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Leveraging blood and tissue CD4+ T cell heterogeneity at the single cell level to identify mechanisms of disease in rheumatoid arthritis

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Abstract

CD4+ T cells have been long known to play an important role in the pathogenesis of rheumatoid arthritis (RA), but the specific cell populations and states that drive the disease have been challenging to identify with low dimensional single cell data and bulk assays. The advent of high dimensional single cell technologies – like single cell RNA-seq or mass cytometry – has offered promise to defining key populations, but brings new methodological and statistical challenges. Recent single cell profiling studies have revealed a broad diversity of cell types among CD4+ T cells, identifying novel populations that are expanded or altered in RA. Here we will review recent findings on CD4+ T cell heterogeneity and RA that have come from single cell profiling studies and discuss the best practices for conducting these studies.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disorder characterized by chronic inflammation of the synovial tissues, leading to joint damage, disability, and increased mortality [1,2]. The pathophysiology of RA involves a complex interplay between multiple

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cell types, including leukocyte populations, synovial fibroblasts, chondrocytes, osteoclasts and others [3]. Multiple lines of evidence drawn from genetic, histologic, and clinical observations point to key role for CD4+ T cells in directing the autoimmune response in RA. Genome-wide association studies (GWAS) have highlighted the major histocompatibility complex (MHC) as by far the strongest contributor to disease heritability, driven by variants in *HLA-DRB1*, *HLA-DPB1, and HLA-B* [4,5]. *HLA-DRB1* and *HLA-DPB1* are components of the MHC class II molecule, which antigen presenting cells use to present antigens to CD4+ T cells. We have further demonstrated that genetic risk alleles outside of the MHC locus also point to a role for CD4+ T cells, as genes associated with these loci are preferentially expressed in effector memory CD4+ T cells [6–8]. In addition, CD4+ T cells are frequently found infiltrating the synovium in RA, often in dense lymphocyte aggregates [9,10]. Importantly, interfering with T cell activation by blocking costimulatory signals with abatacept (CTLA4-Ig) is an effective therapy for clinical RA [3].

While it is clear that T cells play an important role in promoting RA pathology, pinpointing the specific T cell phenotypes or functions that are most relevant in this disease has been challenging. CD4+ T cells are typically categorized by the level of expression of surface and intracellular proteins that reflect functionally distinct cell types [11,12]. However, T cells are highly heterogeneous, displaying diverse combinations of surface markers and effector functions. This heterogeneity makes it difficult to describe T cell infiltrates as bulk populations and has highlighted the value of single cell analyses to resolve this heterogeneity.

Single cell analyses by flow cytometry have contributed major insights into T cell abnormalities in RA [13,14], yet flow cytometry analyses have been hampered the limited number of parameters that can be detected simultaneously, which are often insufficient to adequately assess a diverse T cell population. The recent rapid expansion of single cell technologies has led to a dramatic advance in the ability to study complex populations in large-scale with high dimensionality (Figure 1). This high-dimensional single cell profiling may lead to the identification of specific T cell populations or states that are mechanistically linked to disease and ideal for therapeutic targeting. In this review, we discuss recent advances in single cell immunoprofiling and describe their early application in RA. We will then discuss methodological and bioinformatic considerations to maximize the potential of single cell technologies in its application to define mechanisms of immune-mediated diseases.

Low-dimensional single cell analysis of T cells in RA

Single cell assays have a long history in the field of autoimmunity, beginning in 1969 with the initial use of fluorescent assays to label and sort immune cell populations [15–18]. Cytometry has been thoroughly exploited in the exploration of lymphocyte heterogeneity in RA [19–23]. Subsequent improvements in flow cytometry technology have steadily increased the number of parameters that can be measured for each cell, provided access to cytoplasmic and nuclear protein expression through intracellular staining, and facilitated measurement of cell signaling using antibodies specific for the phosphorylation state of signaling molecules [24]. Flow cytometric analyses of T cells from RA synovial tissue and

fluid have highlighted the dramatic 'activated' phenotype of T cells within the RA joint, consistent with an ongoing autoimmune response directed at the synovium [25,26]. Synovial T cells frequently express CXCR3, suggesting Th1 differentiation, and loss of CD27, suggesting a chronically activated state [27–29].

Immunophenotyping of peripheral blood CD4+ T cells from RA patients has also identified characteristic changes, including expansion of Th17 cells relative to Tregs [30,31], and an expansion of CD28− T cells [22,32]. Unfortunately, studies of peripheral blood T cells in RA have often yielded inconsistent results. For example, the abundance of regulatory T cells (T_{REG}) in RA peripheral blood has been observed to be reduced or expanded compared to healthy controls in different studies [33–37]; in addition, conflicting results have been reported concerning the suppressive capability of T_{reg} cells in RA [38–42]. Inconsistent results can be partially attributed to variation in markers used across different studies or the difficulty of resolving highly heterogeneous populations with bulk cell assays – which advancing single cell technologies might help to obviate. However, some of this inconsistency is rooted in methodological issues that will need to be addressed as investigators begin to apply single cell technologies to autoimmune diseases. Specific issues have included the use of small sample sizes, variability in cohorts, technical noise resulting in batch effects, publication bias, and the lack of principled statistical methodology and criteria.

