Review

Cell
P R E S S

A deep profiler's guide to cytometry

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In recent years, advances in technology have provided us with tools to quantify the expression of multiple genes in individual cells. The ability to measure simultaneously multiple genes in the same cell is necessary to resolve the great diversity of cell subsets, as well as to define their function in the host. Fluorescence-based flow cytometry is the benchmark for this; with it, we can quantify 18 proteins per cell, at >10 000 cells/s. Mass cytometry is a new technology that promises to extend these capabilities significantly. Immunophenotyping by mass spectrometry provides the ability to measure >36 proteins at a rate of 1000 cells/s. We review these cytometric technologies, capable of high-content, high-throughput single-cell assays.

The case for deep profiling

To understand the biological actions of cells and their mechanisms of differentiation, we must understand how phenotype and function are structured across diverse cell types and tissues. This structure can be perturbed by innate or infectious sources, which may drive disease pathogenesis; therefore, understanding it is crucial for identifying treatments and preventions. Great cellular diversity underlies this organization, so measurements taken at the single-cell level that encompass RNA, protein and glycan species ('high content') across many cells ('high throughput') will greatly aid our formulation of a more comprehensive understanding. In many respects, this is walking the path previously trodden by genomics and proteomics – long accustomed to thinking about many target markers per experiment. However, traditional single-cell analysis has focused on many cells and a few parameters per experiment. As we delve into more complex cellular systems, such as cellular signaling networks or T cell functional responses, we must reorient this thinking to consider many parameters in many cells; in essence, 'deep profiling' every single cell from a representative population of cells.

Among well-established technologies for cellular analysis, flow cytometry is unique for its ability to interrogate rapidly multiple biologic signatures (protein epitopes, nucleic acids, ion concentrations) simultaneously within a single cell. Over the past 40 years, since the introduction of the first fluorescence-based flow cytometers, the maximum number of proteins that can be simultaneously measured has progressively increased. These advances can be attributed to parallel achievements in hardware, fluorochromes

and data analysis, and have led to state-of-the-art 20-parameter flow cytometers. Concomitant with this development, our understanding of immunology and stem cell biology has matured tremendously with the discovery of scores of functionally diverse cell populations. Here, we review the development and highlight applications of polychromatic flow cytometry (PFC, 6+ colors). In addition, we review recent advances in a next-generation, 'post-fluorescence' single-cell technology termed mass cytometry, which is theoretically capable of measuring 70–100 parameters. Both fluorescence and mass cytometry have unique and powerful features, as well as unique challenges and limitations. Over the next decade, these complementary technologies will play central roles in dissecting the complex interactions of cells.

The polychromatic era

Technical achievements that led to PFC

The development of PFC required multiple stepwise advancements in hardware and reagents. For example, the earliest fluorescence-based cytometers used arc lamps, developed originally for microscopy, emitting light at a broad spectrum of wavelengths [1]. This light interfered with fluorochrome-derived signals, therefore, arc-lamps were not easily used for multi-color detection. By 1974, in the Herzenberg Laboratory at Stanford University, argon lasers, emitting a single wavelength (488 nm) were used as excitation sources for fluorescein [2]. The high power of these lasers dramatically increased sensitivity, allowing resolution of weakly fluorescent signals [3]. Two-color fluorescence detection, using fluorescein and rhodamine dyes, was followed by adding krypton lasers in the 1970s [4]. Over time, these expensive water-cooled lasers have been replaced with HeNe lasers [5], and eventually solid-state lasers of multiple lines. Such lasers were ideal for excitation of an important new class of fluorochromes made of phycobiliproteins, including phycoerythrin (PE) and allophycocyanin (APC) [6]. The recent use of high-powered lasers specifically tuned to excited PE and APC is crucial to successful PFC, for which sensitivity is a major hurdle [7].

Generally, these engineering achievements slightly predated the introduction of new organic and inorganic fluorochromes. In the late 1980s (Figure 1), the remarkable ability of PE to absorb and transfer energy to other fluorescent molecules was recognized and exploited to produce an array of tandem dyes (e.g. PE-Texas Red, PE-Cy5, PE-Cy5.5, and PE-Cy7) [8,9]. In the 1990s, APC-based tandem dyes were synthesized [9], and a large spectrally resolved series of small organic dyes (known as the Alexa dyes)
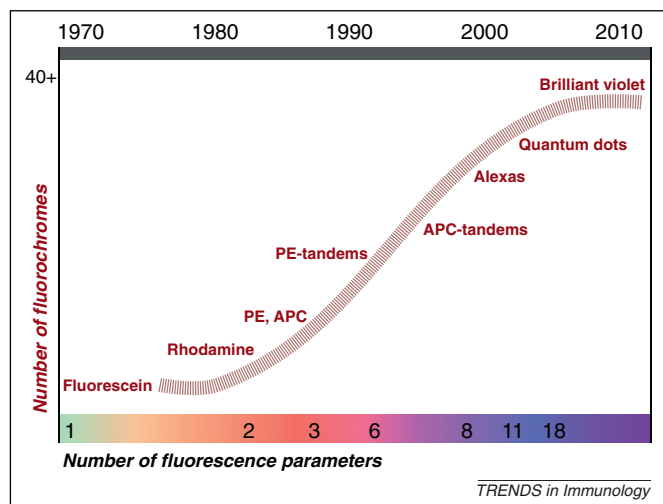
Figure 1. History of fluorochrome development. A timeline showing when the major fluorochromes were introduced, and how this related to the maximum number of parameters that could be simultaneously measured at that time.

