Advances in Clinical Mass Cytometry

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KEYWORDS

• Mass cytometry • High-dimensional • Cytometry • Protein • Signaling
• Infectious disease • Neuroscience • Clinical research

KEY POINTS

- Mass cytometry enables system-level monitoring of immune cells to study cellular mechanisms driving disease conditions.
- Reproducibility, high sensitivity, and robustness of mass cytometry assays make mass cytometer a suitable tool for clinical sample analysis.
- Clinical applications of mass cytometry allow therapeutic decision making and patient stratification.

INTRODUCTION

As patient-tailored therapies are becoming routine, improved methods for comprehensive profiling of responses at the single-cell level are required. Flow cytometry (FCM) is a commonly used tool for this task in clinical research applications including cancer, infectious diseases, cardiovascular disease, and immune monitoring. Development of novel fluorophores, laser technology, and data analysis methods have allowed an increase in the number of parameters able to be measured per cell, doubling each year (Roederer Law for FCM), which has led in development of novel functional and phenotypic assays detecting up to 30 markers per cells.[1–6] Moreover, arrival of spectral FCM that captures the entire emission spectrum of every fluorophore has pushed this limit to 40 to 43 markers per cell.[7,8] However, spectral overlap of fluorescent dyes remains a key limitation for FCM, and although it can resolved mathematically using compensation, with increases in parameters, the spectral overlap remains challenging to remove.[9–14] Additionally, application of FCM for clinical samples remains challenging, because larger sample volumes are required to test several combinations of antigen fluorochromes and to account for spillover correction; additionally, robust standardization of instrumentation is needed to achieve

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reproducibility and comparable results between cytometry experiments.[15,16] Despite this, clinical laboratories rely heavily on FCM for many cellular phenotyping assays.

Mass cytometry or cytometry by time of flight (CyTOF) addresses many limitations of fluorescence and spectral FCM as it utilizes rare earth metal-tagged antibodies and inductively coupled plasma ionization to detect up to 50 parameters per cell.[17,18] Because of the elimination of fluorescent dyes, compensation is usually not required, and even with a limited volume of sample, the maximum number of markers per cell can be measured. However, CyTOF does not eliminate spillover. A minor spillover between CyTOF channels occurs because of metal impurity, oxidation, and mass overlap.[1] With proper experimental design, these limitations can be addressed.[19,20] Compared with FCM panel design, CyTOF panels are relatively easy to design and do not require extensive domain knowledge. Additionally, sample multiplexing of samples eliminates batch effects and leads to comparable results between CyTOF runs. All these features make CyTOF an ideal tool for clinical research.

Here is discussed development of CyTOF assays that enable single-cell detection of surface and intracellular proteins, thus enabling evaluation of cytokine production, signaling pathways, codetection of proteins and ribonucleic acid (RNA), cellular metabolism and epigenetic marks. Then the authors discuss the application of these assays in clinical research across domains including cancer, infectious disease, autoimmune disease, and neuroscience. Finally, how CyTOF could be incorporated in more routine clinical monitoring will be discussed.

CYTOMETRY BY TIME OF FLIGHT-BASED FUNCTIONAL ASSAYS

Since the advent of mass cytometry over a decade ago, assays to profile different cellular features have been developed. Here is an overview of each of these assays. A graphical representation is shown in **Fig. 1**.

Cellular Phenotyping

The human immune system consists of various cell types possessing distinct antigen specificities. Over the years, advancement in bulk transcriptomics, sc-RNA seq, and FCM has revealed novel markers (genes and proteins) defining cell types. Thus, inclusion of more markers can improve resolution into cellular identity. Theoretically, CyTOF can measure up to 100 parameters per cell. Current studies have measured up to 47 markers per cell.[21] That CyTOF can evaluate single cells in high parameters allows detection of novel and rare cell types as shown in studies of gastrointestinal disorders including innate lymphoid and T regulatory cells.[22,23]

