New Technologies for Cellular Analysis

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Abstract Cytometric technologies have been indispensable for understanding biological and pathological processes, and are increasingly used to provide critical information on safety and efficacy in drug development. Highly sophisticated multiparametric cytometry methods are now available to measure treatment-induced changes in the phenotypes and functions of individual cells in heterogeneous populations. Numerous phenotypic and functional cytometry assays have been validated for pharmacodynamic studies in clinical drug trials, and that number is likely to expand as new analytical technologies become available. This chapter will discuss three new cytometric technologies that will likely impact clinical drug development in the near future: Imaging cytometry on a chip; Imaging flow cytometry; and Mass cytometry. Each of these platforms is well-suited to specific aspects of cellular analysis, and combines new technologies with tried and true cytometry methods.

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Key Terms

Depth of field (DOF) The optical distance across which objects are acceptably sharp and in focus varies depending upon microscope hardware and the method of image acquisition. Traditional IFM uses lenses with relatively narrow DOF, whereas in confocal microscopy, samples can be "optically sectioned" and reassembled to represent three dimensional cellular structures in sharply focused two-dimensional images. The latter process is relatively slow and suffers from photobleaching and other untoward effects of sample reanalysis that are required for monitoring intracellular changes over time

Hydrodynamic Focusing In flow cytometry, individual cells in suspension are analyzed. Most flow cytometers rely on a process call hydrodynamic focusing to direct single cells for interrogation to the laser light source. Briefly, the cell suspension is contained in a stream of fluid centered within an outer stream. The two fluids differ enough in their velocity and form a two-layer stable flow. Within the laminar flow, the cells orient with their long axis parallel to the flow

Fluorescence spectral overlap Fluorochromes are excited at one wavelength of light and emit energy at another. The histogram display of the emission spectra from various fluorochromes shows a major peak indicating the wave length where most of the signal will resolve and a shoulder or tail where a smaller portion of the signal can be detected. The optics of a flow cytometer are setup such that the major signal from each fluorochrome is detected in a specific channel. A situation where a small portion of the signal from one fluorochrome overlaps with the detection channel of a second fluorochrome is referred to as fluorescence spectral overlap

Chip Cytometry and Cell Imaging

Laser scanning cytometry (LSC) is an established method for quantifying the fluorescence of immobilized cells, and has been used to characterize patient treatment responses in clinical trials [1]. A variety of LSC platforms have been developed that interrogate cells mounted on slides or cartridges containing the sample. For example, the Imagn2000 instrument from Biometric Imaging has been used to enumerate cluster of differentiation (CD) CD4 and CD8 lymphocytes during treatment of human immunodeficiency virus (HIV) infected patients [2]. This instrument uses a simple 2-color-Photomultiplier Tube (PMT) system and a slide-based cartridge to interrogate cells while minimizing the risk from aerosols. SurroMed markets the SurroScan, a 4-color LSC instrument that uses cartridges with 32 wells for absolute cell counting in small volumes of whole or diluted blood. The CompuCyte iCyte® LSC uses advanced optics and CCD cameras for collection of four fluorescence channels and optical images of cells. This approach allows the examination of smaller quantities of blood while sparing reagents, and may reduce artifactual cell activation in unfixed samples, two useful characteristics for pharmacodynamics studies [3, 4].

Traditional flow cytometry (FC) functional assays can be adapted for LSC, although the total number of cells acquired in LSC is considerably smaller than can be achieved using more traditional flow-based methods. Also, as most LSC instruments are limited to four colors, the detection of rare subpopulations can be difficult, if not impossible using LSC. In further contrast to flow cytometers, which use hydrodynamic focusing to align cells, microfluidic flow cytometers like the Fishman-R and instruments by Zellkraftwerk (also known as "chip cytometers") pass cells through micro-fabricated channels etched onto chips, where they are illuminated by lasers for the measurement of cellular fluorescence. Such miniaturization reduces required sample volumes and reagent costs, and allows the collection of both cytometric data and cell images [5].

