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ABSTRACT

Advanced single-cell analysis technologies (e.g., mass cytometry) that help in multiplexing cellular measurements in limited-volume primary samples are critical in bridging discovery efforts to successful drug approval. Mass cytometry is the state-of-the-art technology in multiparametric single-cell analysis. Mass cytometers (also known as cytometry by time-of-flight or CyTOF) combine the cellular analysis principles of traditional fluorescence-based flow cytometry with the selectivity and quantitative power of inductively coupled plasma–mass spectrometry. Standard flow cytometry is limited in the number of parameters that can be measured owing to the overlap in signal when detecting fluorescently labeled antibodies. Mass cytometry uses antibodies tagged to stable isotopes of rare earth metals, which requires minimal signal compensation between the different metal tags. This unique feature enables researchers to seamlessly multiplex up to 40 independent measurements on single cells. In this overview we first present an overview of mass cytometry and compare it with traditional flow cytometry. We then discuss the emerging and potential applications of CyTOF technology in the pharmaceutical industry, including quantitative and qualitative deep profiling of immune cells and their applications in assessing drug immunogenicity, extensive mapping of signaling networks in single cells, cell surface receptor quantification and multiplexed internalization kinetics, multiplexing sample analysis by barcoding, and establishing cell ontologies on the basis of phenotype and/or function. We end with a discussion of the anticipated impact of this technology on drug development lifecycle with special emphasis on the utility of mass cytometry in deciphering a drug's pharmacokinetics and pharmacodynamics relationship.

Introduction

Single-cell cytometry technologies (flow cytometry and image cytometry) have had a substantial impact on drug discovery and development for the past few decades. Extensive use of cell sorters and analyzers has not only enabled cloning or sorting of pure populations of cells (cell lines, primary cells, and more recently stem cells), they have also enabled researchers to measure the effects of a drug at the single-cell level and thus to better understand its mechanism of action. Whereas until recently, the application of cytometry assays in drug development had largely been limited to targeted measurements of one or a handful of diagnostic markers on single cells, the era of precision medicine is necessitating a more comprehensive multiplexed “single-cell-omics” approach for the discovery and monitoring of cellular biomarkers to track disease, stratify patients, or assess the success of therapy. For example, detailed phenotyping combined with identification of potential signaling nodes that constitute the intended and unintended targets of drugs have been successfully employed in drug screens (Zhang et al., 2009; Ellinger et al., 2014) and clinical studies with signaling inhibitors such as anticancer drugs (Guerrouahen et al., 2009; Tinhofer et al., 2012). As new drugs are developed for targeted therapy of patients stratified using a precision medicine approach, advances in multiplexed single-cell assay technology will be instrumental during the discovery and development phases of drug development. The invention of mass cytometry has opened up new possibilities for such highly multiplexed assays on single cells. Although still in its early stages, mass cytometry has already initiated a transformation of single-cell analysis in both academia and the pharmaceutical industry alike. The initial groundbreaking publications on mass cytometry underscored its utility and amenability for highly multiplexed assays to measure cellular phenotype and functional markers (e.g., intracellular cytokine assays and phospho-signaling fingerprinting of immune cells) (Bendall et al., 2011; Bodenmiller et al., 2012). Mass cytometry is increasingly recognized as a significant step forward in single-cell profiling that has the potential to accelerate biomarker discovery throughout drug development.

Mass cytometers are essentially inductively coupled plasma–mass spectrometers (ICP-MS) that have been adapted for single-cell analysis. Traditionally, ICP-MS has been used for elemental analysis with a high degree of sensitivity and can detect metals at concentrations as low as one part per trillion (one in 1012). In biology and biologic industries, ICP-MS is commonly used to quantify toxic metal contaminants, metal-containing biofactors and proteins, or in the study of metal transport, metallo-drugs, and metal speciation.