became commercially available [10]. With this arsenal of lasers and fluorochromes, PFC graduated through eight (1998) to 11 (2001) colors [11,12]. During this period, violet (405 nm) lasers became available; however, there were few useful violet-excitable fluorochromes for immunopheno-typing. This changed with the introduction of a series of fluorescent, inorganic semiconductor nanocrystals (called quantum dots) in 2004, and led to the current state-of-the-art in PFC 18-color cytometry [13]. Recently, additional violet-excitable fluorochromes were developed based on the Nobel Prize winning discovery of organic polymers that conduct electrons [14]. These dyes and their resonance-energy tandems provide additional, bright options at a variety of wavelengths – for many experiments, they are biochemically more suitable for immunophenotyping than quantum dots [15]; however, they do not increase the number of unique fluorescence parameters that can be measured, and thus 18 colors remains the current maximum.

It is noteworthy that development of 18-color flow cytometry did not arise solely from achievements in laser and fluorochrome technology. Engineering advances in the optics (optimal light collection and delivery to detectors) and signal processing (digital electronics) were important, and continue to be areas of active development. In addition, software was developed to process rapidly the raw data and correct fluorescence spillover between dyes (a process known as 'compensation'). Similarly, tools to display and analyze 20-parameter data, and aggregate complex analyses across hundreds or thousands of specimens have been developed.

In summary, development of PFC required improvements in nearly every component of flow cytometry technology, including chemistry (dye development), hardware and software. All areas are still targeted for continued improvement.

Applications of PFC
Technical advances in flow cytometry have come hand-in-hand with a deeper understanding of hematopoietic cell types and function. The earliest cytometers resolved major

cell lineages, such as T and B cells, allowing characterization of cell-mediated and humoral immunity [16,17]. Later, with the development of monoclonal antibodies in the 1970s [18] and the emergence of the HIV epidemic in the 1980s [19], three-color measurements of T cell subsets (CD4$^+$ helper and CD8$^+$ cytotoxic) became important clinical research and diagnostic tools. Similarly, leukemia and lymphoma typing and staging have become reliant on flow cytometry – first as two-color panels, but now using 8–10 colors for better fidelity [20,21]. In the 1990s, with the availability of additional fluorochromes, naïve and various memory T cell subsets were distinguished [22], and cytokine production was quantified [23]. More recently, advances in PFC instrumentation have provided unprecedented resolution of immune system cells, for example, regulatory T cells [24], follicular helper T cells [25], and T helper 17 cells [26], to name a few. The availability of increasingly complex flow cytometry technology has driven a progressively more detailed understanding of immune cell subsets and functions.

A recent testament to the utility of 15-color experiments is the identification of memory T cells with stem-like properties [27]. These T$_{SCM}$s are phenotypically identical to naïve cells with respect to a number of markers (CD45RA$^+$, CD45RO$^-$, CCR7$^+$, CD62L$^+$, CD27$^+$, CD28$^+$ and CD127$^+$), but the cells express higher levels of CD95 and interleukin 2 receptor b; identification and characterization are only possible by the simultaneous measurement of 12 cell-surface markers. T$_{SCM}$s have enormous proliferative capacity, can reconstitute immunodeficient hosts, and mediate antitumor responses in a humanized mouse model [27]. These properties suggest that T$_{SCM}$s are important in the maintenance of immunological memory.

Indeed, the utility of advanced PFC (15+ colors) is apparent in a variety of other biological settings; however, designing the complex staining panels required is difficult [28]. Yet, there is a demand for even more measurements to be performed on a cell-by-cell basis – for example, the need to characterize the expression of multiple chemokine receptors on T$_{SCM}$s requires adding those markers to an already complex 15-color panel. Similarly, barcoding schemes [29] – which allow cells from different samples or stimulation condition to be mixed for high-throughput analysis – require 2–3 additional fluorescence parameters, as demonstrated in cell signaling studies of healthy and malignant leukocytes.

The need for a higher level of multiparametric analysis of single cells cannot currently be met with fluorescence technologies because of the limitation of the number of spectrally resolvable fluorochromes. This creates an opportunity for new technologies to complement PFC for cellular analysis.

