Flow Cytometry II: Mass and Imaging Cytometry

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INTRODUCTION

The need to refine our understanding of the immune system in order to characterize dermatologic disease pathology and guide therapy necessitates a better understanding of the immune system. One of the most widely employed characterization techniques is flow cytometry. At its core, flow cytometry attempts to profile cellular states at a given point in time. Newer technologies extend the fundamental scope of flow cytometry; mass cytometry provides much more data on the specific state of immune cells, and imaging cytometry describes single-cell dynamics in heterogeneous mixtures. In this article, we introduce and review these two novel extensions to traditional flow cytometry and provide examples that can guide future studies that can benefit from these techniques.

WHAT FLOW CYTOMETRY DOES

- Mass cytometry (CyTOF) and imaging cytometry (ImageStream) are technological offshoots of traditional flow cytometry that allow characterization of multiple simultaneous cellular and subcellular parameters.
- Mass cytometry uses rare earth metal isotopes to label cells that are then read by a mass spectrometer, allowing for more than 35 labels on each cell.
- Imaging cytometry (ImageStream) combines high-resolution light and fluorescent microscopy to capture an image for each event passing through the detector.

LIMITATIONS

- During the mass cytometry (CyTOF) process, the cells are destroyed, making subsequent cell sorting and analysis impossible.
- Imaging cytometry (ImageStream) suffers from fluorescent dye "spillover," which continues to limit its multiplexing capabilities.
- The data analysis for both techniques can be complicated given the highly multiplexed nature of these techniques.

NOVEL TECHNOLOGIES FOR EVALUATION OF HETEROGENEOUS CELL POPULATIONS

In cutaneous diseases, the interaction between the immune system and native local tissue correlates with various disease states, and flow cytometry has played an integral role in the study of immunology. This technique allows for cell type characterization within blood or other tissue samples via simultaneous identification of multiple cellular markers. In this manner, cell populations expressing specific markers can be associated with certain disease states. In flow cytometry, antibodies labeled with fluorescent dyes (fluorophores) bind cellular markers. Antibody-bound cells are then analyzed by a flow cytometer that measures each cell's emitted light as it passes a detector. Cells bound to different fluorophores will emit light at a spectrum specific to the fluorophore, allowing for identification of cell populations of interest (Jahan-Tigh *et al*., 2012).

Multiple antibodies with different fluorophores can be used in the same preparation of cells. However, the potential for emitted spectral overlap ("spillover") among the various fluorophores can result in false-positive readings. Despite software-based correction and advances in fluorophore design, the spillover effect limits the number of antibody targets detectable on a cell. Although up to 20 different antibodies can be studied simultaneously on a single sample preparation, 8- or 9-plex studies are routinely performed in practice (Peters and Ansari, 2011).

Mass cytometry (CyTOF, or "time of flight") and imaging cytometry are two novel extensions of the flow cytometry technology that expand on the cellular characterization capable of traditional flow methods. The focus of this review will be to introduce these techniques and envision potential applications in the field of investigative dermatology.

MASS CYTOMETRY

Mass cytometry, also known as CyTOF, is a variation on mass spectrometry coupled with flow cytometry. This technology employs the resolution capabilities of small-molecule mass spectrometry scaled to the single-cell level. As in flow cytometry, single-cell mass cytometry allows for the simultaneous detection and characterization of multiple internal (e.g., phosphoproteins) and external (e.g., polysaccharides) markers (Bandura *et al*., 2009; Bendall *et al*., 2011). Whereas traditional flow requires the use of fluorophores, mass cytom-

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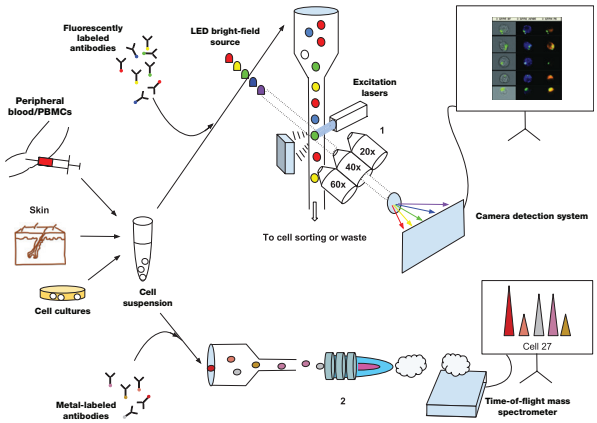