ABBREVIATIONS: ABC, antibody bound per cell; CyTOF, cytometry by time-of-flight; DOTA, 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetra-acetic acid; ICP-MS, inductively coupled plasma–mass spectrometry; NK, natural killer; PE, phycoerythrin; TOF, time of flight.

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The use of ICP-MS in biologic assays was first demonstrated in a study that used conjugated gold clusters to quantify endocytic low-density lipoprotein in cultured cells (Andreou et al., 1998). Adaptation of ICP-MS to an immunoassay format was demonstrated later by Baranov et al. who showed the feasibility of using antibodies tagged to either gold (Au) or lanthanide metal tags in analyzing immune complexes (Baranov et al., 2002; Ornatsky et al., 2006). The same investigators further developed cell-based immune-assays coupled to ICP-MS, a technology that formed the basis for the invention of the first mass cytometer (Ornatsky et al., 2008a,b). Later, Ornatsky et al. (2010) described their groundbreaking invention of the mass cytometer and demonstrated its capabilities for highly multiplexed analysis of cellular markers.

In this brief overview we describe the principles and instrumentation behind mass cytometry by time-of-flight (CyTOF) technology. Subsequently, we will discuss emerging applications in biomarker research in the pharmaceutical industry in the context of the anticipated impact on drug development.

Mass Cytometry: Principles and Instrumentation

CyTOF is essentially an ICP-MS with a time-of-flight detector that is designed for simultaneous quantitative measurements on single cells or bead-based immune-detection assays. CyTOF uses antibodies that are linked to rare earth metals (referred to as tags in this review). The metal tags serve the same function as fluorophores in traditional fluorescence-based flow cytometry. The current state-of-the-art mass cytometer is capable of measuring up to 100 different stable isotopes of rare earth metals, although the current availability of these tags is limited to 40 different rare earth metal tags.

CyTOF can be broadly divided into three main functional units—sample introduction, ICP combustion region, and TOF-MS detector (Fig. 1). A detailed description of the instrument has been discussed in several reviews published previously (Bandura et al., 2009; Ornatsky et al., 2010; Tanner et al., 2013).

Sample Introduction. The sample introduction system performs three main functions: introduction of the cell suspension, pneumatic nebulization of the cell suspension, and evaporation of water from the droplets containing the cells before they are introduced into the plasma. Cells can be introduced either manually or via an autosampler. Upon injection, a narrow capillary tube delivers the cells to a nebulizer, wherein argon gas—based pneumatic nebulization converts the cell suspension into a fine spray of water droplets and releases the droplets (containing cells) into a heated chamber called the spray chamber. The argon gas in the spray chamber (known as make-up gas) carries the nebulized cells through the heated spray chamber and into the plasma. During transit in the heated spray chamber, the water in the cell droplets is evaporated by the combined action of argon gas and heat. The distal end of the spray chamber is connected to a radiofrequency generator/spark plug combination that together generate the plasma. Only about 30–40% of nebulized cells make their way into the plasma and the remaining cells are nonspecifically lost on the walls of the spray chamber. This process results in a 30–40% efficiency of analysis of the injected cell samples.

ICP-MS. Inductively coupled plasma is initiated by the spark plug, which generates ions in the argon gas. The electrons released by the initial spark are accelerated back-and-forth by the alternating magnetic fields generated by the radiofrequency coil. The resulting collisions between the electrons and the argon atoms create further ionization finally resulting in sustained plasma, which is maintained by a continuous supply of argon flow from the torch body. The cells are delivered concentrically into the plasma core, which is maintained at a temperature of 7000 K and at atmospheric pressure.