The post-fluorescence era: mass cytometry
A new platform has been developed that couples flow cytometry with mass spectrometry. This technology, known as mass cytometry, offers single-cell analysis of at least 45 simultaneous parameters without fluorescent agents or interference from spectral overlap (Figure 2). For this, stable (nonradioactive) isotopes of nonbiological, rare
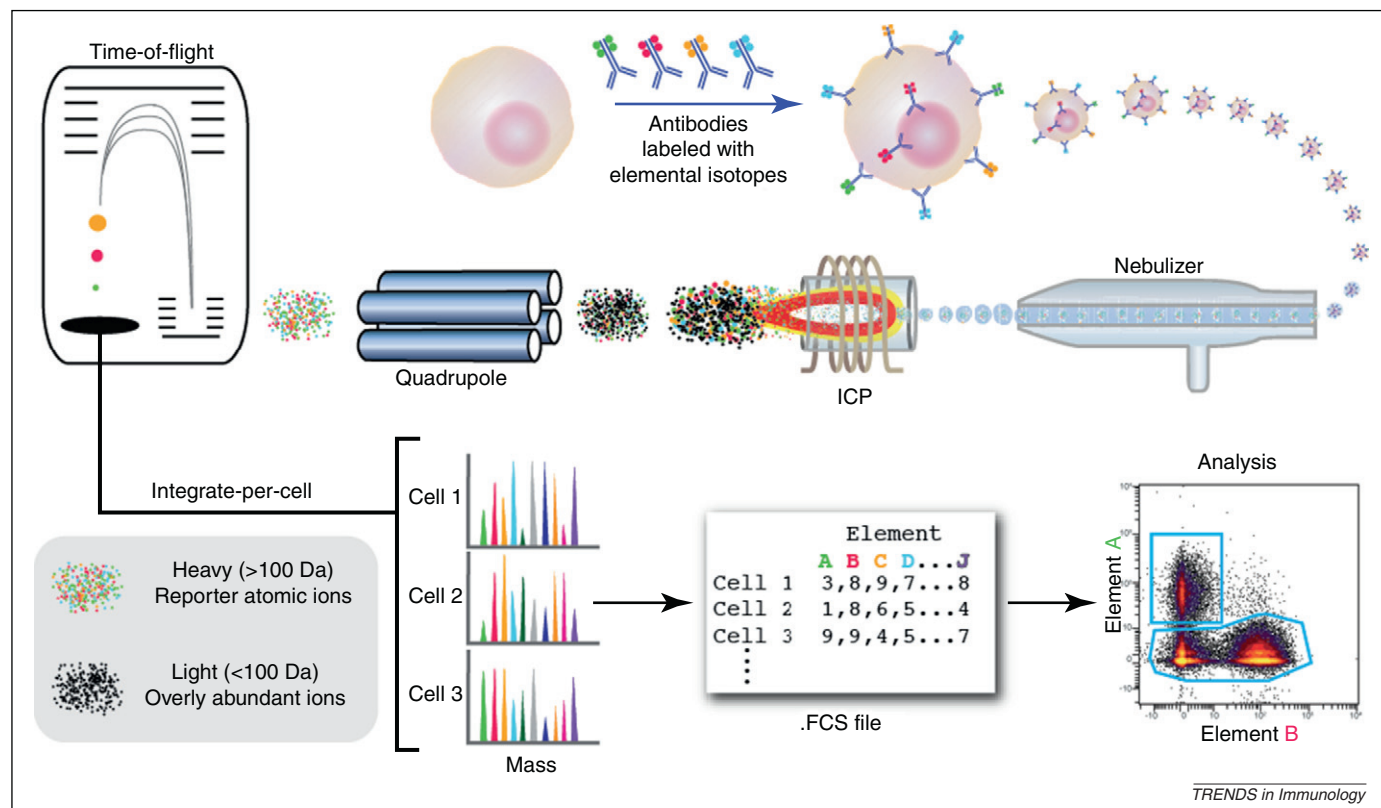
Figure 2. Mass cytometry allows single-cell atomic mass spectrometry of heavy elemental (>100 Da) reporters. Schematic of ICP-MS-based analysis of cellular markers. An affinity product (e.g. antibody) tagged with a specific element binds to the cellular epitope. The cell is introduced into the ICP by droplet nebulization. Each cell is atomized, ionized, overly abundant ions removed, and the elemental composition of remaining heavy elements (reporters) is determined. Signals corresponding to each elemental tag are then correlated with the presence of the respective marker and analyzed using conventional cytometry platforms.

earth metals are used as reporters. By exploiting the resolution, sensitivity and dynamic range of mass spectrometry on a time-scale that allows the measurement of 1000 individual cells per second, this configuration offers a new approach to high-content cytometric analysis.

Elemental mass spectrometry
Inductively coupled plasma mass spectrometry (ICP-MS) is the most advanced and sensitive means of determining the elemental composition of materials [30]. Classically, it has been used for ultra-trace (10^{-15} g/ml) detection of metals and other elements in both environmental (water, soil and air) and clinical (blood and urine) samples. The central component of this system is a high-temperature plasma (~7000 K), which vaporizes the sample, breaks all molecular bonds, and strips one electron from each atom. This creates a cloud of elemental ions, from which the relative abundance of isotopes can be determined. The ability to detect and quantify trace levels of multiple, nonbiologic elements from complex matrices makes ICP-MS an ideal detection tool for biological studies [31–38].

Mass cytometry
Mass cytometry is the adaptation of ICP-MS to single-cell analysis [39], based on the concept that a purified single isotope could be used to tag antibodies, and that these conjugates could be quantified in an ICP-MS detection system. Mass cytometry has essentially the same workflow as conventional flow cytometry (Figure 2). Cells are stained with target-specific antibodies labeled with metal isotopes

(typically lanthanide metals) [37,40]; these are the same antibody clones used in conventional cytometry. Cells are also stained with rhodium- or iridium-conjugated DNA intercalators, providing a baseline for detection and information about DNA content [33]. The use of differential intercalator staining [36] as well as chemical labeling with chelated metals [41,42] provides a viability measure. In the instrument, stained cells are nebulized into single-cell droplets and introduced into the plasma. The resulting charged atomic ion clouds are immediately transferred into the high vacuum of the mass spectrometer.