Figure 1. Schematic representation of both imaging cytometry (top) and mass cytometry (bottom). See text for details. (1) Microscope objective array with three different objective magnifications. (2) Argon plasma torch.

etry uses antibodies conjugated to rare earth transition metals, which are not found in biological systems (Ornatsky *et al*., 2006). More than 35 different purified metal isotopes are employed as labels and more are in development. In practice, this allows for multiplex detection of upward of 27 different antigens on a single cell (Bendall *et al*., 2011).

The initial sample preparation process is similar to that of classic flow cytometry. Single-cell suspensions are prepared from whole tissue, blood samples, bone marrow aspirates, or tissue culture. After incubation with the metal-labeled antibodies, the cells of interest are injected into an argon flow chamber designed to accommodate cell suspensions (Figure 1). The cells are swept up by the argon gas into a specialized compartment, where they are exposed to a plasma torch reaching temperatures of 10,000 K, similar to that found in the atmosphere of the sun. The cells and their metal labels are then vaporized, atomized, and ionized. Importantly, the cell is now represented by an ion "cloud" and the times of flight (TOFs) of the ions from the plasma to a mass spectrometer detector are then measured. Lighter particles (i.e., metals) arrive at the detector earlier than the heavier particles.

The detector measures the metal content found in this cell cloud and can discriminate between the signals of heavier metal antibody isotopes and the metals normally found in biological systems. The CyTOF platform includes software to analyze the TOF and, based on the properties of the ionized cell cloud, the software can detect the heavy metal isotope composition of each cell that is subjected to the spectrometer. The mass spectrometer is able to discriminate between isotopes of a heavy metal differing by only the weight of a neutron, allowing for fine resolution without the spillover effect seen with fluorophore-labeled antibodies. This spillover effect is the term given to the overlap of the emitted spectra of two different fluorophores (e.g., a predominantly red fluorophore gives off some orange signal, which gives a false positive for the presence of the orange fluorophore) and is a major limitation of fluorophore-dependent flow cytometry. Because of the lack of spillover, the probe set design for mass cytometry with a large number of probes (i.e., greater than 30) is much easier than that for traditional fluorescent flow cytometry. Another unique aspect of mass cytometry is a recent technological off-