Upon entering the plasma, the cells are completely vaporized and atomized. The resulting ion cloud exists at the other end of the plasma and expands at diffusion controlled rates on its way to the sampler cone. As the ion cloud passes through three cones of the sampler, skimmer, and reducer, the ions are moved through progressively higher levels of vacuum generated between the cones. The ion cloud is then passed through an ion optics to filter out the neutral and negatively charged ions. The filtered circular beam of ion cloud is thus enriched in positively charged ions and is then passed through an ion guide that converts a circular beam of ion cloud into a beam compatible with the rectangular slit of the orthogonal-acceleration reflectron time-of-flight analyzer. This ion guide consists of two radiofrequency-quadrupoles and is tuned to a low mass cutoff \((m/z = 80)\) to filter out the abundant low mass ions generated from metals and other elements found naturally in cells. Thus, the filtration results in an ion cloud that is considerably less dense than the parent ion cloud and can be manipulated and focused more efficiently. Furthermore, the filtering enables the ICP-MS to be configured to a TOF mass analyzer.

TOF and Data Processing. In the TOF chamber, ions are accelerated at a fixed potential. As a result, the ions travel at speeds proportional to the square root of their masses. In essence, low mass ions travel faster than high mass ions. The ions then impinge on a sheet beam in the detector. Subsequently, ion counts are converted to digital signals using an 8-bit 1-GHz analog-to-digital converter. The detector is set to measure \(m/z\) in the range of 80–200 at a 1-nanosecond sampling resolution.

The ion cloud derived from a cell or bead is measured in 13–15 segments, each with a duration of 20 microseconds. The total duration of a typical cell-derived ion cloud is 200–300 microseconds and is measured as “event length.” Events longer than 300 microseconds are “gated” out of the analysis as doublets (doublets are unseparated ion clouds arising from highly concentrated cell suspensions). The event-length feature is useful in identifying cellular or bead events from background and to discriminate between single events and doublets or cell aggregates. The data can be saved either as text files or the standard flow cytometry FCS 3.0 format that can be analyzed by any standard flow cytometry data analysis software.

Data Normalization and Analysis. Since CyTOF lacks the light scatter (forward and side scatter) measurements for cells, a combination of event length and DNA stain is used to identify cellular events. Typically, cells are stained with two DNA stains; the first DNA stain is a viability stain, whereas the second DNA stain (added at the end of the staining protocol) is used to distinguish cellular events from debris and for discriminating single events from doublets or cell aggregates. The data can be saved either with or without the rhodium-based (Darzynkiewicz, 2010; Darzynkiewicz et al., 2010; Ornatsky et al., 2008b) or Ir-based DNA intercalator (Ornatsky et al., 2008b; Tinhofer et al., 2012), or a platinum-containing compound such as cisplatin (Ellinger et al., 2014). The Rh- and Ir-DNA intercalators stain by reaching pseudo-equilibrium, and neither reach saturation nor are fixable and hence tend to leak out of the cells after staining. In contrast, cisplatin is a fast DNA-labeling stain and is fixable (Ellinger et al., 2014).

Continuous operation of the machine leads to signal drift primarily attributable to changes in plasma temperature and buildup of cellular material in the nebulizer and on the cones. In addition, regular cleaning and maintenance can also lead to day-to-day variation in performance characteristics. To control for signal variation, data normalization beads (typically two-metal or four-metal calibration beads) are spiked into each sample before sample acquisition. Since these beads are stable over long periods of time, they serve as internal standards to correct for signal drift either over several hours of continuous operation or to
correct day-to-day variation. Data are normalized using the “data normalization algorithm” (Finck et al., 2013) as part of the mass cytometer software or as a free downloadable standalone utility. This feature is particularly useful in longitudinal studies wherein sample acquisition and analysis is performed over an extended period of time.

