopathic syndromes and pathologies with incomplete understanding of disease-driving mechanisms [7, 35–39].

Mass cytometric immune profiling of autoimmune diseases revealed pathophysiological modes of action which provide the rationale for new therapeutic strategies, otherwise overlooked [35, 36, 40]. A combination of mass cytometry and machine learning detected a pro-inflammatory monocyte signature in pediatric and adult systemic lupus erythematosus (SLE) patients which was reverted upon blockade of interferon (IFN)/JAK pathway in an in-vitro setting [35, 40]. Likewise, an enhanced responsiveness to IFN-γ has been identified in T-helper and classical monocytes of polyarticular juvenile idiopathic arthritis [17], encouraging the use of Jak inhibitors for the treatment of autoimmune disease entities with an increased IFN signature.

Another application of HDcyto is the identification of blood biomarkers for disease severity. Primary Sjögren's syndrome is a prototype of chronic autoimmune pathology with organ

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inflammation and systemic disease. Mass cytometry analysis of blood and glandular tissue was instrumental to the identification of a 6-cell disease signature present in blood which correlated to inflammatory activity in the tissue, therefore offering a potential diagnostic alternative to invasive biopsies [37]. Similarly, HDcyto showed alterations in distinct clusters of mucosal-associated T cells, NK cells and B cells in peripheral blood associated with chronic graft-versus-host-disease (cGVHD) severity [38]. Importantly, these exploratory results were then also confirmed by low-dimensional flow cytometry, indicating that similar findings not only are reproducible, but that they might be employed on a larger scale for clinical diagnostic purposes. Further studies in this direction hold significant promise to find biomarkers that facilitate diagnosis, help in therapeutic decisions and reduce the need for invasive procedures in diagnosis consolidation [41]. Not only blood, but also tissue samples or other liquid biopsies (e.g. bronchoalveolar lavage) have been subjected to multiparameter profiling by mass cytometry and automated data analysis. Distinct monocyte/macrophage and granulocyte cell populations distinguished asthmatic patients from cystic fibrosis patients and healthy controls. The method proved to be especially useful for dissecting a heterogeneous condition like asthma into groups with different immunological characteristics which, might reflect differences in disease pathophysiologies and could help to make individualized therapeutic decisions in the future [39].

Finally, unbiased algorithm-guided analysis of HDcyto data has revealed unanticipated immune signatures of diseases with incomplete understanding of pathophysiological mechanisms. Narcolepsy, a rare neurological sleep disorder, has been considered an autoimmune disease due to its high HLA-association, but compelling evidence had been lacking until Hartmann et al discovered a disease-associated T cell activation and cytokine signature in blood [42]. The disturbances in the immune system of narcolepsy patients have later been found to –at least in part– be based on the antigenic history of patients T cells [43]. Based on these benchmarking results further studies can delineate the contribution of identified cell phenotypes or functional states to disease and evaluate their potential as therapeutical targets.

Monitoring biomarkers for individualized patient stratification and response to treatment

More recently, the use of HDcyto, together with computational tools, highlighted the translational relevance of HD techniques in the precise identification of biomarkers of response to therapy [5, 8, 11, 38]. A recent study showed how HDcyto can predict the response to anti-PD1 (a monoclonal antibody that targets an inhibitory receptors on activated/exhausted T cells) treatment in different cohorts of patients with melanoma, with crucial implications in terms of patient stratification and precision medicine [8]. HD analysis strategy [8, 44] allowed correlating the $CD14^{+}$ CD16-HLA-DR^{hi} population (classically associated with peripheral blood monocytes), with the clinical response to the treatment to anti-PD1 in patients with metastatic melanoma. Through the systematic bioinformatic analysis and the exhaustive characterization of the peripheral blood cell populations of treated patients, they observed that a higher frequency of classical monocytes in the blood correlates with a greater disease-free survival and better overall survival, proposing these cells as a new predictive biomarker of response to treatment of metastatic melanoma. It is interesting to note that the immunological mechanisms underlying this correlation have not been elucidated, thus demonstrating that this type of analysis can generate new hints to study multiple unsolved biological processes, previously unimaginable.