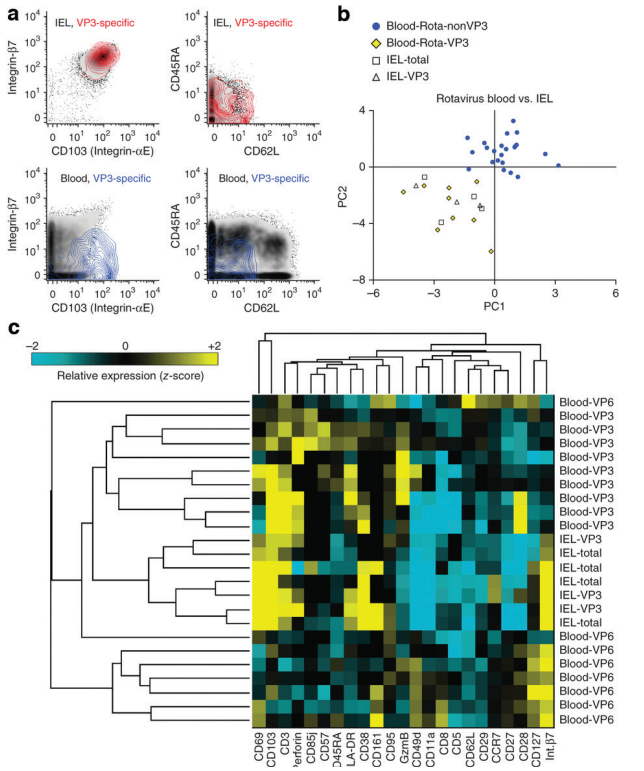

Figure 2. Principal-component analysis of mass cytometry data. (**a**) This group of cytometry fluorescence plots compares CD8⁺ T cells from the intestinal intraepithelial lymphocytes (IELs) that are specific for a rotavirus epitope called VP3 to the same VP3-specific T cells from the peripheral blood (i.e., they are gated on CD8⁺). Cells that are positive for both markers (in this case, CD103 and integrin-β7) are present in the upper right quadrant, whereas those negative for the two markers are present in the bottom left. The red outlines in the two upper panels and the blue outlines of the two lower panels represent the cells that are CD8⁺ and specific for VP3 of all the CD8⁺ cells (CD8⁺ cells are represented by black dots). (**b**) This graph displays the first two principal components (i.e., PC1 and PC2) or the first two groups of variables (e.g., cell markers) that best predict the variance in the data. In this example, each data point represents a donor's cell marker expression data from either peripheral blood or IEL specific for the VP3 epitope. (**c**) Each row in this clustergram is the expression data for a single donor. Only the variables that were calculated to be most important for this set of data as derived from the principal-component analysis are listed along the *X* axis (CD69, CD103, etc.). Each square correlates with the cumulative expression data from millions of that specific donor's T cells that were vaporized after passing through the plasma torch. A separate calculation involving the Euclidian distance between values allows similar samples to be clustered together. Reprinted from Newell *et al*., 2013.

shoot that overcomes the need to put the cells into suspension before analysis by directly labeling thin slices of fixed tissue with metal-conjugated antibodies. Either a laser or an ion beam, depending on the technique, then scans the tissue and releases the metal labels, which are read by an adjacent mass cytometer that is then able to reconstruct a multispectral image of the tissue (Giesen *et al*., 2014; Angelo *et al*., 2014).

In their study, Newell *et al*. (2013) employed mass cytometry to characterize T cells from the blood of 17 healthy donors and intestinal intraepithelial lymphocytes from nine gastric bypass patients. In these experiments, between 16 and 64 million CD8 T cells from each donor were analyzed for their ability to recognize viral epitopes including influenza, Epstein–Barr virus, cytomegalovirus, and rotavirus. Each T cell was stained with 37 heavy metal–labeled antibodies, creating

an incredible amount of data to subsequently analyze. One commonly employed method to handle these data is principal-component analysis, in which multiparametric data (e.g., the various cell surface markers labeled by the antibodies) are reduced to multiple components (e.g., groups of surface markers), with each component being composed of the variables that best predict the distribution of the data (Figure 2), effectively reducing the dimensionality of the data into more manageable parts.

DISADVANTAGES OF MASS CYTOMETRY

One of the important limitations of this technique is the cellular destruction that occurs once the cells reach the plasma torch. This prevents purification of specialized cell populations. Nonetheless, this multiplex system can potentially be used in a workflow that incorporates fluorescence-activated cell sorting (FACS), whereby the CyTOF technique identifies markers of interest in a tissue sample, and FACS isolates the cells of interest in a similar later experiment. Another drawback is that it is slower, with throughput speeds of 500–1,000 cells per second compared to traditional flow cytometry/FACS that can achieve speeds up to 50,000 cells per second.

IMAGING CYTOMETRY

A second offshoot of traditional flow cytometry combines high-throughput cytometry with high-resolution bright-field and fluorescent microscopy. The basic architecture of the apparatus is similar to that of classic flow cytometry whereby the biological solution of interest is labeled with fluorescent antibodies and injected into a liquid stream. The flow of the liquid causes the cells and/or particles to line up until they are struck by an excitatory light source, which causes them to fluoresce. The most common imaging cytometry platform, ImageStream system (Amnis, Seattle, WA), captures image data for each of these events that pass through the detector system using one or more microscope objectives (e.g., 20×, 40×, or 60×). The addition of these microscope objectives to the flow cytometry apparatus is the fundamental difference between classic flow cytometry and imaging cytometry, as illustrated in Figure 1 (top). Once the LED light and the excitation lasers strike each cell, pictures of each cell are taken by a high-speed camera apparatus as the cell passes through the sensors.