Traditional flow cytometry data analysis software such as FlowJo (Treestar Inc., Ashland, OR) and FCSExpress (De Novo Software, Glendale, CA) can be used to analyze data generated from mass cytometers. These software programs are particularly useful for user-guided data analysis. However, detailed data analysis of CyTOF data by the manual gating of a series of bidirectional plots could be a daunting task and could compromise identification of new cell phenotypes or functional markers. To address this limitation, several computer-guided data analysis packages [e.g., Cytobank (Guerrouahen et al., 2009; Zhang et al., 2009), spanning tree progression of density-normalized events (Qiu et al., 2011; Chattopadhyay et al., 2014), viSNE (Amir et al., 2013), principal component analysis (Newell et al., 2012; Amir et al., 2013), flow analysis with automated multivariate estimation (Guilliams et al., 2014), SamSpectral (Wong et al., 2012), and RchyOptimyx (Aghaeepour et al., 2012)] are available and enable unbiased data analysis in multidimensional space. These programs are capable of reducing multidimensional data to two dimensions without loss of single-cell resolution and can be used to identify cell populations, analyze heterogeneity in cell composition, and quantify functional responses at the single-cell level (Finck et al., 2013). A majority of the above software can be used either in a fully automated mode or in a semiautomated mode with user input. Establishment of the mass cytometer technology and the associated data analysis packages calls for a significant investment in infrastructure and training.

Applications of Mass Cytometry in Pharmacokinetics/Pharmacodynamics and Biomarker Discovery

Adoption of flow cytometry by the pharmaceutical industry in the late 20th century revolutionized drug discovery efforts (Nolan et al., 1999). Widespread application of flow cytometry to small-molecule drug and biologics research enabled the development of single-cell functional assays (Chang et al., 2010), cell line differentiation and sorting, biomarker discovery (Kantor, 2002), assessment of drug toxicity (Lappin and Black, 2003), and pharmacokinetic/pharmacodynamic drug profiles (Dieterlen et al., 2011; Vafadari et al., 2012). As pharmaceutical research is becoming increasingly focused on the mechanism of drug-target interactions and its resulting pharmacology, the importance of cytometry techniques that enable single-cell analysis cannot be overstated. The introduction of mass cytometry constitutes a significant advance in multiplexing phenotypic, signaling, and functional biomarkers in single cells. The ability to simultaneously measure up to 40 different cellular measures and the advantage of performing multiplex measurement on limited sample volumes makes mass cytometry uniquely suited to contemporary drug development workflows. In this section we present a few examples of emerging and potential applications of mass cytometry in drug discovery.

**Deep Phenotyping.** Phenotyping refers to measurements that identify constituent cellular subsets in complex mixtures of cells (e.g., peripheral blood preparation, splenocytes, bone marrow aspirates, single-cell preparation of tissues or tumors) by measuring the expression pattern of cell surface markers (i.e., proteins or their glycosylation
patterns) using antibodies conjugated to fluorophores or metal tags. The presence or absence of a protein and its post-translational modifications (e.g., glycosylation) or degree of expression on the cell surface can be used to identify unique cell subsets.

The ability to multiplex up to 40 cellular subset markers in mass cytometry, without a requirement for compensation for overlap in fluorescence signals as needed in conventional flow cytometry, makes mass cytometry an ideal technology to deeply phenotype cells in complex cell populations. This feature was elegantly demonstrated by the seminal paper by Bendall et al. (2011), in which the investigators characterized human bone marrow cells using antibody cocktails that consisted of 13 cell surface subsetting markers in combination with 18 intracellular functional markers. In a more recent study Horowitz et al. (2013) demonstrated the multiplexing power of mass cytometry to characterize human natural killer (NK) cell subsets. This study revealed that the phenotypic diversity of NK inhibitory receptors (largely controlled by genetics) and activating-receptor expression (largely regulated by exposure to environmental cues) produces 6000–30,000 distinct subsets of NK cells in a given individual. This number can be greater than 100,000 distinct subsets in a given donor panel, a result that was heavily underappreciated before the advent of mass cytometry.

Since typical flow cytometry assays employ 3–8 markers, with more advanced laboratories frequently multiplexing up to 12–14 markers, a study for deep phenotyping often requires staining aliquots of samples with multiple cocktails of antibodies. This approach can also pose challenges in clinical studies in which sample volume is often limited. Thus the ability to multiplex up to 40 different markers makes mass cytometry ideally suited for phenotypic characterization of preclinical and clinical samples in limited sample volumes.