High-dimensional analyses reveal an expanded view of CD4+ T cell heterogeneity

The recent development of mass cytometry - a fusion of mass spectrometry and flow cytometry that is capable of the simultaneous acquisition of over 40 parameters on a single cell level – has further extended the dimensionality of single cell cytometric assays [43]. Mass cytometry relies upon staining cells with the same target-specific antibodies that are commonly used in flow cytometry to tag markers of interest; however, in mass cytometry antibodies are labeled with pure, non-radioactive rare earth isotopes instead of fluorescent proteins. After staining, single cells are analyzed by a time-of-flight mass spectrometer by integrating the detection of heavy metal reporter ions to determine expression levels for each labeled antibody [44–46].

Single cell immunoprofiling by mass cytometry has already been used to reveal remarkable heterogeneity within conventional T cell subsets. Wong et al. used mass cytometry to profile CD4+ T cells across eight human tissue types and described 75 different populations, including multiple T_h1 populations for each T_H subset. Many cell populations were tissue-specific and differed based the expression of trafficking receptors and cytokine production [47••]. They observed that certain populations co-expressed "key" cytokines like IFN-γ, IL-4, and IL-17A that are typically restricted to a single CD4+ T_H subset, in line with previous findings highlighting the phenotypic plasticity between CD4+ T_H lineages [48–51], reviewed in [52]. Other studies have taken advantage of high-dimensional single cell mass cytometry analysis to describe multiple populations of T_{REG} and T_{FH} cells [53,54].

While advances in flow cytometry and mass cytometry enable users to define single cells across many parameters, the set of proteins to be measured must be decided *a priori*, limiting the use of these technologies in unbiased discovery studies. In contrast, single cell transcriptomic analysis presents an opportunity to define single cell expression profiles without relying on prior knowledge. Several different single cell RNA-seq (scRNA-seq) methods have been developed over the past decade [55–59] and successfully applied in various immunological studies, such as identifying differentiation pathways in immune cell lineages [60,61], establishing novel transcriptional regulatory networks [62], and revealing functional diversity among lymphoid cell populations [63,64].

Single cell RNA-seq technologies provide an orthogonal approach to cytometry-based methods for establishing CD4+ T cell heterogeneity. As CD4+ T cell subsets are differentiated by their putative functionality, quantifying of transcript expression on the single cell level can be used to identify gene expression programs that underlie those functional divisions. Single cell sequencing of T cells isolated from patients with liver cancer identified 11 distinct CD4 and CD8 T cell populations, some of which were expanded in hepatocellular carcinoma and marked by specific gene signatures [65••]. The functional diversity of natural killer T (NKT) cells is difficult to characterize using cytometry alone; however, single cell RNA-seq analysis revealed differential patterns of gene expression that resolve NKT subsets and indicate potential functions [66]. Single cell transcriptomic profiling is also particularly useful for understanding T cell differentiation and proliferation, as the expression of key transcription factors and other regulatory genes can be easily ascertained and used to assign cells to differentiation trajectories [67,68].

Early high-dimensional analyses of T cells in RA

These same technologies are already being used in RA tissue and blood to define key features of pathogenic CD4+ T cell populations in RA. We have recently applied mass cytometry to evaluate the heterogeneity of CD4+ T cells that infiltrate RA synovium [69]. With this high-dimensional analysis, we identified a T 'peripheral helper' (T_{PH}) cell population that is markedly expanded in RA synovium, constituting ~25% of synovial CD4+ T cells. T_{PH} cells, characterized as PD-1^{hi} $CXCR5^{-}$ CD4+, display a unique capacity to infiltrate inflamed tissues and enhance local B cell antibody production and differentiation into plasma cells. A preliminary single-cell RNA-seq analysis of a single RA synovial sample has also demonstrated multiple T cell subsets, including a population of T_{PH} cells, in the RA T cell infiltrate [70].

In a distinct approach, Ishigaki and colleagues used parallel single cell transcriptomics and T cell receptor (TCR) sequencing to identify and analyze expanded CD4+ T cell clones in RA patients [71••]. Expanded memory CD4+ T cells in both the synovium and periphery are phenotypically similar in expression to senescent T cells, upregulating Granzyme B and downregulating CD28. Intriguingly, the majority of expanded memory T cell clones did not belong to the well-defined T_H1 or T_H17 subsets despite their established association with RA [31,72–74]. Although the findings are limited by the small number of donors studied, this study suggests that as yet undefined CD4+ T cell populations may undergo expansion in RA and may be relevant to RA pathology.