All cellular material is ionized, therefore, atomic ions are produced from elements common in cells (such as carbon, nitrogen and oxygen), along with ions from the argon plasma itself. To resolve the probe ions (e.g. lanthanides) from these overly abundant ions, the mass cytometer is configured as a quadrupole-time-of-flight (qTOF) instrument [30]. The quadrupole acts as a filter allowing only the heavier elemental ions, which consist primarily of the reporter masses, to be quantitated by TOF mass analysis.

For a typical cell, the ion cloud has a lifetime of ~300 μs over which it is measured (scanned) 20–30 times by TOF-mass spectrometry. This lifetime precludes analysis of >1000 cells/s, as single cells cannot be resolved beyond this rate. At lower rates, the system is remarkably robust; there is little measurable signal (background) between cells, as the elemental reporters used are uncommon in a biological context or within the laboratory environment. The amount of each isotopic reporter is quantified for the

Table 1. Comparison of utility and performance of state of the art commercial fluorescence flow cytometry and mass cytometry single-cell analysis platforms.

Technology		Fluorescence flow cytometry	Mass cytometry
Measurement basis		Fluorescent probes	Stable mass isotope probes
Experimental design			
Max no. of measurements		20 (18 fluorescence)	37 (including DNA)
Theoretical no. of subsets[a]		2.6×10^5	1.4×10^{11}
Panel design complexity (no. of probes)	Easy	<8	37
	Moderate	8–12	
	Hard	12–18	
Sensitivity range for different probes[b]		0.1–10	1–2
Sample throughput			
Sampling efficiency		> 95%	< 30%
Measured cells/s		25 000	500–1000
Cells/h		25–60 million	2 million
Commercial reagent cost			
Per probe per test[c]		$2.00–$8.00	$1.50–$3.00

[a]Theoretical number of subsets is the number of distinct cell types determinable, assuming only on or off for each marker; that is, 2^{colors}.

[b]Sensitivity range is in arbitrary units, and compares the rough sensitivity for different probes (fluorescence or ICP-MS) to detect a given epitope on a cell by immunophenotyping.

[c]Estimated based on the price of commercially conjugated reagents or unconjugated antibodies and commercial conjugation kits.

ion cloud of each cell by integrating across all scans for that cell. The resulting summary data are recorded in an FCS file format, so that they can be visualized with conventional flow cytometry software. Originally developed at the University of Toronto [39], the first commercial version of the mass cytometer, called the CyTOF, as well as associated reagents, are produced and distributed by DVS Sciences (www.dvssciences.com).

Comparisons of mass cytometry versus PFC
Although mass cytometry offers a number of unique features compared to PFC, the technology is relatively new and encompasses unique hurdles. PFC has the unique capability to work on live cells, and to be able to recover viable analyzed cells. Beyond this obvious difference, the two technologies are complementary – although there is overlap, each is well-suited to addressing a particular set of questions. The features of each technology are compared below, and summarized in Table 1.

Dimensionality. Years of hardware and reagent advances have preceded the complex, state-of-the-art PFC experiments reported in the past decade. By contrast, the learning curve for the first large mass cytometry experiment was much less steep [42]. This study – predicated on the fact that mass cytometry could exploit and adapt many established PFC principles – examined regulatory cell signaling behavior across hematopoietic cells using two 34-parameter panels, each of which included 31 antibody targets, a DNA intercalator, and measures of viability and cell size. One panel used 31 channels for cellular phenotyping, whereas the other simultaneously analyzed 18 intracellular phosphorylation responses and 13 cell-surface phenotypic markers in response to a variety of immunological perturbations. Less than 1 year later, a 37-parameter study of virus-specific T cell function and phenotype was reported [41]. These experiments used the maximum number of parameters currently accessible by mass cytometry.

To date, conventional PFC has shouldered a major burden in immunology. For instance, mapping the complex immune system requires measurement of a carefully selected set of 12+ markers and fluorophores matched with laser lines to call out the many attendant cell subpopulations of interest. However, as the number of markers reaches 15, designing PFC panels becomes laborious, with a current limit of 18 markers measured simultaneously. In cases in which multiple intracellular events (pathways) must be tracked, PFC cannot simultaneously detail multiple pathways across multiple cell subsets (i.e. on a cell-by-cell basis).

Therefore, the ability of mass cytometry to measure so many parameters, without the loss of sensitivity accompanying compensation, is an important advantage. However, there are a number of cellular qualities mass cytometry cannot yet measure. For example, forward and side scatter (FSC and SSC) are light-based measures of cell size and granularity commonly used in flow cytometry to discriminate large granular leukocytes, lymphocytes, doublets of cells, and cellular debris. These measures, which are used to filter out experimental artifacts or provide broad definitions of cell subsets, are not currently available for first generation mass cytometers. Additionally, small molecule fluorescent reporters for Ca^{2+} flux [43], mitochondrial permeation [44], and cell division (CFSE) [45] do not have metal-reporter equivalents. By contrast, there are also opportunities to measure novel metal parameters at the single-cell level including: platinum (cisplatin – a cancer drug), barium (magnetic resonance imaging contrast reagent), iodine (radioactive iodine therapy – for thyroid ablation), and gold (for experimental autoimmune therapy).