**Cellular Barcoding and Sample Multiplexing.** Cell barcoding in cytometric analyses was first developed for fluorescence-based flow cytometry (Krutzik et al., 2008, 2011). The technique involves barcoding cells with a unique combination of amine-reactive fluorescent dyes that are not used as fluorescent tags on antibodies. Since each cell population has a unique fluorescent barcode, the different cell populations can be mixed together and stained with a cocktail of fluorescently tagged antibodies in a single tube. Using a combination of different intensities of three different fluorescent amine-reactive dyes, 62–216 unique barcodes can be generated, providing sufficient barcode choices to label each well of a 96-well plate (Krutzik et al., 2008).

Bodenmiller et al. (2012) extended the barcoding technique to mass cytometry by developing amine-reactive DOTA polymer–based metal-chelating reagents. Using seven metals in a binary fashion, the investigators generated 128 unique metal-based barcodes to uniquely label each well of a 96-well plate. The wells were subsequently combined into one tube for further sample processing and data collection. The resultant data were deconvoluted to yield results for 96 distinct sample treatments.

Cell barcoding offers several advantages for high-throughput assays, including a dramatic reduction in data acquisition time, ~100-fold reduction in antibody volumes, and minimal experimental variation introduced by sample handling (Krutzik et al., 2008). Considering the application to the drug discovery development continuum, this technique can be combined with functional assays (e.g., phospho-signaling and intracellular cytokine assays) to enable fast and efficient screening of drugs during discovery, and can be extended to multiplexing assays in longitudinal clinical studies. Since current mass cytometers can multiplex up to 40 different metals tags, dedicating a few channels for cell barcoding still leaves upwards of 30 different channels for cellular phenotypic and functional markers.

**Functional Assays**

**A. Cell Signaling State Characterization.** Antigen- or cytokine-induced phospho-signaling pathways (Hale and Nolan, 2006; Perez and Nolan, 2006) have been used as sensitive and early markers of cell activation in clinical studies to characterize cancers (Lim et al., 2012), measure intended targets (Strumberg et al., 2005; Crump et al., 2010; Brandwein et al., 2011; Pallis et al., 2012), and assess the off-target effects of drugs (Powers et al., 2011). However, it is increasingly recognized that a full appreciation of the nature and extent of cellular activation can be best assessed by simultaneously mapping several intersecting signaling pathways in single cells (Bonilla et al., 2008). To date, phospho-protein analysis by flow cytometry has been the tool of choice and may continue to be the choice for targeted phospho-protein measurements in clinical trials. However, the limited numbers of fluorophores available and the incompatibility of protein fluorophores to alcohol-based permeabilization in phospho-protein assays limit the ability of multiplexing to simultaneously measure multiple phosphorylation targets. In contrast, the metal tags used in mass cytometry are not affected by alcohol-based permeabilization reagents, making mass cytometry more compatible with highly multiplexed phospho-signaling assays in early drug discovery, biomarker development, and assessing biologics immunogenicity.

Several studies have highlighted the versatility of mass cytometry to analyze the signaling state of immune cells. In one study the investigators analyzed the signaling state of normal human bone marrow cells in response to external cytokine stimuli in the presence or absence of signaling inhibitors (Bendall et al., 2011), whereas in a different study the same investigators used cell barcoding strategies to perform pharmacological measurements of kinase activation and inhibition in cell lines upon exposure to cytokine stimuli in the presence or absence of kinase inhibitors (Bodenmiller et al., 2012). In a recent Immunological Genome Project collaborative study investigators used the multiplexing power of mass cytometry to combine several cell surface markers with 15 markers of the cell-signaling state across 15 time points to elucidate the transcriptional landscape of αβ T-cell differentiation (Darzynekiewicz, 2012). The success of these studies was singularly dependent on the ability to perform high-dimensional measurements on single cells, which required multiplexing upwards of 20–30 independent cellular measurements. Furthermore, clinical trials often necessitate adoption of technology that can multiplex upwards of 20–30 markers on limited sample volumes. Thus the high number of simultaneous cellular measurements, the ability to multiplex samples by cell barcoding, and virtual absence of the need for compensation make mass cytometry uniquely suited for kinetic or single time-point assessment of the signaling state of cells.