In a different study, Bengsch et al. performed an elegant epigenetic-guided mass cytometry approach to define core T cell exhaustion-specific genes and clusters with expression patterns [45]. Combinatorial co-expression of phenotypic and transcription factors identified nine distinct CD8 exhausted T cell clusters, which were differently represented in healthy controls, cancer and HIV patients. These results hinted for a role in HD profiling of $CD8^{+}$ T cell exhaustion to monitor disease progression or response to therapy. A similar study by Brummelman et al. [18] shows how HD single cell analysis of $CD8^{+}$ T cells from patients with lung cancer were enriched in tumors compared with cancer-free tissues and blood. HD analysis strategy permitted to find variations in the frequency of $CD8^{+}$ T cells $CD69^{+}$ $CXCR5^{+}$ (with characteristics of stem-like cytotoxic $CD8^{+}$ T cells) prevalent within early (Stage I) versus late stage (StageII-IVA) disease in samples of patients with lung carcinoma; therefore, that the abundance of $CXCR5^{+}$ $CD8^{+}$ T cells in the tumor negatively correlates with disease progression.

In synthesis, HDcyto has a strong potential for immunomonitoring and identificationof novel biomarkers, such as new cell populations, which are of relevance for individualized response to treatment and the stratification of patients.

Characterization of tissue-specific atlas for the investigation of local pathology

The unique features of HDcyto do not only apply to the investigation of liquid biopsies in pathological conditions, but also to the depiction of cellular heterogeneity *per se*, revealing new details on developmental paths and functions of immune populations [12, 46, 47].

Using mass cytometry, Wong et al., performed a thorough characterization of lymphocytes across eight non-lymphoid and lymphoid human tissues [48]. By analyzing the functional and trafficking markers together with cytokines expression profile, the authors found tissue- and cell type-specific signatures of distinct resident T cell subpopulations (TRM). Similarly, using multiparameter flow cytometric approach, Thome et al., described differential features of T cells in pediatric versus adult human blood, lymphoid and mucosal tissues [49]. Specifically, they reported differences in frequency and activation status of T cell subpopulations (including TRM cells) demonstrating an early differentiation and regulation in T-cell compartment.

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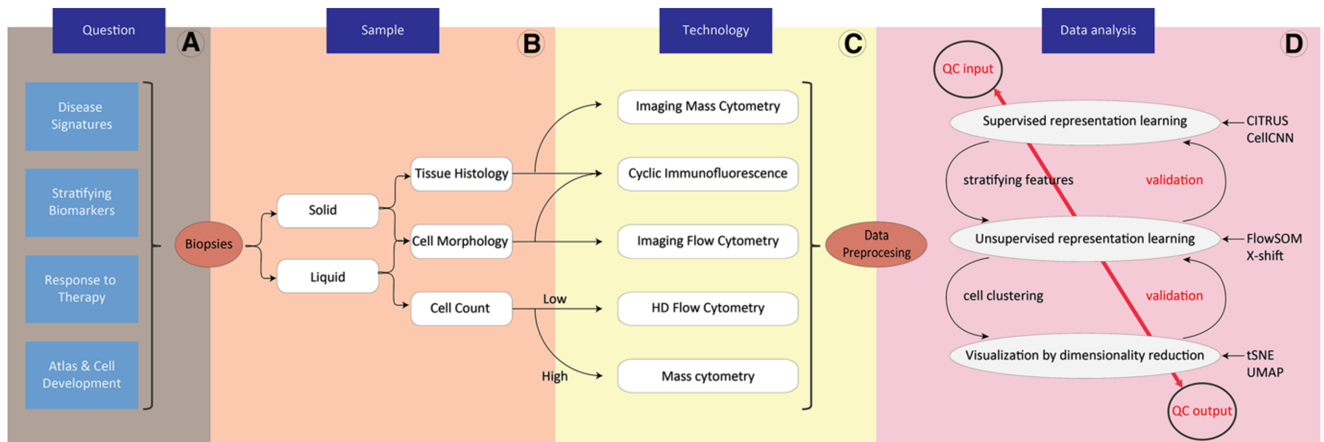

Figure 1. Overview of HDcyto experimental design with clinical samples. (A) Example of questions that can be addressed by HDcyto. Both factors, (B) the sample characteristics, and (C) the most suitable HDcyto technology will help selecting the algorithm to choose lX, (D) Schematic design of integrated data analysis . QC = data quality control.

The classification of innate lymphoid subsets (ILCs) and dendritic cells (DCs) in human would have not been possible without HD technologies, due to their complex and largely overlapping phenotypical characteristics. The simultaneous measurement of phenotypic molecules, cytokines and transcription factors across mucosal and non-mucosal tissues in healthy and diseased individuals identified distinct subsets of ILC2, ILC3 subsets and intraepithelial (ie)ILC1-like cells. Interestingly, HD profile of (ie)ILC1-like cells and NK cells shared expression of few surface antigens and cytokines, suggesting NK cells plasticity under influence of tissue environment [50]. Conversely, HDcyto expanded the taxonomy of DCs in different tissues, highlighting a heterogeneity within plasmacytoid and conventional DCs which include both precursors as well as mature DC [13, 51].