Sensitivity. Currently, the sensitivity of lanthanide-tagged antibodies is lower than that of the most popular fluorescent reporters [36,42] (Figure 3). The primary reason for this limitation is the chelating polymer [37,40] common to the commercially available probes. This

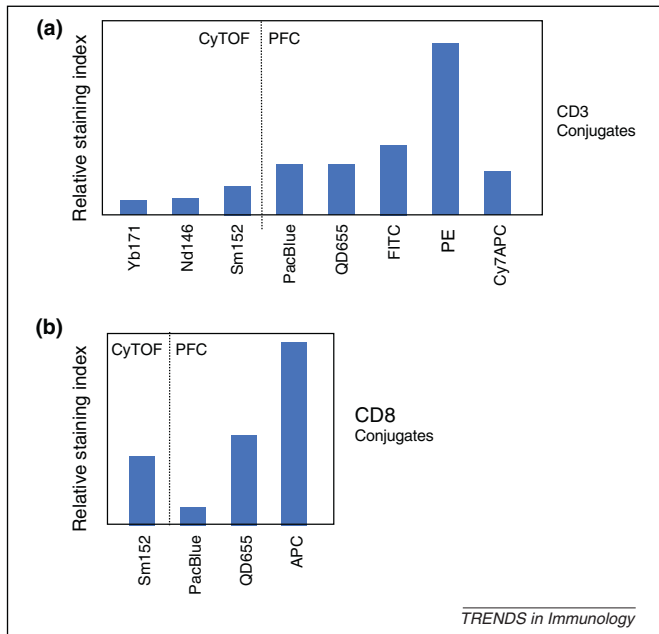**Figure 3**. Performance of various metal isotopes and fluorochromes. **(a)** Relative staining indices, calculated as described in [15], for a CD3 antibody conjugated to Yb171, neodynium 146 (Nd146), or samarium 152 (Sm152) and analyzed by CyTOF, or Pacific Blue (PacBlue), Quantum Dot 655 (QD655), fluorescein isothiocyanate (FITC), PE, or Cy7APC and analyzed by PFC. Staining indices by CyTOF are low. **(b)** Relative staining indices for mouse anti-human CD8 (clone RPA-T8) conjugated to Sm152 and analyzed by CyTOF, and conjugated to PacBlue, QD655, or APC and analyzed by PFC. Staining indices for CD8 conjugates were considerably higher than anti-CD3 antibodies, and CyTOF conjugates had better performance than PacBlue (PFC) conjugate. Note, low staining indices may be acceptable for many applications, because background staining can be low by CyTOF.

polymer allows a maximum of around 100 metal reporter ions (M^{3+}) to be attached to an antibody molecule, creating a ceiling on signal levels until alternative probes can be developed.

Although this has not prevented the measurement of many popular cytometric targets [36,41,42], it could preclude analysis of those with low signal-to-noise ratios. By contrast, the low background signals in mass cytometry (where elemental isotopes are not naturally found in cells) compared to flow cytometry (with inherent autofluorescence of cells), can balance this deficiency and thereby have a significant advantage in multiplexed measurement of subtle regulatory changes (e.g. protein phosphorylation [42]). In any event, an important advantage of mass cytometry is the similarity in the sensitivity across all lanthanide-based reporters – varying only 2-fold in sensitivity across isotopes from the lower to upper mass range. By contrast, the sensitivity of various fluorochromes can differ widely (10–50-fold) in flow cytometry and is a primary hurdle to development of multicolor panels [28].

Usability. The CyTOF mass cytometer provides three orders of magnitude of resolution between adjacent detection mass channels; pragmatically, two adjacent metal isotopes can differ in abundance by $\sim 10^3$ before spectral overlap arising from imperfect resolution of masses needs to be corrected by compensation (i.e. 0.1% spillover). As noted, the signal response for the majority of the lanthanide metal isotope reporters falls within approximately twofold of one another. Compared to fluorescence, where changing a single parameter might require redesign of an

entire analysis panel to avoid spectral overlap issues, these qualities of mass cytometry significantly simplify experimental panel design. Although mass cytometry all but eliminates spectral overlap issues, there can still be signal interference/overlap from isotopic impurities in the metal reporters (usually + and/or – 1 Da) and oxidation (+16 Da) of the reporter ions during analysis [30,34]. Notably, a wider variety of tagged antibodies are available for flow cytometry than for CyTOF; however, kits are available for in-house conjugation of even small quantities of purified antibody.

Quantitation. Both fluorescence [46] and mass-based [30,36,47] measurements are quantitative when the proper controls are used. The CyTOF mass cytometer is linear across almost four orders of magnitude regardless of the number of parameters measured. Fluorescence flow cytometers typically have a range of at least five orders of magnitude where linearity is often a function of the photon amplifiers. Practically speaking, this working dynamic range for fluorescence detection can be compromised both by autofluorescence as well as spectral spillover. Nevertheless, both mass and fluorescence cytometers use detection technologies that are highly mature, and thus are highly accurate. In terms of precision, for any given reagent, fluorescence measurements will be somewhat better than mass cytometry in theory; however, in practice this precision is already better than biological variation and so is probably irrelevant.