**B. Intracellular Cytokine Analysis.** Intracellular cytokine assays are widely used as functional markers of immune activation in assessing: 1) disease state, 2) therapeutic response, 3) drug immunogenicity, 4) response to vaccination, or 5) autoimmune disease status. Several studies have established the necessity to use multiparameter (>9-color) flow panels to accurately identify the cellular and functional phenotype of immune cells (De Rosa et al., 2001; Roederer et al., 2004). Whereas multicolor flow cytometry panels may continue to be commonly employed for intracellular cytokine staining assays worldwide, the sample limitations and technical expertise required to run complex multicolor flow cytometry experiments can be challenging (McLaughlin et al., 2008a,b). Even the use of prestandardized panels can only allow for the measurement of not more than three cytokines in a panel when combined with several surface markers (Maecker, 2009). The higher capacity of multiplexing and virtual
absence of fluorescence-style compensation in mass spectrometry makes it an attractive alternative in preclinical and clinical studies that aim to extract maximum biomarker measurements from a limited-volume sample. A well-designed mass cytometry panel that includes a comprehensive set of surface phenotyping markers, activation markers, and cytokines would not only be immensely beneficial in assessing the primary endpoints of the study but would also enable early detection of undesirable immune cell activation and cytokine secretion in response to therapy.

C. Cell Cycle Analysis. Cell cycle analysis is a useful tool in measuring cell activation, malignancy, or as a marker for cell health. Cell cycling assays by flow cytometry have traditionally used DNA and RNA intercalating dyes (e.g., propidium iodide, 7-AAD, pyronin Y) in combination with bromodeoxyuridine assays that mark replicating cells (Darzynkiewicz, 2010; Darzynkiewicz et al., 2010). A recent study adapted mass cytometry to analyze stages of the cell cycle in both cell lines and mitogen-stimulated peripheral blood mononuclear cells, and demonstrated the feasibility of cell cycle analysis by mass cytometry (Behbehani et al., 2012). This study combined stage-specific cell cycle protein markers (e.g., cyclins, retinoblastoma proteins, and phosphohistones) with DNA dyes (Ir-based DNA intercalators and iodine deoxyuridine) to identify individual stages of the cell cycle. Although this strategy opens new possibilities for simultaneous assessment of the cell cycle and detailed cellular phenotype in complex mixtures of cells (e.g., blood from cancer patients), further optimization of mass cytometry techniques is required to achieve the depth of cell cycle analysis currently available using flow cytometry protocols (Darzynkiewicz, 2012).

D. Receptor Quantification. Quantification of cell surface receptor or protein levels [measured as antibodies bound per cell (ABC)] is central to theoretical modeling and simulation of drug pharmacokinetics and pharmacodynamics. Often ex vivo measurements on representative cell lines or primary cells from healthy donors are used to predict the kinetics of drug binding to the intended target, for example, a monoclonal antibody directed against a membrane-bound receptor, and the subsequent internalization. Currently, several flow cytometry–based assays are available for the quantification of cell surface antigens of which the Quantibrite method (BD Biosciences, San Jose, CA) is most widely used. This bead method requires an antibody conjugated to phycoerythrin (PE) at a ratio of one PE molecule per molecule of antibody. Often this requires custom conjugation of therapeutic antibodies to PE and subsequent purification of desired conjugates, which results in low yields of the conjugated antibody.