HDcyto expanded also our knowledge of human immunology and provided a unique resource to study the role of leukocytes in pathological conditions; for instance, the characterization of tumor microenvironment (TME). One of the first immune landscapes of tumors were described in clear cell renal cell carcinoma (ccRCC) and lung adenocarcinoma [2, 52]. In both studies researchers analyzed the TME of lesions and normal tissue using mass cytometry, IHC/IF techniques and single cell RNA sequencing data. The authors identified different T cell subpopulations in ccRCC and lung adenocarcinoma lesions through automated data analysis [2, 52]. Each T cell subpopulation was characterized by the expression of immunomodulatory receptors (PD-1, 4-1BB, ICOS, CTLA-4, TIM-3, LAG-3 and others) and activation markers (CD38, HLA-DR). Chevrier et al., associated for the first time CD38 expression with cells exhaustion [2]. Lavin et al., further analyzed the functional status and cytokine production in T cells [52]. They showed that lung adenocarcinoma lesions had lower expression of granzyme B and IFN-γ among cytolytic $CD8^+$ T cells and NK cells, and a significant expansion of regulatory T cells (Tregs) compared to healthy lung tissue. Another indicative population of lung adenocarcinoma TME were NK cells, which showed reduction in frequency and cytolytic activity (low expression of granzyme B, IFN-γ and CD57).

Besides the characterization of lymphocytes, Chevrier et al., focused on describing the myeloid compartment of TME. Authors identified 17 subtypes of tumor-associated macrophages in ccRCC lesions and displayed clusters on the diffusion map to emphasize transition conditions in developmental paths. Additionally, distinguished macrophage populations were associated with clinical outcome of ccRCC [2]. The role of myeloid cells in lung adenocarcinoma TME is crucial as well, as it was shown that $PPAR\gamma^{hi}$ macrophages and DCs were associated with pathology [52]. There are several studies that implemented HDcyto and made it "the technique of choice" in the investigation of clinical samples [53–55].

New algorithms for high-dimensional data mining

In the previous sections we provided an overview over technological advancement in HDcyto and the different fields of development in human research. The increased usage and success of high throughput technologies would not have been possible without the concurrent advance of bioinformatic platforms that provide computational tools required for the deconvolution and interpretation of an otherwise intelligible set of results. Currently, we can identify a threefold effort in the development of innovative algorithms for data visualization, classification of cell population based on similarity, and the unbiased identification of stratifying signatures (Fig. 1).

Most of the currently available tools for data visualization rely on dimensionality reduction, which is similar to a principle component analysis. One popular way to visualize HDcyto data is obtained by t-distributed stochastic neighbor embedding (t-SNE) [56, 57], which is now implemented in different platforms (e.g. R, MatLab, Pyton). This algorithm applies a non-linear reduction that accounts for pair-wise similarity among all cells. This allows capture of the overall measurement-informativity and project it in a 2/3-dimensional plot. Albeit implementations of

