development scientists—for translating biomarker discovery into clinical reality.

The idea of systematically triaging a large number of candidate biomarkers to a subset of manageable size that can be rigorously analyzed hardly seems notable. What then distinguishes these studies from previous efforts in biomarker development? The answer lies in the combination of the experimental systems selected, the strategy of including only the best-characterized specimens, the use of comprehensive mass spectrometry approaches with the latest innovations in quantification, and a level of validation that exceeds that seen in most earlier studies[7].

Previous efforts to validate biomarkers have been thwarted by the bias and artifacts that result from the genetic and epigenetic variability of patients: gender, ethnicity, age, diet and environmental factors all contribute to a level of biological complexity beyond the scope of what can be typically interrogated. Whiteaker *et al.*[2] avoid the complexity that arises from the heterogeneity of cancers by using a simple, defined mouse model of breast cancer. This system provides duplicative biological samples sufficient to reach the high number of mass spectrometry measurements required to achieve the statistically necessary number of biological and technical replicates. A unique strength of the approach taken by Addona *et al.*[3] is to use each patient as their own control, thus considerably reducing genetic and nutritionally induced variability[8]. Admittedly, this design circumvents the complexity that undermines most biomarker discovery investigations. However, it does seem useful for certain kinetic analyses, for example, those involving planned perturbations to elicit physiological effects (e.g., exercise testing) or tests of drugs or diet. Moreover, together with the work of Whiteaker *et al.*[2], it shows that the analytical performance of targeted proteomic strategies is up to the challenge of triaging large numbers of biomarker candidates and circumventing dedicated immunoassays.

Whether the discovery strategies proposed by Whiteaker *et al.*[2] and Addona *et al.*[3] will become the mainstay of clinical biomarker development remains to be seen. We have no doubt that they will be adopted by many groups, at least in the near future. Regardless, the papers emphasize that mass spectrometry is becoming increasingly attractive for clinical diagnostics and has the potential to displace ELISAs, the current gold standard for biomarker validation. Specific quantitative immunoassays are not available for the majority of human proteins, and such assays are often prohibitively expensive to develop and difficult to multiplex. The cost per data point is already much lower for an SRM assay than for multiplexed ELISAs, and certain mass spectrometers even permit both quantification of proteotypic peptides and confirmation of peptide sequences as an added confidence measure. Validated SRM assays might also reach the market quickly as they do not require clinical reagents such as antibodies.

Neither Whiteaker *et al.*[2] nor Addona *et al.*[3] claim that their biomarker validation pipelines are optimized in terms of performance, cost or speed. Moreover, the clinical utility of their final candidates remains to be determined. Optimization of sample preparation and of separation and quantification algorithms will undoubtedly lead to future improvements, as will mass spectrometers with enhanced linear dynamic range for ion detection. Other label-free methods for proteomic quantification have been described recently[9–11] that are well suited for the early steps in biomarker discovery and clinical proteomic studies, and these may also play a role in improving biomarker discovery.

Finally, it is important to highlight the potential of combinations of biomarkers, each with imperfect sensitivity and specificity, to provide improved discriminatory and diagnostic power. Many pathologies, even when categorized as similar, are complex and have multiple etiologies, especially at the molecular level. They are therefore better defined by disease signatures than by single biomarkers[12,13]. Given that very few single biomarkers are likely to have the high sensitivity and specificity necessary to make diagnosis and treatment decisions, the field would do well to relinquish its somewhat myopic focus on single proteins and explore the predictive power of biomarker panels. This approach requires a fundamentally different clinical study design based on multivariate predictive signatures. Cooperation between clinical researchers, biostatisticians and regulatory authorities will continue to be indispensable for addressing these and other future challenges.

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Next-generation flow cytometry

Matthew R Janes & Christian Rommel

Mass cytometry dramatically enhances the dimensionality of fluorescence-based flow cytometry for phenotypic analysis of heterogeneous cell populations.

Unequivocal identification of rare cell types using a panel of diagnostic features usually requires simultaneous measurement of multiple biomarkers on a per cell basis[1]. However, for conventional fluorescence-based flow cytometry, the spectral overlap of fluorochrome markers complicates the measurement of multiple parameters for a single cell. In a recent paper in *Science*, Bendall *et al.*[2] increase the dimensionality of conventional flow cytometry by coupling it with atomic mass spectrometry through the use of antibodies tagged with stable isotopes of transition elements. They use the approach, named mass cytometry[3], to simultaneously measure 34 distinct cellular parameters in human bone marrow samples on a single-cell basis. This provides an unprecedented view of hematopoietic differentiation and the relationships between immunophenotype, cell signaling and drug action.