Sampling and throughput. Mass cytometry throughput is limited to about 1000 cells/s [39]; commercial fluorescence cytometers can operate at rates 25–50 times faster. In addition, cell injection and cleaning routines are time-consuming, increasing run times per sample. Moreover, unlike flow cytometry, where nearly all the cells introduced into the instrument are analyzed, the nebulization of single-cell droplets into the ICP is currently inefficient, allowing the measurement of about 30% of the cells in the samples. However, this loss can be overcome when a high number of analytical parameters are desired per sample – perhaps requiring one sample by mass cytometry and multiple runs on PFC. Moreover, this loss is stochastic so it does not appear to introduce sampling bias. Overall, the current generation mass cytometer can process about 8 samples/h, with about 250 000 processed events per sample (i.e. 2 million events/h). This analysis rate defines the lower limit for rare event detection that can be achieved, although as shown in Bendall *et al.* [42], this does not compromise detection of important, rare cell populations if one is willing to collect enough cells over a sufficient time frame.

Experimental. Overall, the current reagents available for mass cytometry may be utilized best for investigating intracellular regulatory molecules (where autofluorescence can be highly confounding), or in situations in which a high number of simultaneous measurements are needed. Rare event analysis, detailed phenotyping requiring the measurement of low abundant cellular targets or light-scatter properties and/or rapid analysis of individual samples for the time being may be better suited for fluorescence cytometry. For instance, improvements such as *in situ* single-cell amplification techniques using DNA branched

chains [48,49], polymers with more chelator sites allowed per antibody, or lanthanide nanocrystals might overcome certain sensitivity issues, whereas more efficient sampling interfaces with integrated optics for measuring light-based reporters are already on the horizon (see below).

Future developments in the post-fluorescence era
As has occurred throughout the history of flow cytometry, improvements in mass cytometry are likely to dramatically increase its utility. Current efforts are focused on several areas: throughput, mass and dynamic range, and development of additional and more sensitive metal isotope probes. Although the low cellular acquisition rate is a physical limitation of mass cytometry, a prototype auto-sampler has been introduced. This will help wash-out time between samples and allow automated acquisition. In addition, sample multiplexing techniques currently used in flow cytometry, such as fluorescent cell barcoding (FCB) [29,50], are also being adapted to mass cytometry. Use of barcoding, even in a binary approach, allows up to 128 conditions to be multiplexed using seven parameters (2^7), potentially leaving 30 or more parameters for single-cell measurement.

Conventional commercial ICP-MS instruments have a linear range of almost 10^9; however, for the CyTOF mass cytometer the range is 10^4 across a measurement window large enough to include all lanthanide isotopes. Interestingly, this is not an instrument limitation, but a limitation in the speed of computer hardware [40]. Without limitations in the speed of data digitization, the current CyTOF mass cytometer could actually measure all known nonbiological transition metals with a linear dynamic range of $\sim 10^6$ per cell. Future generations of instrumentation should be much improved in this regard.

Similarly, there are enough unique nonbiological elemental isotopes to move mass cytometry measurements well beyond 50 simultaneous parameters per cell (Figure 4) in the future. Two important hurdles accompany such development efforts. First is the effective attachment of the reporter isotopes to antibodies. Currently, reagents use polymeric chelators, such as DTPA (diethylene triamine pentaacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), which show a preference for metals with a positive charge of three in solution (e.g. the lanthanide series). The utilization of chelators targeting +2 or +4 metal ions (i.e. nonbiological transition metals Cd, Te, Pd, Ag, Sn, Os and Hf) could provide more than 20 additional measurement parameters. Second is the availability of pure forms of additional isotopes. For many elemental isotopes, purifying out contaminating isotopes becomes prohibitive, and many are available only with >5% contamination. Although this contamination does not affect the ability to quantify the probe, it does reintroduce the need for compensation and probable associated difficulties in panel development.

Increased sensitivity of mass cytometry instrumentation, as with all mass spectrometers, is expected to improve incrementally with subsequent generations. Improvement of sensitivity through reagent technology represents the most immediately promising area of mass cytometry analysis. With commercial chelating polymers, it is possible to

attach on the order of 100 metal atoms per antibody. Given the low ion transmission efficiency (1 in 10^4 ions reach the detector), at least 100 molecules must be present (theoretically) on a cell before a signal is observed, although, in practice, this limit of detection (LOD) is considerably higher. However, probes can be constructed with substantially higher metal content, reducing the LOD drastically. For example, solid metal nanoparticles, such as quantum dots [51], can serve as reporters in mass cytometry as well, and can contain as many as 10^5 atoms of a given metal. With the utility of quantum dots [42] and lanthanide nanoparticles [52,53] already demonstrated in mass cytometry, their optimized application could increase the number of atoms bound per antibody, thus lowering the LOD, increasing signal intensity, and providing better resolution of cell populations with low levels of target protein expression. Ultimately, tools like these may allow detection of single molecules on single cells.

Analysis of multiplexed, multiparametric data
Rapid increases in the numbers of measurable single-cell parameters, both in flow and mass cytometry, have brought a daunting increase in the complexity of the data. Analysis of flow cytometry data is typically manual, performed in one or two dimensions at a time by selecting subsets of interest from parent populations. This approach is not scalable, and suffers from individual user bias (Figure 5a). Moreover, it requires prior knowledge of the cell type of interest, so unexpected cell types may be overlooked.