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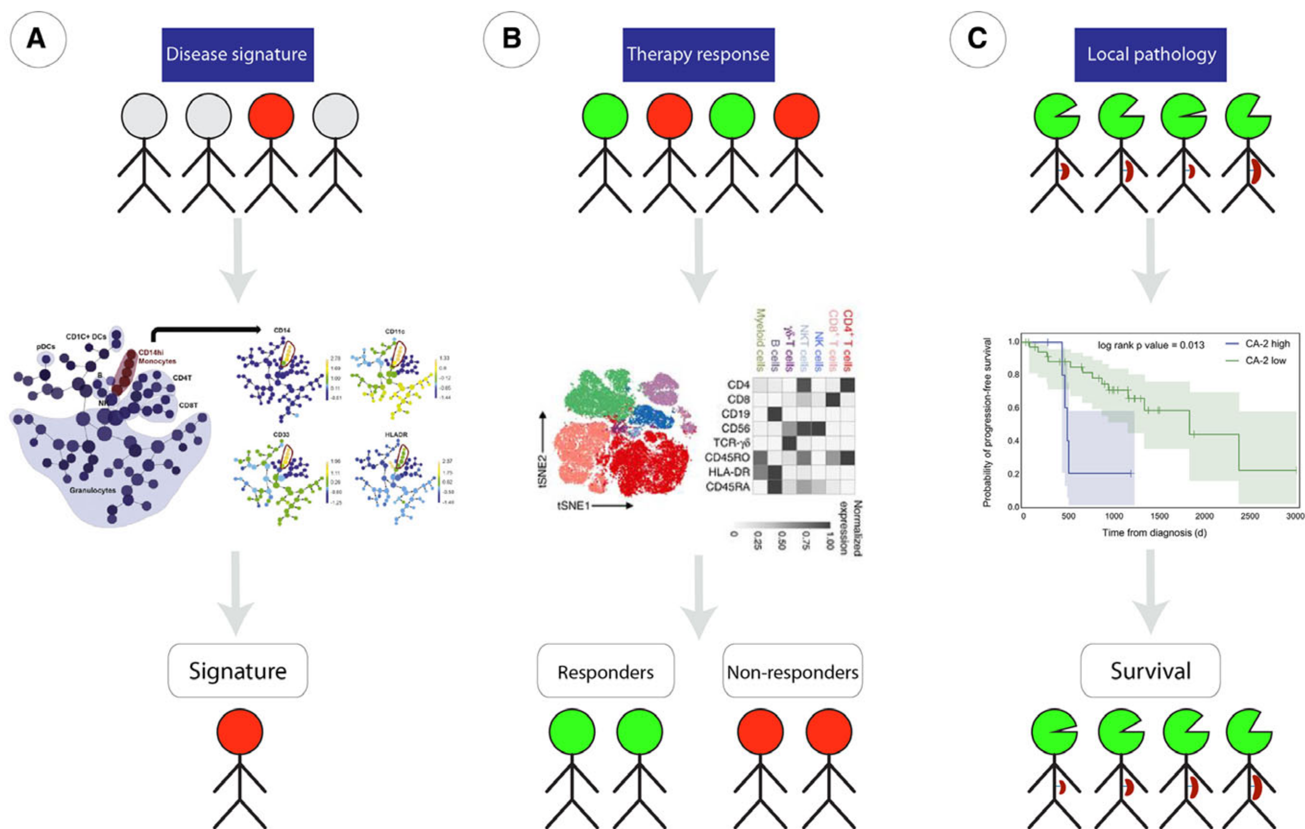

Figure 2. Overview of different clinical questions addressed using HDcyto approaches. (A) *O'Gorman et al.* [40] used supervised machine learning to identify a pro-inflammatory monocyte signature characteristic of SLE patients (B) *Krieg et al.* [8] made use of a systematic immune profiling to identify a subset of CD14highHLA-DRhigh monocyte that allows the stratification of melanoma patient upon checkpoint-inhibitor therapy (C) *Chevrier et al.* [2] found an association between a specific immune asset and the disease survival in patients with clear cell renal cell carcinoma by the HDcyto investigation of tumor infiltrating cells.

the algorithm allows now multi-core processing (R package *multicore.R.tsne*), t-SNE are still amenable of long computational time, which often requires even important subsampling of the entire dataset. Implemented in Python, the uniform manifold approximation and projection (UMAP) algorithm offers a valuable alternative to t-SNE, given the reduced computational time, similar embedding properties and the possibility of including additional data on existing paths [58].

Data visualization is not devoid of complex parameterization, which can be visually misleading if a rigorous approach is not used. For this reason, unsupervised representation learning algorithms, such as FlowSOM and X-shift, provide a better alternative for the unbiased classification of cell-clusters. Numbers of different algorithms have been developed to capture the heterogeneity of different cell populations, and reviewed elsewhere [59]. More recently, the power of neural network and self-organizing maps (SOM) were exploited to develop FlowSOM [60]. All analyzed cells are iteratively assigned to 100 nodes of the SOM, which are then arranged in a minimal-spanning tree. While a hierarchical metaclustering function is already implemented, cluster stability and possible overfitting should be further assessed using consensus matrices and the elbow point of k over cluster numbers. Another powerful tool, X-shift, has been developed to combine an innovative clustering algorithm together with cutting-edge visualization resources [61]. This stand-alone application is based on t-weighted k-nearest-neighbor (kNN) density estimation to define clustering-centroids, which can then be visualized through the graphical interface via nested divisive marker trees as well as force-directed layouts.

Newly developed supervised machine learning offers now the opportunity to directly interrogate large datasets for stratifying features based on classifiers. Several algorithms are now available for this purpose, with more and more development and features. Cluster identification, characterization, and regression (CITRUS) [62] is now also implemented in different platforms such as Cytobank. While graphical user interfaces are very handy, they are amenable of lack of flexibility (e.g. data preprocessing is hampered, lack of control over preclustering). Recently, CellCNN has overcome this limitation by building associative filters on-the-fly without preclustering steps, therefore identifying even rarer subsets of stratifying cells [63].