Since mass cytometers can be quantitative, in theory the measurement of receptors on the cell surface can be achieved by direct staining with metal-conjugated antibodies without the need for standard beads or conjugation at a predetermined ratio of metal tag to antibody (e.g., PE needs to be conjugated 1:1 to the staining antibody). Once the ratio of metal bound–antibody molecule is accurately determined using traditional ICP-MS, ABC can be calculated from the staining intensity on cells and the transmission coefficients for the metal tag in the mass cytometer, a number which is determined prior to the ABC measurement.

Wang et al. compared traditional flow cytometry and mass cytometry–based methods to quantify the CD4 number (ABC) of T cells from live or cryopreserved human peripheral blood mononuclear cells (Wang et al., 2012). The ABC for CD4 obtained by mass cytometry was consistent with the number obtained with Quantibrite bead–based flow cytometric methods, thus demonstrating the utility of mass cytometry in receptor number quantification. Whereas ABC determination by Quantibrite bead–based methods does not allow for the simultaneous measurement of multiple markers in a single assay, with mass cytometry, in contrast, every metal channel can theoretically be used for ABC measurements independent of the other metal tags. This allows for the multiplexing of ABC quantification against either single or multiple receptors in a complex mixture of cells.

### Potential Role of Mass Cytometry in Drug Development

Mass cytometry has numerous emerging and potential applications in various stages of drug research. These assays include drug screening, deep phenotyping, biomarker discovery, and pharmacodynamic marker discovery. All assays on the mass cytometer require cells to be fixed as cells are introduced into the machine in water to minimize salt buildup on cones. Whereas this feature is advantageous in minimizing exposure to biologically hazardous material, it precludes assays that require use of live cells. Thus, while flow cytometry can be used to assay metabolites such as reactive oxygen species or calcium signaling, such assays cannot be currently performed on a mass cytometer.

In the discovery and preclinical phases currently, flow cytometry is widely employed in screening compounds for hits in high-throughput systems via cell lines with reporter proteins, sorting (cloning) cells during cell line development, and functional assays and biomarker discovery. Whereas flow cytometry will continue to be used for targeted screening within a compound library, mass cytometry is anticipated to play a major role in elucidating the mechanism of action of a lead compound selected after a prescreen for potential therapeutic benefit. For example, in screening for kinase inhibitors, mass cytometry could be used to more fully elucidate the pharmacology of the inhibitors by measuring the kinetic parameters of the primary target and simultaneously measuring the off-target effects of the inhibitors on other kinases as well as pathway changes up- or downstream of the target. In other words, mass cytometry can enable researchers to create a kinase inhibition fingerprint of the potential

### TABLE 1

Comparison of features of mass cytometry and flow cytometry

<table>
<thead>
<tr>
<th>Feature</th>
<th>Flow Cytometry</th>
<th>Mass Cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of biomarkers that can be multiplexed</td>
<td>3–18 Parameters</td>
<td>Up to 40 parameters (the instrument is capable of multiplexing 100 biomarkers)</td>
</tr>
<tr>
<td>Compensation</td>
<td>Fluorescence correction absolutely required</td>
<td>No fluorescence-style correction required. However, correction for isotopic impurities in metal tags and oxides is needed</td>
</tr>
<tr>
<td>Status of cells</td>
<td>Both live and fixed cells can be analyzed</td>
<td>Cells are required to be fixed prior to analysis</td>
</tr>
<tr>
<td>Cell sorting</td>
<td>Yes (in sorters)</td>
<td>No</td>
</tr>
<tr>
<td>Efficiency of analysis of injected samples</td>
<td>&gt;95%</td>
<td>30–40% of injected cells are analyzed</td>
</tr>
<tr>
<td>Speed of acquisition</td>
<td>Up to 60,000 cells/s</td>
<td>Up to 1000 cells/s</td>
</tr>
<tr>
<td>High-throughput compatible</td>
<td>Yes (with a 96-well format)</td>
<td>Yes (with a 96-well format)</td>
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therapeutic, which can play a major role in go or no-go decisions during early drug research. Furthermore, mass cytometers also allow multiplexing signaling assays with deep phenotyping and other functional assays and thus enable researchers to conduct the preclinical assessment on complex mixtures of primary cells isolated from blood or other tissues. This is expected to become a key contribution to translational pharmacology efforts as the therapeutic and target are transitioned into clinical development.