Intracellular Signaling

Cell signaling pathways are vital for cell-cell communication and coordinate cellular proliferation and differentiation.[24,25] Signaling events are often mediated by kinases and phosphatases to regulate phosphorylation events. Deregulation of signaling leads to abnormal cell physiology and disease. The multiparameter, multiplexed nature of CyTOF makes it a natural choice for studying signaling pathways. CyTOF has been applied to study signaling networks and cell-cell communication following drug stimulation, to examine the relationship between pairs of phosphorylation sites to infer signaling networks, and to track signaling dynamics during phenotypic transitions.[26,27] Over the years, researchers have utilized this approach to study cancers including leukemia, glioblastoma, and ovarian cancers[28–30] CyTOF-based signaling analysis has been coupled with transient protein overexpression such as green fluorescent protein (GFP) and red fluorescent protein (RFP) to identify novel signaling mechanisms

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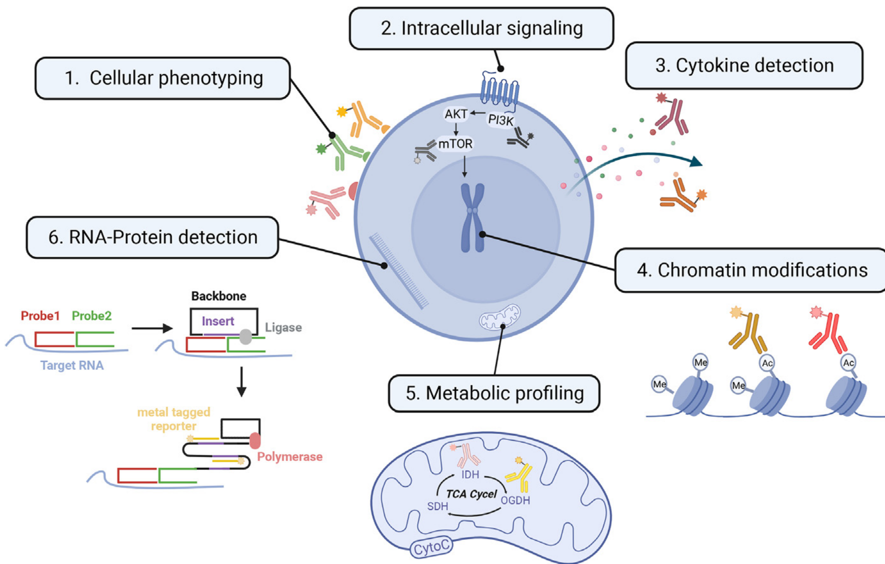

Fig. 1. Cellular view of CyTOF-based functional assays. CyTOF assays require a validated antibody to detect a target of interest. These antibodies can be used to identify molecules across various assays. Broadly, these assays can be divided into 6 subgroups: (1) phenotyping, (2) signaling pathways; (3) cytokine detection assay, (4) chromatin modification, (5) metabolic profiling, (6) RNA-protein detection.

associated with cancer progression and drug response.[31,32] Tape and collogues developed a thiol-reactive organoid barcoding in situ (TOBis) method to use CyTOF to study signaling networks between healthy and cancerous organoid cultures.[33]

Cytokine Detection

Cytokines are intracellular proteins, and their production is often regarded as a marker of immune cell function. Decades of research suggest that nearly all diseases are directly or indirectly linked with cytokine-mediated immune system activation.[34–36] Evaluating cytokine production in single immune cells from a cell mixture like PBMCs can reveal a wide range of effector functions. Such heterogeneity is better understood utilizing single-cell technologies. Microengraving-based and FLUOROSpot assays allow profiling of cytokines but are limited to 4 parameters because of spectral overlap.[37,38] FCM with proper compensation and CyTOF allows measurement of 5 to 14 cytokines per panel.[39,40]

Chromatin Modifications

Histone protein modifications including acetylation, methylation, and ubiquitination regulate chromatin structure.[41] Chromatin regulation is tightly linked with gene expression, which in turn influences cellular phenotype and function. Deregulation of histone modifications are linked with various disease conditions. Traditional methods (chromatin immunoprecipitation, Western blot) to evaluate histone modifications are performed on the bulk population. Immunohistochemistry and immunofluorescence quantify chromatin modifications in single cells but are low throughput and labor-intensive. These challenges can be addressed using epigenetic landscape profiling using cytometry by time of flight (EpiTOF), which utilizes CyTOF to detect a broad range of histone modifications using metal-tagged antibodies.[42] Applied to healthy peripheral blood mononuclear cells, EpiTOF has detected cell-type specific chromatin

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profiles. For instance, natural killer cells were found to fewer chromatin modifications compared with other immune cell types.