Complications associated with low cell numbers and inadequate numbers of fluorescence channels can be further aggravated by chip-to-chip variations in microfluidics channels and optical properties. The Zellkraftwerk instrument [6] is one of the newest iterations of chip cytometers, and seeks to address these issues via a combination of LSC technology, advanced microfluidics, and methods for staining and restaining of cells in a sample (Fig. 1) [7]. Similar to traditional LSC, this instrument captures and scans cells on a fixed slide (Fig. 2). Re-staining of cells is accomplished by serially measuring stained cell fluorescence, then photo bleaching the initial sample to allow re-staining with noncompetitive antibodies for

Fig. 1 Zellscanner ONE. Reproduced with kind permission from Zellkraftwerk

measurement of additional antigens. Thus, despite the limited number of fluorochromes that can be measured using this instrument, the ability to re-assay samples expands the effective number of parameters queried. It is important to consider the photo stability of a given fluorophore when designing re-staining protocols, since many of the newer dyes are designed to be resistant to photo bleaching.

Some characteristics of these platforms are quite useful for clinical trials support, but other factors serve to limit their utility. For example, the narrow field of view and low sample volumes used in these methods means that these instruments generally have a reduced capacity for rare event detection, which can be required for determining minimal residual disease status in hematological malignancies. Furthermore, in the absence of additional manufacturing quality controls, inter-chip variability may limit the reproducibility of chip cytometry in longitudinal pharmacodynamic studies. Platform availability at contract labs can also present challenges for clinical trials support. Individual contract labs may be sufficient for smaller clinical trials, but as a drug progresses through development, the need for additional instruments and greater regulatory oversight become paramount, and may require the participation of larger contract labs.

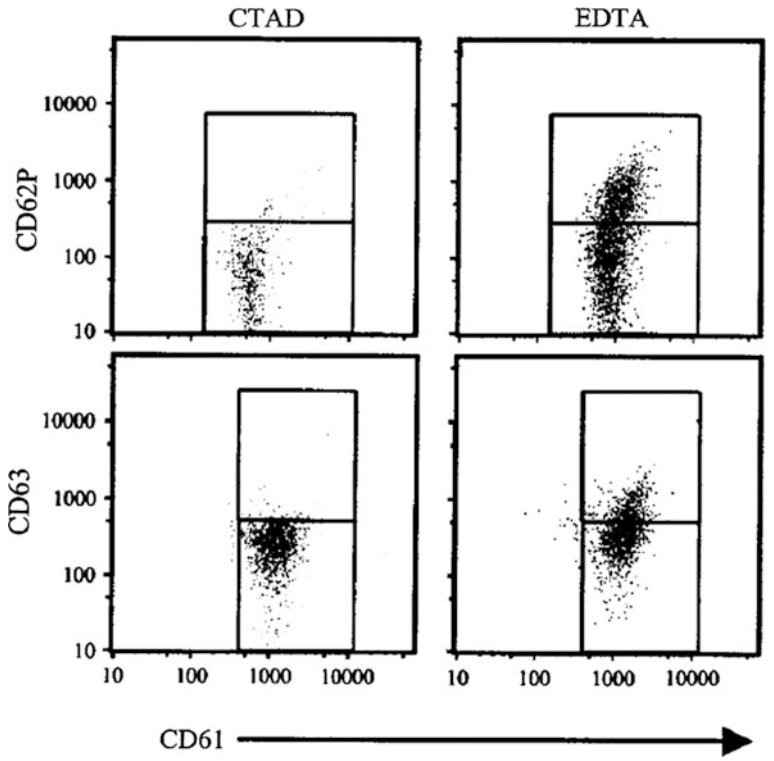

Fig. 2 The use of LSC to measure resting and activated platelets. Platelet rich plasma was prepared from either EDTA or CTAD blood and CD61+ platelets were examined for the expression of the platelet activation markers CD63 and C62P. Reproduced from Wyant et al. with permission

Imaging Flow Cytometry

Traditional FC and automated immuno-fluorescence microscopy (IFM) have proven useful for phenotypic screening and multiparametric cell profiling in early phase drug development [8]. FC and IFM can also improve our understanding of exposure-response relationships in animal studies and clinical trials, but their respective methodological advantages are to a large extent mutually exclusive. FC, for example, provides reliable information regarding the bulk fluorescence of specifically gated cell populations, but has relatively low single-cell resolution, and provides limited spatial information about biomarker micro-anatomical distribution, relative abundance, or normalized activation status, limiting our understanding of complex biological signaling. IFM provides high resolution fluorescence data, and can be used to monitor cell morphology and localize fluorescence signals in specific cells over time. Though amenable to automation, IFM has much lower throughput and provides less statistical power than FC, offers a limited capacity for analyzing

suspension cells, suffers from susceptibility to photo bleaching, and is subject to observer bias through the selection of visual fields. IFM systems also typically offer a limited number of simultaneous excitation sources and imaging modes, limiting the size of multiplex panels.