With imaging cytometry, each event (e.g., cell, cell fragment, parasite) has an associated set of pictures, in contrast to traditional flow cytometry in which no cell images are captured. In classic flow cytometry with 400- to 500-nm commercially available systems, these events are usually single cells passing through the detector apparatus. However, the newest ImageStream model, ImageStream^X Mark II, is capable of detecting subcellular particles down to sizes of 20 nm when combined with fluorescent data (Headland *et al*., 2014). This increased capture resolution dramatically increases the potential applications of imaging cytometry to include experiments elucidating cell–cell interactions, cell signaling localization, colocalization of multiple intracellular targets, apoptosis, transfection efficiency, cell–therapeutics interactions, and cell–pathogen interactions.

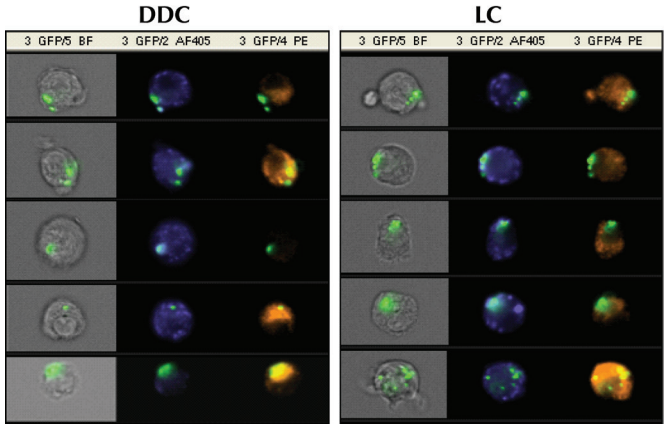

Figure 3. Imaging cytometry output. Each image is multispectral (composed of separate wavelengths of light), which allows only certain channels to be viewed simultaneously. The columns show cell images in gray and display two channels of information: one is the bright-field (BF) or traditional light microscopic image, which is shown as gray, and the second channel displays green information representing the fluorescence collected from the green-fluorescent protein-producing *Staphylococcus aureus*. Overlaying the two channels shows the location of the bacteria inside each cell. The columns with blue images demonstrate the fluorescence of the Alexafluor 405 (AF405) dye conjugated to the early endosomes, and the reddish-orange images display the fluorescence of the phycoerythrin (PE) dye marking lysosomes. Reprinted from van der Aar *et al*., 2013. DDC, dermal dendritic cell; LC, Langerhans cell.

The ability of imaging cytometry to separate cellular populations and colocalize internal cellular processes is illustrated by van der Aar *et al*. (2013), who compared the antigen presentation capability of dermal dendritic cells to that of Langerhans cells. In one experiment, *Staphylococcus aureus* bacteria engineered to express green fluorescent protein were incubated with both Langerhans cells and dermal dendritic cells. This mixture of cells was then labeled with antibodies that bind to the membrane-bound vesicles formed after the cells internalize the bacteria. Two markers were chosen: one that is found on vesicles early after their formation, appropriately named EEA-1 (early endosomal antigen-1), and a second that marks lysosomes, LAMP-1 (lysosomal-associated membrane protein-1). The EEA-1 antibody was conjugated to a blue fluorophore, and the LAMP-1 antibody was conjugated to a red fluorophore. Using the overlay of the green, blue, and red fluorophores, the investigators were able to calculate the proportion of Langerhans cells with *S. aureus* in endosomes and lysosomes compared to dermal dendritic cells (Figure 3). The experiment showed that after an equivalent amount of time with the bacteria inside the cells, the dermal dendritic cells had processed more of the bacteria into lysosomes than had the Langerhans cells, demonstrating the superior antigen-processing capabilities of dermal dendritic cells.

DISADVANTAGES OF IMAGING CYTOMETRY

Like mass cytometry, this technique is slower than traditional flow cytometry and, depending on the microscope objective used, can range from 1,200 cells per second with the 60× objective to 4,000 cells per second with the 20× objective. Because this technology uses fluorescent dyes, it contin-

ues to be subject to signal spillover, which limits the number of simultaneous targets that can be read on a single cell. Newer organic polymer dyes capable of conducting electricity and which produce a more intense fluorescence with less spillover represent an attempt to address this limitation (Chattopadhyay *et al*., 2012).