Metabolic Profiling

Cellular metabolism drives immune cell activation, proliferation, differentiation, and effector functions.[43,44] Typically, the characterization of metabolites within immune cells is done using bulk assays such as mass spectrometry or extracellular flux analysis.[45] To evaluate these features on individual cells, researchers have developed CyTOF-based assays to identify metabolic states through development of antibody panels to measure components of glycolysis, mitochondrial respiration, metabolic transporters or enzymes. These have been applied to the study of immune cells in the setting of normal activation or following chimeric antigen receptor (CAR) T cell treatment.[46,47] The application of these assays to a broad range of diseases will enable identification of metabolic alterations associated with disease that can serve as biomarkers or targets for personalized therapy.

Ribonucleic Acid-Protein Detection

Single-omics methods have provided a plethora of evidence regarding the benefits of profiling RNA and protein from a single cell. Application of these methods is shedding light on mechanisms driving treatment failure. Although these methods are sequencing based, CyTOF can also be utilized for per cell codetection of RNA and proteins. In 2016, Frei and colleagues proposed the proximity ligation assay for RNA (PLAYR) method to quantify more than 40 transcripts and proteins.[48] Briefly, cells are permeabilized, and 2 DNA oligonucleotide probes are added that are designed to hybridize at 2 adjacent regions of a target transcript. Each of these probes contains 1 region for selective hybridization to its cognate target RNA sequence and another region that acts as a template to bind and circularize 2 additional oligonucleotides called insert and backbone. When insert and backbone hybridized to the probe pair, they are amplified to produce concatenated complementary copies. Finally, the amplified products are detected using a metal-labeled oligonucleotide. This detection of transcripts can be multiplexed with traditional protein targeted antibodies to enable codetection of mRNA and protein from individual cells.

CYTOMETRY BY TIME OF FLIGHT IN COMBINATION WITH MULTI-OMICS

A primary goal of clinical studies is to extract the maximum amount of information from limited cellular material. The advent of single-cell transcriptomics (sc-RNA seq), epigenomics single-cell sequencing assay for transposase-accessible chromatin sequencing (scATAC seq), and multi-omics such as cellular indexing of transcriptomes and epitopes by sequencing (CITE seq), has enabled researchers to detect single or multiple omics layers. These methods provide a comprehensive view of heterogeneous clinical samples. Yet these methods are relatively low-throughput, subject to cell type loss, and expensive.[49] To tackle these challenges, researchers often utilize CyTOF to first phenotype cell populations, then FACS sort populations of interest for multi-omics assays[50] or integrate CyTOF data with data from other single-cell modalities.[51,52]

CLINICAL APPLICATIONS OF CYTOMETRY BY TIME OF FLIGHT

Cancer

Initial applications of CyTOF were focused on profiling cell signaling networks, phenotypic identification of tumor cells, and decoding the tumor-immune microenvironment.[28,53,54] These studies identified novel cellular features within patient groups

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considered to be distinct as per clinico-pathological schemes. The advent of machine learning algorithms and novel data analysis approaches have enabled the identification of features predicting disease outcomes or correlating with prognosis. Davis and colleagues performed CyTOF analysis on 60 primary diagnostic B-cell precursor acute lymphoblastic leukemia (BCP-ALL) samples to identify cells predictive of relapse in a diagnosis sample.[55] The authors developed a machine learning model to identify cells predictive of future relapse and identified the features of these cells for further study. Similarly, Leelatian and colleagues introduced a machine learning algorithm, Risk Assessment Population IDentification (RAPID), to identify phenotypically distinct cell-types correlating with patient survival in glioblastoma, a deadly brain tumor.[29] RAPID detected 4 glioblastoma-negative prognostic (GNP) populations enriched for S100 B, SOX2, p-STAT3, and p-STAT5 expression whose abundance was predictive of poor patient outcomes and 5 glioblastoma positive prognostic (GPP) populations enriched with EGFR and CD44 expression whose abundance was associated with improved overall survival.