Imaging flow cytometry (IFC) is a hybrid method that combines the statistical power of multiparametric FC with the spatial and morphological discrimination of fluorescence microscopy, enabling the simultaneous capture of multi-mode imagery (i.e., bright field, dark field, and fluorescence images) [9]. Excellent reviews of IFC have recently been published that provide a good introduction to this technology and specific applications of relevance to drug development [10–14]. Other publications provide useful comparisons of flow imaging with static methods like the CellTracks® system [Johnson and Johnson] [15], highlighting the key technological advances that have driven the broad acceptance of IFC as a tool for both basic and applied research. Over 350 publications describe IFC studies, and the number of novel applications of IFC in basic research is steadily increasing. The utility of IFC is further exemplified in the widespread adoption of imaging cytometers made by Amnis/EMD Millipore, who ten years ago marketed their first of several generations of multispectral imaging cytometers (Fig. 3) [16].

In IFC cells are loaded in suspension into a hydrodynamically focused fluid stream that passes through a flow cell for illumination and detection of specific spectral and morphological characteristics. Here, key differences between FC and IFC begin to emerge. First, in IFC sample loading speed and volume are carefully

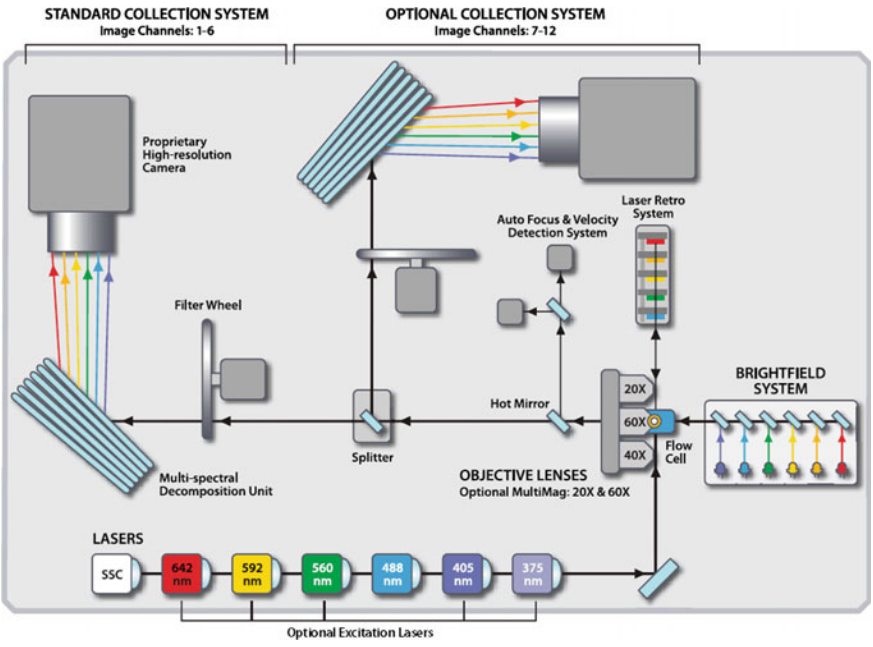

Fig. 3 Schematic of Amnis. Reproduced with kind permission from EMD Millipore

controlled to allow synchronized, time delayed integration (TDI) of optical signals generated after illumination with a bright field light source and at least one laser. In this approach, cells are tracked and images are captured by panning across the flow cell using a high numerical aperture objective. Transmitted and scattered light and cellular fluorescence are captured and spectrally deconvoluted on multichannel CCD cameras. Alignment of these data allows the simultaneous capture of fluorescence data and sharp images of each cell, allowing unbiased quantification of spatial information about target molecules in snapshots of individual cells (Fig. 4).

IFC allows refinements in gating strategies to include cellular aspect ratio, cell diameters, and cell volumes in addition to the traditional light scattering and fluorescence-based gating used in FC. In IFC, individual events are also captured as static visual images that can be integrated with fluorescence data at relatively high resolution, providing an essentially infinite number of options for cell classification, and allowing definitive visual confirmation of event gating. Newer Amnis® IFC instruments can be modified to provide additional functions like extended depth of field imaging for detecting fluorescent puncti over a wider focal range, a useful feature for studies of DNA ploidy and cell-cell interactions [13, 16–18]. This combination of features provides superior discrimination of subtly different populations in heterogeneous cell mixtures.