For such analyses, Flowjo (www.treestar.com) is a popular stand-alone software package for flow cytometry. It contains features that simplify and standardize multi-sample analyses in which samples can be grouped and analyzed together, with tools such as density-based (magnetic) gating to reduce the effects of staining variability. It also includes a wide variety of graphical outputs for visually summarizing multi-dimensional experiments (e.g. polychromatic plots). Cytobank (www.cytobank.org) is a collaboration-centric, web-based analysis platform that has features considered common to flow analysis platforms, has a web-based data sharing and repository function for community-based analysis [54], and 'omics-styled heat map features for integrated analysis of phospho-flow studies – essential for intracellular signaling systems.

Although these latter platforms provide the means to analyze and summarize manually large sets of samples, in terms of population frequencies and expression levels, they do not provide a mechanism to identify overall trends. To address this, SPICE [55] goes a step further to help identify larger trends across user-defined cell populations in large study groups. Still, this approach relies on prior knowledge of existing cell populations. This is problematic for identifying unexpected changes in unanticipated cell populations within complex systems.

To this end, several analysis platforms have recently been developed to assist the search for small changes in n-dimensional datasets. The problem is illustrated in Figure 5a where, as the number of measured parameters increases, seeing the shape of the information requires huge sets of conventional 2D plots. It is akin to the problem

Figure 4. Periodic table summarizing the feasible elemental reporters for single-cell mass cytometry measurement in a biological matrix. Colored elements (green, blue, gray) are those with at least one (relatively) stable isotope having an atomic mass >100 Da. Green elements have been demonstrated in estimating DNA content and cell size [33,42]; blue elements have been conjugated to antibodies for cell-based mass cytometry measurements using either a chelating polymer [37,40] or semiconductor nanocrystals – Qdots [42]; and gray elements have not been published in mass cytometry studies yet but are readily analyzed by ICP-MS. These are future development targets.

of the blind men and the elephant – each seeing with their hands only a small part (a tail, the trunk) of the whole elephant and thereby not encompassing a view of the entire elephant. Now, imagine the problem with a 40-dimensional elephant for which we are equally blind because we cannot humanly see in more than 3D at best. Therefore, approaches are required to enable human-interpretable 2D visualizations that require minimal or readily managed user supervision.

Sam SPECTRAL [56] and density reduction [57] clustering approach this problem by offering different approaches to cluster cells based on the expression levels of various parameters. However, unlike SPICE, they do not provide mechanisms to compare directly these clusters across large sample sets. FLAME (flow analysis with automated multivariate estimation) [58] (Figure 5b) also performs unsupervised clustering, providing tabular summaries tools to compare clusters between samples visually. Most recently, spanning-tree progression analysis of density-normalized events (SPADE) (www.cytospade.org) has provided a platform to analyze large cohorts of samples in which cells are clustered in multidimensional space and then reduced to a 2D representation using a minimum spanning tree algorithm [59] (Figure 5d,e). This 2D model represents the relative relation between each cell cluster in all samples; can be used to interrogate the expression of various parameters between clusters; and provides the ability to compare clusters across samples. SPADE is now an integrated application in Cytobank.