Besides predicting cell types and disease features, CyTOF has been used to study cancer immunotherapies such as CAR T-cell therapy. CAR T treatment has shown remarkable improvement for patients with B-cell malignancies.[56,57] June and colleagues performed functional and molecular characterization of CAR T cells from 2 chronic lymphocytic leukemia patients over the period of 10 years.[58] The data suggested 2 major phases of CAR T therapy response: an initial phase dominated by CD8 CAR T cells or CD4-CD8-Helios^{high} $\gamma\delta$ CAR T and a long-term second phase represented by Ki67+ and CD38+HLA-DR + CD95+ cytolytic CD4 T cells. In the second study, Good and colleagues focused on biomarkers associated with successful CAR T treatment in large B-cell lymphoma (LBCL).[59] The authors detected populations associated with progressive disease (PD) and complete remission (CR) at day 7 after infusion. Responding patients demonstrated a T follicular helper (T_{FH}) cell-like population (PD1+CD57+CD4 CAR T cells) and CD57+ Blimp-1+T-bet + CD8 CAR T cells, while patients with PD demonstrated an immunosuppressive T-regulatory cell-like population.

More recently, CyTOF, together with bulk and single-cell transcriptomics, imaging mass cytometry, pharmacoscopy, and 4i drug response profiling (4i DRP), has been utilized in an observation clinical trial called the Tumor Profiler (TuPro) Study for in-depth tumor analysis in order to support clinical decision making.[60] Two-hundred forty tumor samples from acute myeloid leukemia, ovarian cancer, and melanoma patients over 3 years were collected and analyzed. Preliminary results of this study demonstrated the potential of these technologies to support clinical decision making.

Infectious Disease

Infections induce broad immune responses involving the innate and adaptive arms of a healthy immune system. Mass cytometry has been widely used to study immune response to infection with human immune virus,[61–63] Ebola virus,[64] hepatitis B virus,[65] gamma herpes virus,[66] dengue virus,[67,68] and Zika virus.[69] More recently CyTOF has been employed to compare malaria-[70] and helminth-[71] induced immune response between ethnic cohorts from European, African, and Indonesian cohorts. The authors found distinct immune signatures of T-helper and innate lymphoid cells correlating with each ethnic cohort. In general, application of CyTOF to study cell types contributing to viral and pathogen responses have deepened understanding of the immune response to infection and will ultimately aid in the development of treatments and vaccines.

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Mass cytometry has been utilized to study the immunopathology of severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV-2). These studies were broadly focused on 2 aspects: identifying markers associated with dysfunctional immune systems[21,72] and stratifying patients with early and delayed immune responses.[73,74] Based on severity of disease, patients with SARS-CoV-2 can be classified clinically into: moderate, severe, and critical cases.[75] Studies suggested that compared with healthy donors, patients infected with SARS-CoV-2 in all clinical severity categories have increased frequencies of B cells, naïve CD4 T, and CD4+CD8+ T cells, while frequencies of naïve, memory and effector CD8 T cells were decreased. Activated CD8 T cells, dendritic cells, and macrophages were observed in moderate cases, and these cells were seen in severe and critical cases but with an exhausted phenotype. Additionally, researchers have also applied CyTOF to study immune responses following the administration of mRNA-based vaccines developed by Moderna (mRNA-1273) and Pfizer/BioNTech (BNT162b2).[76,77]

Autoimmune Disease

Inflammatory cells play a vital role in autoimmune disease progression and severity. Various research groups have exploited the multidimensional capability of CyTOF to understand immune regulation for rheumatoid arthritis (RA),[78,79] systemic lupus erythematosus (SLE),[80,81] spondyloarthritis,[82] multiple sclerosis (MS),[83,84] inflammatory bowel disease (IBD),[85] and celiac disease.[86] Some of these studies performed by independent groups identified distinct cell types associated with disease risk. Two independent studies of RA patients identified CD4 T cell-mediated pathogenesis associated with disease outcomes.[78,87] To understand the role of T cells in autoimmune conditions such as CeD, SLE, and systemic sclerosis, Davis and colleagues employed metal conjugated HLA-DQ tetramers and CyTOF. Their analysis revealed that the antigen-specific T-cell phenotype was similar to that published in RA by Rao and colleagues.[78,88] This phenotype was enriched in multiple conditions including CeD, SLE, and systemic sclerosis. Together, these studies show the power of high-parameter phenotyping to identify complex cellular features correlating with disease pathogenesis that can be used for diagnostic purposes or can serve as potential targets for therapeutic interventions.