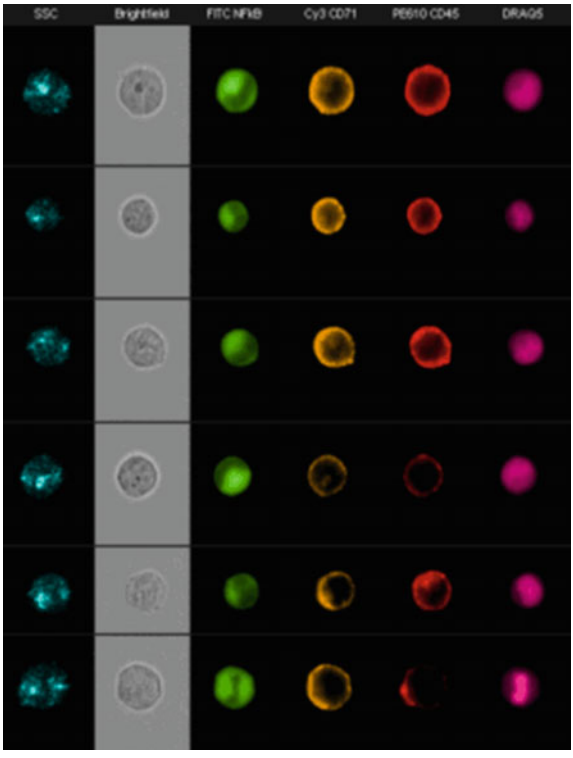

Fig. 4 Amnis cellular images. Reproduced with kind permission from EMD Millipore

IFC reagent selection, sample preparation and data acquisition are very similar to that of traditional FC, meaning that established FC methods are often readily adaptable to IFC. Numerous functional FC assays have been modified for IFC, including the quantification and correlation of shape change and other morphological characteristics with the cell cycle and other cellular events [11, 19]; phagocytosis assays [20–22]; analysis of DNA damage and repair and other events associated with cell death and autophagy [23]; cell-cell and cell-particle interactions and the exchange of cytoplasmic contents [24]; co-localization of intracellular and cell surface epitopes [25]; monitoring of protein interactions and trafficking to organelles [25]; and spot counting for ploidy determination and other applications of in situ hybridization [26]. Though suitable for discovery research and many academic purposes, most published IFC applications are only preliminarily validated, and can be poorly suited to drug development clinical trials. For example, IFC data acquisition is slower than traditional cytometry, and under the most common configurations IFC data files can be hundreds of times larger than files from a traditional flow cytometer. The learning curve for IFC data analyses is significant for those without experience in advanced microscopy, and the high cost of purchasing multiple IFC instruments puts backup instruments out of reach of most labs. This is a critical hurdle for drug development, since backup instruments can be required for regulatory compliance in support of clinical trials.

CyTOF Mass Cytometry

In the past decade, technological advances in fluorescent probes, cytometry instrumentation, and data analysis software have enabled "high dimensional flow cytometry" where 20 parameters (18 fluorescent-labeled probes and two light scatter properties) of data can be collected from an individual cell. High-dimensional flow cytometry allows for a far more in depth cellular characterization and dissection of more refined cellular subsets. Indeed, the ability to measure up to 20 parameters of data on a single cell has been critical in enabling advances in research focusing on hematopoiesis, complex immune responses, and intracellular regulatory signaling networks. Using the existing instrumentation and fluorescent probes, the number of parameters is unlikely to increase beyond twenty. This upper limit is largely due to the overlap in the emission spectra of the fluorescent probes resulting in the detection of one fluorophore in multiple detector channels and the challenges in compensating for this overlap. An innovative, relatively new technology, mass cytometry, or CyTOF® [Fluidigm], can extend the capability of highly multiparametric analysis well beyond 20; already studies using more than thirty-parameter analysis have been published [27].

