Research Techniques Made Simple: Experimental Methodology for Single-Cell Mass Cytometry

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Growing recognition of the complexity of interactions within cellular systems has fueled the development of mass cytometry. The precision of time-of-flight mass spectrometry combined with the labeling of specific ligands with mass tags enables detection and quantification of more than 40 markers at a single-cell resolution. The 135 available detection channels allow simultaneous study of additional characteristics of complex biological systems across millions of cells. Cutting-edge mass cytometry by time-of-flight (CyTOF) can profoundly affect our knowledge of cell population heterogeneity and hierarchy, cellular state, multiplexed signaling pathways, proteolysis products, and mRNA transcripts. Although CyTOF is currently scarcely used within the field of investigative dermatology, we aim to highlight CyTOF's utility and demystify the technique. CyTOF may, for example, uncover the immunological heterogeneity and differentiation of Langerhans cells, delineate the signaling pathways responsible for each phase of the hair cycle, or elucidate which proteolysis products from keratinocytes promote skin inflammation. However, the success of mass cytometry experiments depends on fully understanding the methods and how to control for variations when making comparisons between samples. Here, we review key experimental methods for CyTOF that enable accurate data acquisition by optimizing signal detection and minimizing background noise and sample-to-sample variation.

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Description: This article, designed for dermatologists, residents, fellows, and related healthcare providers, seeks to reduce the growing divide between dermatology clinical practice and the basic science/current research methodologies on which many diagnostic and therapeutic advances are built.

Objectives: At the conclusion of this activity, learners should be better able to:

- Recognize the newest techniques in biomedical research.
- Describe how these techniques can be utilized and their limitations.
- Describe the potential impact of these techniques.

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[a] *The abbreviation "CyTOF", in addition to being the name of this technique, is also the name of a commercial product that enables researchers to use the method. The authors are in no way endorsing any specific commercial products*

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Abbreviations: CyTOF, mass cytometry by time-of-flight; MCA, metal-conjugated antibody; MCB, mass-tag cellular barcoding

SUMMARY POINTS

- The success of mass cytometry experiments is dependent on well–thought-out goals, a detailed experimental design, and practice with CyTOF technology and protocols.
- When designing custom metal-conjugated antibody (MCA) panels, account for target antigen abundance and signal crosstalk.
- Custom MCAs need to be thoroughly validated and titrated.
- Staining protocols may need to be tested to optimize marker signal detection. Metal nucleic acid intercalators should be included in the experiment for accurate single-cell identification and live:dead discrimination.
- To minimize sample-to-sample variation, it is important to normalize samples based on bead standards and/or a sample barcoding strategy.

INTRODUCTION

Growing recognition of the complexity of interactions within cellular systems has fueled development of new technologies capable of a broad, holistic scope of analysis. In mass cytometry by time-of-flight (CyTOF)[a], cells are probed with metal-conjugated antibodies (MCAs). Tagged cell suspensions are then passed through a droplet nebulizer to enter into argon plasma, where individual cells are atomized and ionized, and abundant common ions are removed. Then, time-of-flight mass spectrometry detects the ionized metal tags through 135 detection channels and can measure over 45 parameters in each cell (Bandura et al., 2009) (Table 1). Mass cytometry has been described in detail in previous reviews (Bendall et al., 2012; Doan et al., 2015). Despite the innovative applications of this technique, it is currently scarcely used within the field of investigative dermatology. CyTOF technology may lead to a greater comprehension of cutaneous cellular phenotype heterogeneity, development, hierarchy, and relationship to other tissues. CyTOF may allow simultaneous study of cell state (such as proliferation, hypoxia, or enzymatic activity) and deeper understanding of expression of mRNA transcripts, cytokines, growth factors, or transcription factors within cell subsets. Examples of questions that may be addressed using this technique are abundant. Which signaling pathways of innate lymphoid cells effectively regulate immune homeostasis or contribute to autoimmunity? Which proteolysis products of keratinocytes promote skin inflammation? Which cancer cells have predictive value for early diagnosis, prognosis, development of drug resistance, or relapse?

This review aims to highlight CyTOF's novelty and utility, demystify the technique, and provide guidance on design of the multistep experimental methodology that requires detailed understanding and planning to ensure accurate and consistent results. We will focus on specific considerations needed when designing a panel of desired markers, optimizing the staining protocol, performing metal conjugation of antibodies, and barcoding multiple samples.

Table 1. Summary description of mass cytometry technology, advantages and limitations

Advantages	Limitations
• Possible to simultaneously analyze over 45 parameters (e.g., 40 antibody-tagged markers, cell viability, and DNA content)	• Cells are destroyed through the CyTOF process; thus, it is not feasible to further culture or analyze cells after data acquisition
• Possible to study cell death, cytokine production, and cell signaling simultaneously	Slow sample throughput (maximum of 2,000 events/second), whereas flow cytometry can operate 25–50 times faster
• Minimal background noise from signal overlap or endogenous cellular components	• Some cellular properties cannot be measured (e.g., pH or ion concentration)
• Cost per probe per test ≈ $1.50–$3.00[1]	• Low efficiency (only 30–60% of cells of a sample are measured)
• Cost per analyzed cell ≈ 0.005 cents[2]	• CyTOF's multiplexed, high-dimension data requires new analysis tools
• A single dataset can be analyzed simultaneously by various analysis methods to test multiparameter hypotheses	

Abbreviation: CyTOF, mass cytometry by time-of-flight.

CyTOF allows the characterization and quantification of over 40 markers simultaneously on millions of individual cells. Metal-tagged antibodies are used to label multiple internal and external cellular markers of interest, which can be quantified by time-of-flight mass spectrometry at a single-cell resolution. It can lead to unprecedented breakthroughs of understanding the complex differentiation process and interaction between cell subpopulations, new cell types, functional profiles, and biomarkers.

[1]Estimated based on the price of commercially conjugated reagents or unconjugated antibodies and commercial conjugation kits, in contrast to $2.00–$8.00 for fluorescent flow cytometry (Bendall et al., 2012).

[2]The cost of reagents, disposables, and data acquisition, in contrast to ~$22 to measure cells by single-cell RNA sequencing using the Fluidigm C1 system (Fluidigm, San Francisco, CA) and molecular identifiers (Spitzer et al., 2016).

EXPERIMENTAL DESIGN

Mass cytometry experiments include precise and lengthy multistep protocols that generate immense amounts of data at single-cell resolution (Figure 1). It is therefore imperative to establish a meticulous experimental strategy and to have clear objectives at the onset of each experiment. After defining specific experimental aims, it is important to define the types of cells that will be studied, experimental conditions, comparative groups, and controls. In some cases, FACS or magnetic-activated cell sorting is needed to enrich for rare subsets of cells to avoid long CyTOF acquisition times (sample throughput: flow cytometry = 25,000 vs. CyTOF = 500–2,000 cells/second). At least 300 events of the rarest population should be acquired for analysis.

Watanabe et al. (2015) recently used mass cytometry to compare the relative functional capacities (including TNF-α, IL-2, IL-4, IL-13, IFN-γ, IL-17, IL-22, and IL-10) of skin-tropic (CLA^+) central memory, migratory memory, and effector memory T cells from human blood. T cells were isolated from peripheral blood of healthy individuals and stimulated with phorbol 12-myristate 13-acetate and ionomycin. The opportunity to simultaneously study those many markers from a single sample enabled the authors to conclude that effector