Overall, data visualization and clustering algorithms represent potent tools to explore HDcyto data, while cutting-edge supervised machine learning will provide a further automatization for the investigation of large datasets.

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Concluding remarks

After years of technology and analytical tool development, we have finally reached the verge of the breakthrough of HDcyto in precision medicine (Fig. 2). By conveying interdisciplinary expertise in bioinformatics, biology and clinical medicine, HDcyto technological platform is now delivering new unanticipated insights on a broader spectrum of physiological and pathological conditions. However, an efficient and broader application of HDcyto is still hampered by limitations which cannot be overlooked. The identification of relevant questions with appropriate controls, a rigorous scientific methodology delivering high quality data and the deep understanding of the programming behind automated algorithms assume now a tremendous importance, given the complexity of datasets. For this reason, we think that it is utterly important to provide an appropriate multidisciplinary training to a new generation of researchers, whose role will require them to disentangle convoluted results into intuitive conclusions.

Overall, HDcyto has finally fulfilled the theoretical gap between technological development and clinically relevant findings. With the consolidation of HDcyto platforms and the implementation of analytical algorithms, we expect that in the near future we will observe a strong increase in translational studies that will increase our understanding of human biology and pathophysiology.

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References

1 **Cavrois, M., Banerjee, T., Mukherjee, G., Raman, N., Hussien, R., Rodriguez, B. A., Vasquez, J. et al.**, Mass cytometric analysis of HIV entry, replication, and remodeling in tissue CD4$^+$ T cells. *Cell Reports* 2017. **20**: 984–998.

2 **Chevrier, S., Levine, J. H., Zanotelli, V. R. T., Silina, K., Schulz, D., Bacac, M., Ailles, L. et al.**, An immune atlas of clear cell renal cell carcinoma. *Cell* 2017. **169**: 736–749 e18.

3 **Chew, V., Lai, L., Pan, L., Lim, C. J., Li, J., Ong, R., Chua, C. et al.**, Delineation of an immunosuppressive gradient in hepatocellular carcinoma using high-dimensional proteomic and transcriptomic analyses. *PNAS* 2017. **114**: E5900–e9.

4 **Giesen, C., Wang, H. A., Schapiro, D., Zivanovic, N., Jacobs, A., Hattendorf, B., Schüffler, P. J. et al.**, Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat. Methods* 2014. **11**: 417–422.

5 **Gullaksen, S. E., Skavland, J., Gavasso, S., Tosevski, V., Warzocha, K., Dumrese, C., Ferrant, A. et al.**, Single cell immune profiling by mass cytometry of newly diagnosed chronic phase chronic myeloid leukemia treated with nilotinib. *Haematologica* 2017. **102**: 1361–1367.

6 **Hamlin, R. E., Rahman, A., Pak, T. R., Maringer, K., Mena, I., Bernal-Rubio, D., Potla, U. et al.**, High-dimensional CyTOF analysis of dengue virus-infected human DCs reveals distinct viral signatures. *JCI Insight* 2017. **2**.

7 **Hartmann, F. J., Bernard-Valnet, R., Queriault, C., Mrdjen, D., Weber, L. M., Galli, E., Krieg, C. et al.**, High-dimensional single-cell analysis reveals the immune signature of narcolepsy. *J. Exp. Med.* 2016. **213**: 2621–2633.

8 **Krieg, C., Nowicka, M., Guglietta, S., Schindler, S., Hartmann, F. J., Weber, L. M., Dummer, R. et al.**, High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nat. Med.* 2018. **24**: 144–153.

9 **Lu, Y., Biancotto, A., Cheung, F., Remmers, E., Shah, N., McCoy, J. P., Tsang, J. S. et al.**, Systematic analysis of cell-to-cell expression variation of T lymphocytes in a human cohort identifies aging and genetic associations. *Immunity* 2016. **45**: 1162–1175.

10 **Schulz, D., Zanotelli, V. R. T., Fischer, J. R., Schapiro, D., Engler, S., Lun, X. K., Jackson, H. W. et al.**, Simultaneous multiplexed imaging of mrna and proteins with subcellular resolution in breast cancer tissue samples by mass cytometry. *Cell Systems* 2018. **6**: 25–36.e5.

11 **Wei, S. C., Levine, J. H., Cogdill, A. P., Zhao, Y., Anang, N. A. S., Andrews, M. C., Sharma, P. et al.**, Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. *Cell* 2017. **170**: 1120–1133.e17.

12 **Evrard, M., Kwok, I. W. H., Chong, S. Z., Teng, K. W. W., Becht, E., Chen, J., Sieow, J. L. et al.**, Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking, and effector functions. *Immunity* 2018. **48**: 364–379.e8.