CyTOF® mass cytometry, essentially a hybrid between flow cytometry (Cy) and time-of-flight (TOF) mass spectrometry, is based on the concept that isotopically pure heavy metal reporter elements could be conjugated to cellular probes (most commonly monoclonal antibodies) which could then be quantified in an inductively

coupled plasma mass spectrometry (ICP-MS) detection system [27]. CyTOF® provides at least three orders of magnitude of resolution between adjacent detection channels, thus the use of heavy metal probes rather than fluorescent-labeled probes practically eliminates the need for compensation, removing the parameter restrictions and other technical challenges associated with fluorescence spectral overlap compensation.

CyTOF® mass cytometry has a workflow somewhat similar to that of high-dimensional flow cytometry in that the labeled heterogeneous populations of cells are individually analyzed. Unlike flow cytometry where single cells are interrogated by a laser light source, in mass cytometry cells are nebulized into single-cell droplets and introduced into the plasma, where they are completely vaporized into component elemental ions (Fig. 5) [28]. The cloud of atomic ions for each single cell is extracted into the ion optics and time-of-flight regions of the mass cytometer where the ions are separated by mass. To resolve the probe ions from the abundant cellular and antibody ions, the mass cytometer is configured as a quadrupole–time-of-flight (qTOF) instrument. The quadrupole acts as a filter allowing only the heavier ions (probe) to be quantitated by TOF mass analysis. The masses corresponding to the metal-tagged probes are counted in discrete time-separated detector channels reminiscent of fluorescence emission detection in

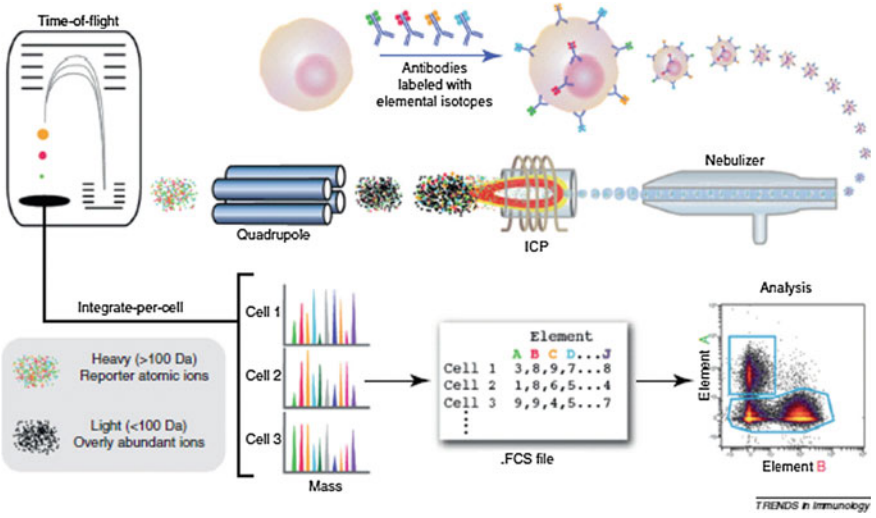

Fig. 5 Schematic of ICP-MS-based analysis of cellular markers. An affinity product (e.g., antibody) tagged with a specific element binds to the cellular epitope. The cell is introduced into the ICP by droplet nebulization. Each cell is atomized, ionized, overly abundant ions removed, and the elemental 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. Reprinted from Bendall et al. [28], Copyright (2012), with permission from Elsevier

the appropriate detector PMT. Much as fluorescence emission is proportional to the level of antibody binding, the intensity of the heavy metal signal detected in each channel is directly proportional to the number of specific probe-derived ions striking the detector and thus the number of antibodies originally bound per cell.

As with any new and highly novel technology, the challenges with CyTOF® mass cytometry are only beginning to be identified. To date, there are a limited number of formal comparisons of mass cytometry and polychromatic flow cytometry [30] and issues regarding sensitivity for some surface antigens and cell loss rates during acquisition are beginning to be discussed [31, 32]. Minor challenges regarding reagent availability are likely to decrease as the technology is more widely utilized. The primary challenge is that currently there are no quality assurance and normalization protocols [33]. The technology lacks high-throughput capabilities and changes in instrument performance are evident after a few hours of acquisition. Between run fluctuations have been reported. Instrument standardization and monitoring are essential in order for this technology to be of value outside of the basic sciences research arena and drug screening applications. A normalization algorithm based on prominent features or "landmarks" in raw flow cytometry data was recently used to correct for instrument variability [34]. Another challenge is the organization and analysis of the high-dimensional data. Although the files are saved in the .fcs file format to allow gating in any flow cytometry data analysis package, traditional methods of sequential, Boolean gating would not allow maximum utilization of high-dimensional data sets. Fortunately, several interesting comparative multivariate analysis packages have been applied to CyTOF® data such as SPADE, PAC, and viSNE. SPADE (Spanning-tree Progression Analysis of Density-normalized Events) [35], was the first to appear. It is a clustering algorithm, which allows identification of low-density clusters and displays the relatedness of clusters via a dendrogram [35]. Principle components analysis (PCA), is a long-standing computational technique, [36] which separates a group of events according to their measured attributes and has recently been applied to CyTOF® data. PCA allows for the clustering of cells that are phenotypically distinct from other cells. viSNE is a recently described algorithm for high-dimensional data analysis [37] in which individual events are displayed on a two-dimensional map which preserves the multi-dimensional separation [38, 39].