In the clinical phase of drug development, as the lead therapeutic enters clinical trials the focus often shifts to measuring defined biomarkers of therapeutic efficacy, disease severity, or unintended effects of the therapeutic. The ability to multiplex over 40 cellular measurements gives mass cytometry a unique advantage in measuring the multiple biomarkers in limited sample volumes. Deep phenotyping combined with functional assays such as intracellular cytokine assays and basal levels of critical phospho-proteins levels (e.g., transcription factors such as signal transducers and activators of transcription, nuclear factor-κ B) linked to disease or cellular dysfunction can be used in patient stratification to identify target populations for therapy.

In essence, mass cytometry is anticipated to accelerate discovery of pharmacodynamic markers for dose optimization, toxicological markers to assess drug safety, and biomarkers of disease severity and therapeutic efficacy in proof-of-concept studies. Successful application of mass cytometry in drug development will require a thorough comparison with current standard technologies and rigorous validation of biomarkers assays, ultimately leading to an expected increased adoption in pharmaceutical industry.

Flow Cytometry or Mass Cytometry: Which One to Choose and When

Mass cytometry is in its infancy and is yet to be extensively adopted for use in drug development. The establishment of mass cytometers entails significant investment in infrastructure and training of personnel for operation and maintenance of the instrument. Although the workflow for sample preparation and staining is very similar to the workflow currently used in flow cytometry assays, optimization of workflow is still required to achieve high-quality results.

A majority of commonly used antibodies for phenotyping and intracellular cytokine and phospho-signaling assays are commercially available as ready-to-use metal-conjugated antibodies. These are available either as standalone reagents or as preformulated kits (11-marker mouse phenotyping kit, 17-marker human phenotyping kit, and an 11-marker T cell deep phenotyping kit) that can be either used independently or further combined to yield a >30-marker cocktail. In addition, commercially available antibody-labeling kits provide the flexibility to generate custom metal-conjugated antibodies. Almost all antibodies validated for use in flow cytometry can be used in mass cytometry, although the staining indices (a function of efficiency of conjugation and the sensitivity of the instrument) could be dramatically different between the two technologies. This can limit the use of certain antibody-metal conjugates in mass cytometers, whereas their fluorescently conjugated counterparts function well in flow assays (Chattopadhyay et al., 2014). Thus, development of an antibody panel for the mass cytometer requires significant time and effort in reagent standardization and optimization.

We hold the opinion that flow cytometry and mass cytometry are complementary technologies, each with unique advantages and limitations (see Table 1). The choice of the technology depends on several factors, including degree of multiplexing desired, whether the assay requires analysis of live cells or fixed cells, downstream workflows using sorted cells, availability of appropriately tagged reagents (fluorophore-tagged or metal-tagged), sample size, desired speed of acquisition, access to instrumentation, and availability of expertise for data analysis. For clinical studies, the study design, single- or multisite data acquisition, and good laboratory practice certification for instrumentation would further influence the choice of technology.

Mass cytometry promises to impact the mechanistic study of biologics, preclinical assessment of drug toxicity, and unwanted immunogenicity, phospho-signaling analysis, and biomarker discovery for the biopharmaceutical industry. On the basis of early publications and the high level of interest in this technology in academia and industry alike, we project an increasing role for mass cytometers in drug development in the near future. Early adoption, rigorous validation, and an open collaborative communication between academia and industry were instrumental in advancing instrumentation, reagent availability, and assay development for flow cytometry. We believe mass cytometry would also benefit from a similar approach.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Atkuri, Stevens, Neubert.

References