13 **Guilliams, M., Dutertre, C. A., Scott, C. L., McGovern, N., Sichien, D., Chakarov, S., Van Gassen, S. et al.**, Unsupervised high-dimensional analysis aligns dendritic cells across tissues and species. *Immunity* 2016. **45**: 669–684.

14 **Mrdjen, D., Pavlovic, A., Hartmann, F. J., Schreiner, B., Utz, S. G., Leung, B. P., Lelios, I. et al.**, High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in health, aging, and disease. *Immunity* 2018. **48**: 599.

15 **See, P., Lum, J., Chen, J. and Ginhoux, F.** A single-cell sequencing guide for immunologists. *Front Immunol.* 2018. **9**: 2425.

16 **Hwang, B., Lee, J. H. and Bang, D.** Single-cell RNA sequencing technologies and bioinformatics pipelines. *Experimental & Molecular Medicine* 2018. **50**: 96.

17 **Throm, A. A., Moncrieffe, H., Orandi, A. B., Pingel, J. T., Geurs, T. L., Miller, H. L., Daugherty, A. L. et al.**, Identification of enhanced IFN-gamma signaling in polyarticular juvenile idiopathic arthritis with mass cytometry. *JCI Insight* 2018. **3**.

18 **Brummelman, J., Mazza, E. M. C., Alvisi, G., Colombo, F. S., Grilli, A., Mikulak, J., Mavilio, J. et al.**, High-dimensional single cell analysis identifies stem-like cytotoxic CD8$^{(+)}$ T cells infiltrating human tumors. *J. Exp. Med.* 2018. **215**: 2520–2535.

19 **Fulwyler, M. J.** Electronic separation of biological cells by volume. *Science* 1965. **150**: 910–911.

20 **Chattopadhyay, P. K., Gaylord, B., Palmer, A., Jiang, N., Raven, M. A., Lewis, G., Reuter, M. A. et al.**, Brilliant violet fluorophores: a new class of ultrabright fluorescent compounds for immunofluorescence experiments. *Cytometry A* 2012. **81**: 456–466.

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21 **Mair, F. and Prlic, M.** OMIP-044: 28-color immunophenotyping of the human dendritic cell compartment. *Cytometry A* 2018. **93**: 402–405.

22 **Nolan, J. P. and Condello, D.** Spectral flow cytometry. *Curr Protoc Cytom* 2013. Chapter 1:Unit1 27.

23 **Schmutz, S., Valente, M., Cumano, A. and Novault, S.** Spectral cytometry has unique properties allowing multicolor analysis of cell suspensions isolated from solid tissues. *PLoS One* 2016. **11**: e0159961.

24 **McGrath, K. E., Bushnell, T. P. and Palis, J.** Multispectral imaging of hematopoietic cells: where flow meets morphology. *J. Immunol. Methods* 2008. **336**: 91–97.

25 **Grimwade, L. F., Fuller, K. A. and Erber, W. N.** Applications of imaging flow cytometry in the diagnostic assessment of acute leukaemia. *Methods* 2017. **112**: 39–45.

26 **Hennig, C., Adams, N. and Hansen, G.** A versatile platform for comprehensive chip-based explorative cytometry. *Cytometry A* 2009. **75**: 362–370.

27 **Hummert, M. W., Alvermann, S., Gingele, S., Gross, C. C., Wiendl, H., Mirenska, A., Hennig, C. et al.**, Immunophenotyping of cerebrospinal fluid cells by Chipcytometry. *J. Neuroinflammation* 2018. **15**: 160.

28 **Chevrier, S., Crowell, H. L., Zanotelli, V. R. T., Engler, S., Robinson, M. D. and Bodenmiller, B.** Compensation of signal spillover in suspension and imaging mass cytometry. *Cell Systems* 2018. **6**: 612–620 e5.

29 **Han, G., Spitzer, M. H., Bendall, S. C., Fantl, W. J. and Nolan, G. P.** Metal-isotope-tagged monoclonal antibodies for high-dimensional mass cytometry. *Nat. Protoc.* 2018. **13**: 2121–2148.

30 **Han, G., Chen, S. Y., Gonzalez, V. D., Zunder, E. R., Fantl, W. J. and Nolan, G. P.** Atomic mass tag of bismuth-209 for increasing the immunoassay multiplexing capacity of mass cytometry. *Cytometry A* 2017. **91**: 1150–1163.

31 **Bodenmiller, B., Zunder, E. R., Finck, R., Chen, T. J., Savig, E. S., Bruggner, R. V., Simonds, E. F. et al.**, Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators. *Nat. Biotechnol.* 2012. **30**: 858–867.