Over the past two decades, fluorescence-based flow cytometry has become a mainstay of clinical research. Antibodies designed to tag surface markers are color-coded with fluorochromes and passed in single file across the path of a laser that causes the dye molecules to emit a burst of light. Although hundreds, if not thousands, of fluorescent dyes are currently available for such measurements, the spectral overlap between these options routinely limits the capacity of flow cytometry to simultaneous quantification of only six to ten parameters.

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Combining the use of multiple lasers with panels of antibodies tagged with either dyes or quantum dots that fluoresce at different wavelengths has allowed up to 17 distinct antigens to be identified simultaneously from a sample[4]. However, the complications posed by signal overlap and cellular autofluorescence require a considerable amount of optimization for this level of multiplexing.

Bendall *et al.*[2] transcend this technical plateau by labeling cells with a cocktail of 31 epitope-specific antibodies, each conjugated to different isotopes of transition elements. (e.g., gadolinium, ytterbium, neodymium, samarium and dysprosium) through metal-chelating coupling reagents. Three additional parameters—DNA content, cell viability and cell length—provide a total of 34 parameters. They then spray single-cell droplets from the mixture of labeled cells through an argon plasma–filled inductive coil heated to a temperature of >5,500 K. Each cell is vaporized and ionized into its atomic constituents, and the number of each type of isotope-tagged antibody is quantified by time-of-flight mass spectrometry (**Fig. 1**, top). Because transition (many of which are so-called rare earth) elements are found at only trace levels in cells, the readout is much sharper than current alternatives that are plagued by cell-derived autofluorescence. The technology, which theoretically enables simultaneous evaluation of >100 parameters, is now commercially available as the CyTOF platform (DVSSciences, Markham, ON, Canada).

The increase in the number of cellular parameters that can be measured using mass cytometry necessitates new methods of data analysis. Flow cytometry data are typically interpreted by hierarchical gating analysis: two markers are used to group the cells into subpopulations, which are then further subdivided based on the levels of other pairs of markers. The choice of marker pairs and the boundaries between cell populations are not always easy to define. The method of Bendall *et al.*[2] circumvents the need for subjective pairwise analysis by clustering all of the high-dimensional data at once, identifying populations of cells with similar patterns of cellular markers. These populations are then visualized using a tree structure in which similar populations are placed close to each other. The size of each population is indicated in the tree plot by the size of the circle. Finally, 'lighting up' the tree with colors that show cell populations expressing specific markers (such as CD3 to identify T cells) allows specific regions and branches of the tree to be associated with biologically relevant cell types (**Fig. 1**, bottom left). Parsing the data in this manner presents it more clearly and informatively than is possible using the dot blots traditionally used for flow cytometry analyses.