Instead of clustering, there are other approaches that can leverage the complexity of n-dimensional single-cell datasets to lower dimensionality to biologically meaningful observations – for example, identifying the simplest combination of markers with biological or clinical relevance. Principle components analysis (PCA) has been used classically to calculate linear vectors through all measured parameters, thus identifying those combinations that describe the most variance in the data and relations between samples. However, this method is not generally useful to immunophenotyping data, because of the general lack of correlations of expression in most markers. To address this, FlowType [60], a new R-package, takes n-dimensional data, automatically defines populations, and exhaustively stratifies all possible combinations of markers, comparing them across different samples. The idea is to identify the simplest population from the n-dimensional dataset that differs between samples. This, SPADE, and similar packages are available through the Bioconductor project (www.bioconductor.org), and are being evaluated using common datasets against predefined performance metrics
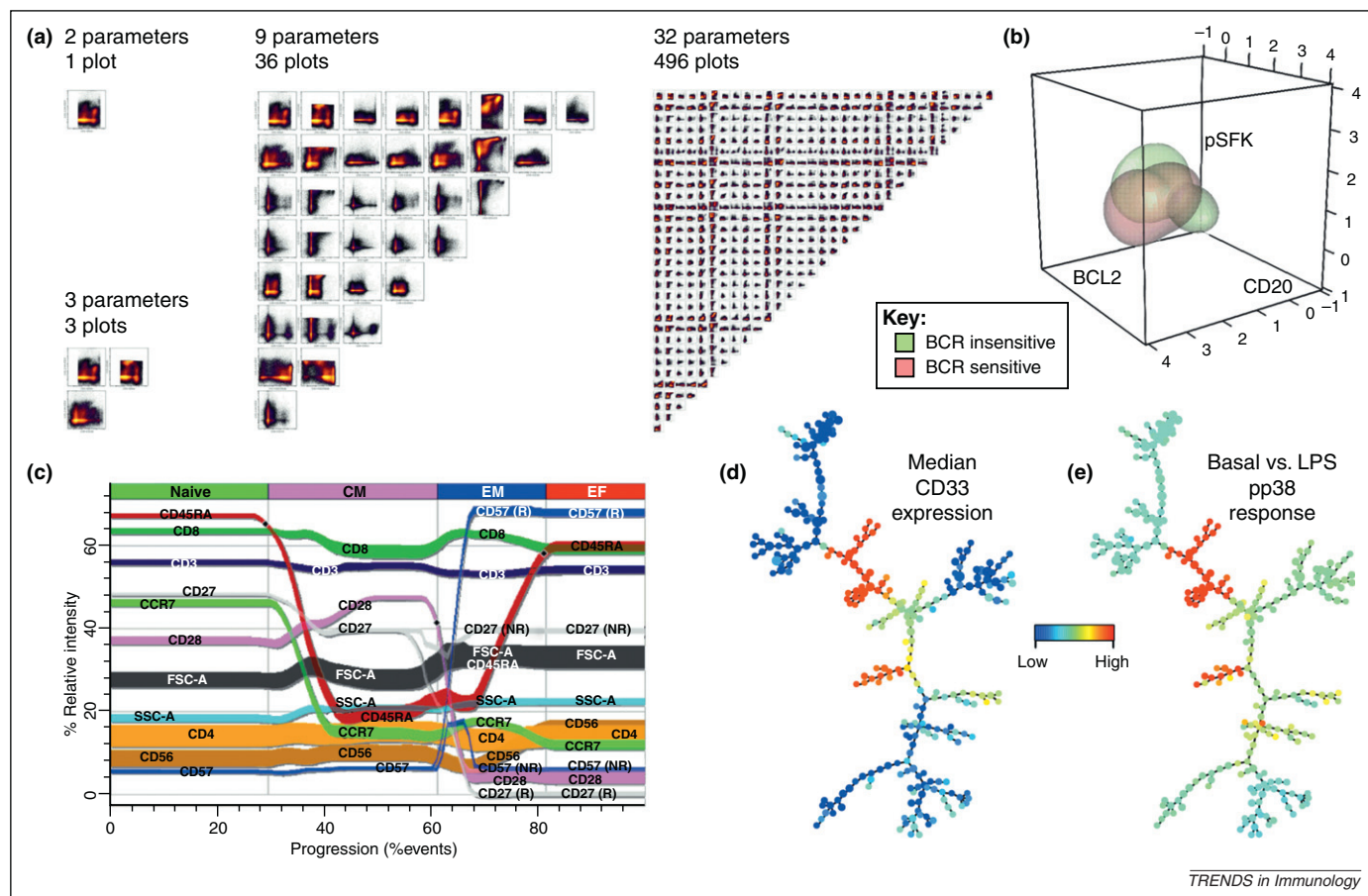
Figure 5. Complexity of multidimensional single-cell analysis. **(a)** Human-interpretable 2D scatter plots are not a scalable solution in single-cell analysis. As the number of single cell parameters increases, the number of unique 2D plots increases exponentially. **(b)** FLAME analysis of B lymphocytes. Clustered cell events graphically displayed for visual comparison of receptor signaling cell populations. **(c)** Gemstone analysis of CD8 T cells showing progression of phenotypic markers including branching expression of markers such as CD57. **(d and e)** SPADE plots representing clusters of cells in normal human bone marrow: expression of CD33 (monocytes) across all cell clusters **(d)**; level of phosphor p38 (pp38) measured in response to LPS stimulation and normalized to an unstimulated (basal) control **(e)**.

through the FlowCAP initiative (Flow Cytometry: Critical Assessment of Population Identification Methods – http://flowcap.flowsite.org/). Similarly the parameters expressed by a single cell can be linked to each other, on a cell-by-cell basis, to construct relationship networks or classifiers. This approach, which uses Bayesian inference, has been particularly useful to examine T cell receptor signaling, revealing interactions between regulatory phosphoproteins without biochemical interrogation [61], as well as investigating cell signaling feedback mechanisms [62,63].

Lastly, Gemstone (www.vsh.com) works differently to create a 2D summary of n-dimensional single cell datasets – exploiting the continuous expression patterns of various parameters (Figure 5). It employs probability state modeling to organize and visualize cell populations relative to one another [64]. Although this analysis usually requires *a priori* knowledge of the relationship between at least some of the markers measured, it still visually summarizes all cells in a given sample and can reveal cell subsets and relations that other tools may not.

Many of these approaches take into account an important problem in multiparametric analysis: the fact that simultaneous measurement of multiple markers can provide more information than is ultimately necessary for understanding the biology of a disease. Cells could be parsed too finely, into functionally redundant subsets.

Given this possibility, it is important to consider how many parameters must be measured simultaneously to address effectively a particular hypothesis. To arrive at such conclusions, however, high-dimensional, hypothesis-generating experiments, and the tools to analyze and distill them, will be necessary.

Concluding remarks
Over the past 40 years, continual improvement in single-cell analysis technologies has driven our investigation and understanding of immunology and stem cell biology. Pushing the multiparameter limits of fluorescence-based analysis has led to unprecedented studies of regulatory signaling in both the healthy and diseased hematopoietic system. It has also identified many distinct immune cell subsets – most of which have no assigned function. Now, next-generation, mass cytometry instrumentation will probably drive the next stage of deep profiling in mapping biological mechanisms of normal development; the role of multiple cell subsets in carrying out appropriate immune responses; and how any malfunction in these causes disease. Both fluorescence and mass cytometry will continue to be critical tools in cell biology for the foreseeable future; their complementarity with other single-cell applications and future improvements has the greatest promise for future discovery.

Conflict of interest

Acknowledgments

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