32 **Mei, H. E., Leipold, M. D., Schulz, A. R., Chester, C. and Maecker, H. T.** Barcoding of live human peripheral blood mononuclear cells for multiplexed mass cytometry. *J. Immunol.* 2015. **194**: 2022–2031.

33 **Zunder, E. R., Finck, R., Behbehani, G. K., Amir el, A. D., Krishnaswamy, S., Gonzalez, V. D., Lorang, C. G. et al.**, Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm. *Nat. Protoc.* 2015. **10**: 316–333.

34 **Schulz, D., Zanotelli, V. R. T., Fischer, J. R., Schapiro, D., Engler, S., Lun, X. K., Jackson, H. et al.**, Simultaneous multiplexed imaging of mRNA and proteins with subcellular resolution in breast cancer tissue samples by mass cytometry. *Cell Systems* 2018. **6**: 531.

35 **O'Gorman, W. E., Kong, D. S., Balboni, I. M., Rudra, P., Bolen, C. R., Ghosh, D., Davis, M. M. et al.**, Mass cytometry identifies a distinct monocyte cytokine signature shared by clinically heterogeneous pediatric SLE patients. *J. Autoimmun.* 2017. **81**: 74–89.

36 **Throm, A. A., Moncrieffe, H., Orandi, A. B., Pingel, J. T., Geurs, T. L., Miller, H. L., Daugherty, A. L. et al.**, Identification of enhanced IFN-γ signaling in polyarticular juvenile idiopathic arthritis with mass cytometry. *JCI insight* 2018. **3**.

37 **Mingueneau, M., Boudaoud, S., Haskett, S., Reynolds, T. L., Nocturne, G., Norton, E., Zhang, X. et al.**, Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren's signature correlating with disease activity and glandular inflammation. *J. Allergy Clin. Immunol.* 2016. **137**: 1809–21e12.

38 **Stikvoort, A., Chen, Y., Rådestad, E., Törlén, J., Lakshmikanth, T., Björklund, A., Mikes, J. et al.**, Combining flow and mass cytometry in the search for biomarkers in chronic graft-versus-host disease. *Frontiers in Immunology* 2017. **8**: 717.

39 **Yao, Y., Welp, T., Liu, Q., Niu, N., Wang, X., Britto, C. J., Krishnaswamy, S. et al.**, Multiparameter single cell profiling of airway inflammatory cells. *Cytometry Part B: Clinical Cytometry* 2017. **92**: 12–20.

40 **O'Gorman, W. E., Hsieh, E. W. Y., Savig, E. S., Gherardini, P. F., Hernandez, J. D., Hansmann, L., Balboni, I. M. et al.**, Single-cell systems-level analysis of human Toll-like receptor activation defines a chemokine signature in patients with systemic lupus erythematosus. *J. Allergy Clin. Immunol*. 2015. **136**: 1326–1336.

41 **Chen, Y. B. and Cutler, C. S.** Biomarkers for acute GVHD: can we predict the unpredictable? *Bone Marrow Transplant*. 2013. **48**: 755–760.

42 **Hartmann, F. J., Bernard-Valnet, R., Quériault, C., Mrdjen, D., Weber, L. M., Galli, E., Krieg, C. et al.**, High-dimensional single-cell analysis reveals the immune signature of narcolepsy. *J. Exp. Med*. 2016. **213**: 2621–2633.

43 **Latorre, D., Kallweit, U., Armentani, E., Foglierini, M., Mele, F., Cassotta, A., Jovic, S. et al.**, T cells in patients with narcolepsy target self-antigens of hypocretin neurons. *Nature* 2018. **562**: 63–68.

44 **Goswami, S., Basu, S. and Sharma, P.** A potential biomarker for anti-PD-1 immunotherapy. *Nat. Med.* 2018. **24**: 123–124.

45 **Bengsch, B., Ohtani, T., Khan, O., Setty, M., Manne, S., O'Brien, S., Gherardini, P. F. et al.**, Epigenomic-guided mass cytometry profiling reveals disease-specific features of exhausted CD8 T cells. *Immunity* 2018. **48**: 1029–1045.e5.

46 **Horowitz, A., Strauss-Albee, D. M., Leipold, M., Kubo, J., Nemat-Gorgani, N., Dogan, O. C., Dekker, C. L. et al.**, Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci. Transl. Med.* 2013. **5**: 208ra145–208ra145.

47 **Bendall, S. C., Davis, K. L., Amir el, A. D., Tadmor, M. D., Simonds, E. F., Chen, T. J., Shenfeld, D. K. et al.**, Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development. *Cell* 2014. **157**: 714–725.