With the large number of parallel measurements per cell, CyTOF® mass cytometry is potentially a very powerful new tool for drug discovery and development. It could allow for the identification of multiple parallel translational pathway responses to agonist/antagonist intervention. In the past, high-dimensional flow cytometry was sometimes referred to as proteomics at the single cell level; with the potential to measure 100 parameters per cell CyTOF® mass cytometry would more closely achieve that goal. It is easy to imagine that the technology might be used for hypothesis generating experiments much in the way as gene chips. For example, samples collected before and after therapeutic intervention could be stained with a variety of CD markers for analysis of changes in the clustering patterns.

Conclusions

FC is considered to be the optimal technology for the analysis of large numbers of heterogeneous cellular populations. In the drug development process, FC has been applied to drug screening and lead compound characterization, preclinical biomarker and pharmacodynamic studies, and to patient stratification drug response outcomes during clinical trials [40]. The value of established cytometry platforms in many of these applications is increasingly obvious and, once they are properly validated, some of the technologies described here are likely to help fill existing gaps in experimental methodologies and instrumentation. The challenges before us may seem daunting, but as was the case for FC assays these past two decades, it is likely that the emergence of additional novel technologies will aid the evolution of established methods onto new cytometry platforms. The improved ability to characterize immune cell phenotypes and functional responses to therapy afforded by these emerging technologies is likely to spur additional advances in cancer immunotherapy, autoimmunity research, and the diagnosis and treatment of chronic viral diseases. As cytometers continue to shrink [41] and cytometry expands into the realm of molecular diagnostics [27], it is increasingly likely that cytometry will play a major role in the optimization of personalized drug therapies and health care delivery in lesser developed nations.

Summary Box

- Cytometric technologies are indispensable for understanding biological and pathological processes, and are increasingly used to provide information on safety and efficacy in drug development.
- Highly sophisticated multiparametric cytometry methods are now available to measure treatment-induced changes in the phenotypes and functions of individual cells in heterogeneous populations.
- Laser scanning cytometry (LSC) is an established method for quantifying the fluorescence of immobilized cells, and has been used to characterize patient treatment responses in clinical trials.
- Microfluidic flow cytometers like the Fishman-R and instruments by Zellkraftwerk (also known as “chip cytometers”) pass cells through micro-fabricated channels etched onto chips, where they are illuminated by lasers for the measurement of cellular fluorescence. Such miniaturization reduces required sample volumes and reagent costs, allows the collection of both cytometric data and cell images, and may reduce artifactual cell activation in unfixed samples.
- Imaging flow cytometry (IFC) is a hybrid method that combines the statistical power of multiparametric FC with the spatial and morphological discrimination of fluorescence microscopy.

- IFC allows refinements in gating strategies to include cellular aspect ratio, cell diameters, and cell volumes in addition to the traditional light scattering and fluorescence-based gating used in FC.
- CyTOF® mass cytometry, essentially a hybrid between flow cytometry (Cy) and time-of-flight (TOF) mass spectrometry, is based on the concept that isotopically pure heavy metal reporter elements could be conjugated to cellular probes (most commonly monoclonal antibodies) which could then be quantified in an inductively coupled plasma mass spectrometry (ICP-MS) detection system.
- CyTOF® provides at least three orders of magnitude of resolution between adjacent detection channels, thus the use of heavy metal probes rather than fluorescent-labeled probes practically eliminates the need for compensation, removing the parameter restrictions and other technical challenges associated with fluorescence spectral overlap compensation.

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