Neuroimmune Disorders

Applications of CyTOF are not limited to the peripheral immune system. A growing body of literature highlights the unique immune environment of the central nervous system (CNS) where immune cells have varied roles in immune defense, tissue homeostasis, and neurologic conditions such as Alzheimer disease (AD) and multiple sclerosis (MS).[89–93] A CyTOF study revealed that CNS resident immune cells can be distinguished from peripheral immune cells by expression of CD44.[94] The brain parenchyma is enriched with immune cells, mainly microglia, a specialized macrophage accounting for up to approximately 10% CNS cells. Studying murine microglia using CyTOF, Mrdjen and colleagues and Ajami and colleagues reported that microglia undergo dramatic changes with respect to functional and phenotypic markers in amyotrophic lateral sclerosis and encephalomyelitis.[95,96] To better understand the role of microglia in MS, Böttcher and colleagues profiled microglia from 5 brain regions and found that in disease conditions, microglia demonstrate an activation phenotype with upregulation of CD68, CD86, CD45, and CX3CR1.[83] These studies demonstrate the utility to use CyTOF to monitor immune composition within the CNS.

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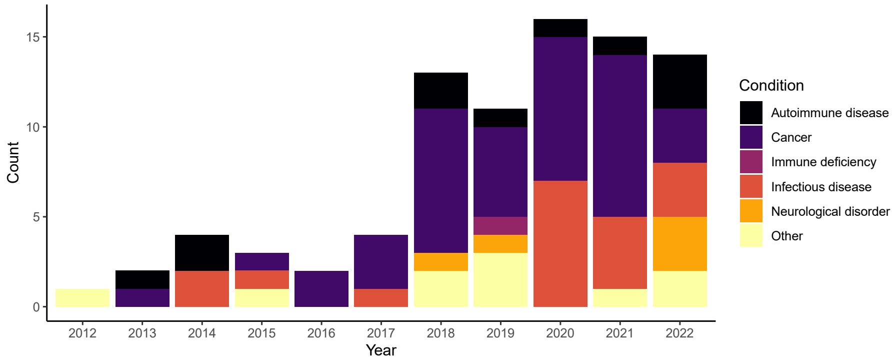

Fig. 2. CyTOF use in clinical trials. Bar graph depicting increasing number of clinical trials reported since 2012 utilizing CyTOF for correlative analyses. The data were collected from https://clinicaltrials.gov/ using "CyTOF" as a keyword.

FUTURE DIRECTIONS

CyTOF has significantly increased the ability to profile cellular populations at the single-cell level. This is beneficial particularly when the samples have low cell numbers such as in clinical biopsies. As of 2022, more than 80 clinical trials were reportedly using CyTOF (**Fig. 2**) to perform correlative assays.

Further, standardized protocols proposed by multicenter and multicohort studies suggested lower inter- and intrainstrument variability, reproducibility, sensitivity, and robustness of CyTOF-based analyses.[97,98] These facilitated researchers to include CyTOF within their state-of-the-art analytical platforms to acquire samples from clinical trials and collaborate across institutions and laboratories. The Cancer Immune Monitoring and Analysis Centers-Cancer Immunologic Data Commons (CIMAC-CIDC) network (https://cimac-network.org/) established by National Cancer Institute aims to identify biomarkers of cancer immunotherapies across clinical trials and uses CyTOF as a core assay in the context of these trials.[99] The Immunophenotyping Assessment in a coronavirus disease 2019 (COVID-19) Cohort (IMPACC) launched by National Institutes of Health (NIH) utilized CyTOF and other proteomic and transcriptomics technologies to identify biomarkers associated with effective COVID therapeutics.[100] As genomic and exome sequencing are routine tests for genetic disorders, the infrastructure proposed by these networks can be utilized as a part of clinical decision making and patient stratification.

SUMMARY

CyTOF offers system-level monitoring of the immune system in conjunction with novel biological assays to decode immune mechanisms driving disease conditions. In the past decade, researchers have exploited the high-dimensional capabilities of CyTOF to discover disease associated cell-types and cellular features that are linked to outcomes and identified biomarkers for future treatment.

CLINICS CARE POINTS

- Clinical mass cytometry overcomes challenges faced by traditional flow cytometers such as fluorescent spillover and requirement of larger sample volumes and reproducibility.

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- With improved standardized operating protocols, mass cytometry can be utilized to aid routine clinical decision making.

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STATEMENTS AND DECLARATIONS

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