48 **Wong, M. T., Ong, D. E., Lim, F. S., Teng, K. W., McGovern, N., Narayanan, S., Ho, W. Q. et al.**, A high-dimensional atlas of human T cell diversity reveals tissue-specific trafficking and cytokine signatures. *Immunity* 2016. **45**: 442–456.

49 **Thome, J. J. C., Bickham, K. L., Ohmura, Y., Kubota, M., Matsuoka, N., Gordon, C., Granot, T. et al.**, Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. *Nat. Med.* 2015. **22**: 72.

50 **Simoni, Y., Fehlings, M., Kløverpris, H. N., McGovern, N., Koo, S.-L., Loh, C. Y., Lim, S. et al.**, Human innate lymphoid cell subsets possess tissue-type based heterogeneity in phenotype and frequency. *Immunity* 2017. **46**: 148–161.

51 **See, P., Dutertre, C.-A., Chen, J., Günther, P., McGovern, N., Irac, S. E., Gunawan, M. et al.**, Mapping the human DC lineage through the integration of high-dimensional techniques. *Science* 2017. **356**.

52 **Lavin, Y., Kobayashi, S., Leader, A., Amir, E. D., Elefant, N., Bigenwald, C., Remark, R. et al.**, Innate immune landscape in early lung adenocarcinoma by paired single-cell analyses. *Cell* 2017. **169**: 750–765.e17.

53 **Kinchen, J., Chen, H. H., Parikh, K., Antanaviciute, A., Jagielowicz, M., Fawkner-Corbett, D., Ashley, N. et al.**, Structural remodeling of the human colonic mesenchyme in inflammatory bowel disease. *Cell* 2018. **175**: 372–386.e17.

54 **Azizi, E., Carr, A. J., Plitas, G., Cornish, A. E., Konopacki, C., Prabhakaran, S., Nainys, J. et al.**, Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. *Cell* 2018. **174**: 1293–1308.e36.

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55 **Olin, A., Henckel, E., Chen, Y., Lakshmikanth, T., Pou, C., Mikes, J., Gustafsson, A. et al.**, Stereotypic immune system development in newborn children. *Cell* 2018. **174**: 1277–1292.e14.

56 **Amir el, A. D., Davis, K. L., Tadmor, M. D., Simonds, E. F., Levine, J. H., Bendall, S. C., Shenfeld, D. K. et al.**, viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat. Biotechnol.* 2013. **31**: 545–552.

57 L. VDM. Accellerating t-SNE using tree-based algorithms. *The Journal of Machine Learning Research* 2014.

58 **McInnes LaH, J.** UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. ArXiv e-prints 2018.

59 **Mair, F., Hartmann, F. J., Mrdjen, D., Tosevski, V., Krieg, C. and Becher, B.** The end of gating? An introduction to automated analysis of high dimensional cytometry data. *Eur. J. Immunol.* 2016. **46**: 34–43.

60 **Van Gassen, S., Callebaut, B., Van Helden, M. J., Lambrecht, B. N., Demeester, P., Dhaene, T. and Saeys, Y.** FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry A* 2015. **87**: 636–645.

61 **Samusik, N., Good, Z., Spitzer, M. H., Davis, K. L. and Nolan, G. P.** Automated mapping of phenotype space with single-cell data. *Nat. Methods* 2016. **13**: 493-496.

62 **Bruggner, R. V., Bodenmiller, B., Dill, D. L., Tibshirani, R. J. and Nolan, G. P.** Automated identification of stratifying signatures in cellular subpopulations. *PNAS* 2014. **111**: E2770–E2777.

63 **Arvaniti, E. and Claassen, M.** Sensitive detection of rare disease-associated cell subsets via representation learning. *Nature communications* 2017. **8**: 14825.

64 **Gregori, G., Patsekin, V., Rajwa, B., Jones, J., Ragheb, K., Holdman, C., Robinson, J. P. et al.**, Hyperspectral cytometry at the single-cell level using a 32-channel photodetector. *Cytometry A* 2012. **81**: 35–44.

65 **Han, Y., Gu, Y., Zhang, A. C. and Lo, Y. H.** Review: imaging technologies for flow cytometry. *Lab Chip* 2016. **16**: 4639–4647.

66 **Lin, J. R., Izar, B., Wang, S., Yapp, C., Mei, S., Shah, P. M., Santagata, S. et al.**, Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. *eLife* 2018. **7**.

67 **Bodenmiller, B.** Multiplexed epitope-based tissue imaging for discovery and healthcare applications. *Cell systems* 2016. **2**: 225–238.

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