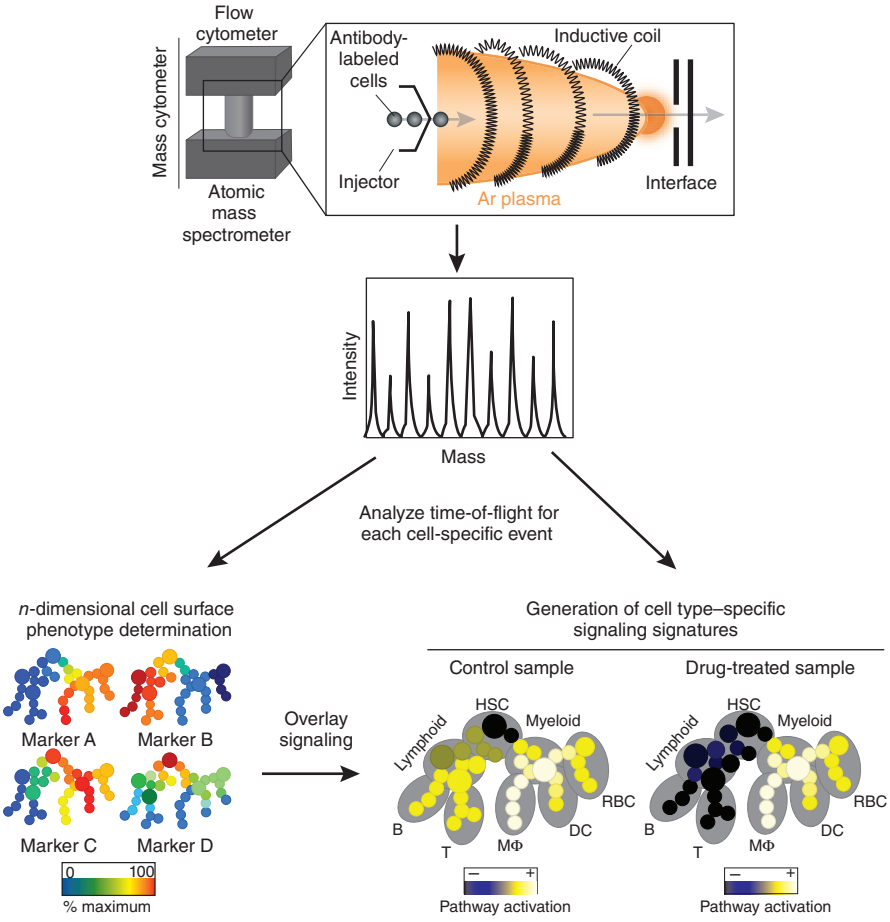

Figure 1 Mass cytometry permits high-dimensional immunophenotyping and dissection of signaling behavior in single cells. The approach (top) combines technologies from flow cytometry and atomic mass spectrometry and involves the use of antibodies tagged with distinct elemental isotopes to provide an elemental mass spectrum for each cell in the sample. Bendall *et al.*[2] use the approach to discriminate between cell types (bottom left) and to dissect intracellular signaling pathways in response to a drug (bottom right). HSC, hematopoietic stem cells; Mϕ, macrophage or monocyte; RBC, red blood cells; DC, dendritic cells; B, B cells ; T, T cells.

Bendall *et al.*[2] use CyTOF and their novel data analysis method to study hematopoiesis in healthy human bone marrow mononuclear cells. Hematopoiesis provides an excellent test case to demonstrate the potential of the approach, as the continuum of cell-surface antigens that define stages along the developmental lineage has been especially refractory to resolution using conventional flow cytometry. The more refined analysis enabled by mass cytometry leads the authors to propose that certain cell populations, such as naive T cells, may be more heterogeneous than previously believed. The ability to measure many cell surface markers in conjunction with markers of intracellular signaling (changes in phosphorylation) allows Bendall *et al.*[2] to map a system-wide perspective of distinct signaling responses across multiple cell subtypes. Using distinct biological stimulae, both in the presence and absence of selective inhibitors, they observe unanticipated similarities and differences in signaling behaviors across different cell lineages. For instance, mature B cells and myeloid cells responded differently to stimulation by interleukin 3 (IL3) despite similar abundance of the IL3 receptor in both cell types[2]. The technology is especially well suited to identifying changes in cellular signaling that coincide with the progression of development. For instance, the coordinated phosphorylation response to B cell receptor activation increases at distinct stages of phenotypic progression along the axis of B cell development, especially as the cell line reaches maturity[2].

Besides its likely value in basic research for following intracellular signaling networks at the single-cell level, mass cytometry analysis holds enormous potential for high-content drug screening and biomarker-based monitoring of drug activity, selectivity and efficacy in patients. As a more precise and more multiplexed format

than fluorescence-based flow cytometry, it promises to vastly improve how we measure the responses of individuals to drug treatments and how we compare the selectivity of drugs across multiple biochemical pathways, not only *in vitro* but also potentially using freshly fixed blood or bone marrow samples. Experiments involving the drug dasatinib[2] exemplify the utility of mass cytometry for these types of analyses (**Fig. 1**, bottom right). Dasatinib inhibited specific stimulus-induced responses in particular subsets of immune cells[2]. Monitoring of so-called 'sensitive' or 'insensitive' response signatures could be invaluable in exploring drug specificity and potency, and potentially suggest the use of a drug to treat diseases that share aberrant signaling signatures.

Several drugs, such as p110δ (an isoform of PI3K)[5], SYK[6], JAK[7] or BTK[8], are believed to potently and selectively target aspects of immune signaling in a manner that depends on the cell type and/or developmental stage. These are advancing through clinical development to target an array of immune-mediated disorders that include rheumatoid arthritis, multiple sclerosis, inflammatory or allergic respiratory diseases, and myeloid proliferative disorders. Comparative single-cell analyses using mass cytometry may be invaluable in understanding the mechanisms of these drugs, especially in the context of selectively inhibiting the target in specific cell types, for different disease indications, or in different patient populations. Mass cytometry may provide insight into mechanisms of drug resistance, or reveal avenues for combination therapy. It may also find applications in the detection of rare circulating tumor cells in the blood of cancer patients[9].

Like any analytical tool, mass cytometry has its limitations. Not least of these is that, unlike conventional flow cytometry, analysis involves cell destruction. Bendall *et al.*[2] also acknowledge that mass cytometry may not be as sensitive as fluorescence detection using the most quantum-efficient dyes, although the overall range of sensitivities between mass cytometry isotopes is much smaller than that seen for fluorescent dyes. It also remains to be seen how easily it can be scaled for parallel analysis of hundreds of samples with reproducibility reported across a range of operators in different locations. Notwithstanding the scope for its improvement, mass cytometry seems poised to make substantial contributions in multidimensional, high-throughput biology.

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