SUMMARY

The future of flow cytometry is bright, with new fluorescent dyes, more metal labels, and an ever-expanding set of applications under development. These technologies provide deep profiling of single cells and small-molecule quantification—techniques that can advance our understanding of cutaneous disease and drive development of novel therapeutics.

CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of the Duke University School of Medicine and Society for Investigative Dermatology. The Duke University School of Medicine is accredited by the ACCME to provide continuing medical education for physicians. To participate in the CME activity, follow the link provided. Physicians should only claim credit commensurate with the extent of their participation in the activity.

To take the online quiz, follow the link below:
http://continuingeducation.dcri.duke.edu/research-techniques-made-simple-journal-based-cme-rtms

SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2015.263.

REFERENCES

Angelo M, Bendall SC, Finck R *et al*. (2014) Multiplexed ion beam imaging of human breast tumors. *Nat Med* 20:436–42

Bandura DR, Baranov VI, Ornatsky OI *et al*. (2009) Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. *Anal Chem* 81:6813–22

Bendall SC, Simonds EF, Qiu P *et al*. (2011) Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* 332:687–96

Chattopadhyay PK, Gaylord B, Palmer A *et al*. (2012) Brilliant violet fluorophores: a new class of ultrabright fluorescent compounds for immunofluorescence experiments. *Cytometry A* 81:456–66

Giesen C, Wang HA, Schapiro D *et al*. (2014) Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat Methods* 11:417–22

Headland SE, Jones HR, D'Sa AS *et al*. (2014) Cutting-edge analysis of extracellular microparticles using ImageStream(X) imaging flow cytometry. *Sci Rep* 10:5237

Jahan-Tigh RR, Ryan C, Obermoser G *et al*. (2012) Flow cytometry. *J Invest Dermatol* 132: e1

Newell EW, Sigal N, Nair N *et al*. (2013) Combinatorial tetramer staining and mass cytometry analysis facilitate T-cell epitope mapping and characterization. *Nat Biotechnol* 31:623–9

Ornatsky O, Baranov VI, Bandura DR *et al*. (2006) Multiple cellular antigen detection by ICP-MS. *J Immunol Methods* 308:68–76

Peters JM, Ansari MQ (2011) Multiparameter flow cytometry in the diagnosis and management of acute leukemia. *Arch Pathol Lab Med* 135:44–54

van der Aar AM, Picavet DI, Muller FJ *et al*. (2013) Langerhans cells favor skin flora tolerance through limited presentation of bacterial antigens and induction of regulatory T cells. *J Invest Dermatol* 133:1240–9

QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

For each question, more than one answer may be correct.

1. Which flow analytical technique uses rare earth metal–labeled antibodies?

A. FACS.
B. Mass cytometry (CyTOF).
C. Principal-component analysis.
D. ImageStream.

2. What is an advantage of the ImageStream technique over traditional flow cytometry?

A. It allows for separation of different cell subpopulations in heterogeneous cell mixtures.
B. It captures high-resolution image data for each event passing through the system.
C. It obtains absolute quantification data for subcellular compartments.
D. It can be used on paraffin-embedded tissue sections.

3. As a dermatologist conducting research, you are interested in separating $CD4^+$ lymphocytes from peripheral blood as quickly as possible. Which technique would be preferred?

A. Phase-contrast microscopy.
B. ImageStream.
C. FACS.
D. Mass spectrometry.

4. One of the advantages of CyTOF-based studies is the following.

A. It allows for high-throughput multiplex analysis of protein expression data in single cells.
B. It allows for high-resolution image data collection of single-cell events.
C. It allows for cells to be sorted after staining and data acquisition.
D. It utilizes high-resolution fluorescence data.

5. What do flow cytometry, CyTOF, and ImageStream have in common?

A. They all employ fluorescence-based techniques.
B. Cells are lysed first and subsequently labeled with antibodies of interest.
C. These techniques utilize inductively coupled plasma.
D. Whole-cell suspensions are first labeled with antibodies of interest.