One potential benefit of characterizing the extent of CD4+ T cell diversity with high-dimensional analyses is that it may provide a means to differentiate between pathogenic and non-pathogenic variants of known T cell subsets. For example, single cell RNA-seq was used to define a spectrum of pathogenicity for T_H17 cells isolated from mice with experimental autoimmune encephalomyelitis (EAE) and identify key genes involved in the process [75]. Similarly, immunoprofiling of T_{REG} cells in RA described the discovery of a novel senescent-like T_{REG} cell population characterized by the loss of CD28 expression and increased numbers of double stranded DNA breaks. Compared to standard T_{REG} cells, $CD28^-$ T_{REG} cells had impaired suppressive function and produced higher amounts of proinflammatory cytokines IFN-γ and TNF [76•].

Identifying biomarkers through cell phenotyping

As the diversity, precision, and cost of therapeutics in RA has increased, the importance of being able to determine the option best-suited for a given patient up front has become increasingly clear. There is now a major need for biomarkers to predict response to therapies with distinct mechanisms of action; however, efforts using multiplexed cytokine profiling and genetic variation have not yet led to clinically applicable tools [77,78]. The increased resolution of single cell assays is an asset for revealing disease biomarkers, as the ability to characterize the diversity of lymphocyte populations can be leveraged to monitor the abundances of multiple populations longitudinally or in a case-control context. Changes in the frequency of disease-associated populations that can be easily measured in peripheral blood can be used as a powerful readout of disease state in less accessible compartments.

Several studies have suggested the potential ability to identify specific lymphocyte populations whose peripheral frequencies are predictive of treatment response in order to guide therapeutic decisions. Tracking CD4+ T cell populations by flow cytometry in patients with early RA receiving methotrexate and healthy controls revealed that higher abundances of naïve CD4+ T cells are significantly associated with increased chances of remission [79]. Response to treatment with tocilizumab, an IL-6 receptor inhibitor, is associated with higher baseline frequencies of natural killer ($CD3^-$ $CD56^+$) cells [80] and higher increases in the frequencies of T_{REG} cells in the periphery [81]. A case-control study of RA patients and healthy controls demonstrated that IL-10+ producing $LAG3^+$ T_{REG} cells are specifically increased after treatment with abatacept, and that the magnitude of this increase is correlated with the strength of response [82]. Immunoprofiling studies have also revealed changes in the function of lymphocyte populations in response to therapy: for example, RA patients who respond well to anti-TNF treatment have higher production of GM-CSF from T cells [83]. Response to TNF inhibition therapy is also associated with a higher abundance of CD8+ T cells that are specifically reactive to apoptotic epitopes [84]. Studies such as these fuel hope for the development of predictive cellular biomarkers, though none have been prospectively validated and adopted for use clinically to date.

The Future of Single Cell Immunoprofiling

Recent advances in availability and throughput have made single cell technologies a practical choice for conducting immunoprofiling studies to understand mechanisms of

disease and define predictive biomarkers. The application of these methods in RA include the profiling of blood, as many studies that we refer to above already have done, but also performing immunoprofiling in human tissue. We and others are pursuing these goals in Accelerating Medical Partnerships Rheumatoid Arthritis/Systemic Lupus Erythematosus (AMP RA/SLE network; URL: https://www.niams.nih.gov/Funding/Funded_Research/AMP_RA_Lupus/), which involves obtaining, disaggregating, and performing single cell profiling on synovial tissue from cases and controls to query both immune infiltration and stromal adaptions. For human immunology to successfully leverage the large quantities of observational data that emerge from single cell queries of the immune system, we will need to develop and reliably apply robust statistical methods and study design principles in single cell studies. Taking full advantage of the power of single cell analysis will require overcoming technical, methodological, and bioinformatic challenges.

Among the many considerations that must be taken into account when designing single cell immunophenotyping experiments, one of the most prominent is determining how to handle batch effects. Here we use the term 'batch' to refer to a set of samples processed together in a single experimental run, and the term 'batch effect' to refer to variation in a dataset caused by technical variation in the processing of different batches of samples. Large-scale microarray assays powerfully illustrated the dramatic effects that differences in machine sensitivity, preparation or handling of samples, or protocol variations can have on the results of transcriptomic analyses [85–87]. Single-cell technologies such as mass cytometry and scRNA-seq are even more vulnerable to confounding from batch effects due to extensive intra-individual and inter-individual heterogeneity of expression among single cells. Application of single cell profiling to human tissues, where cases and controls may respond differently to sample processing and manipulation, could provide an additional source of batch effects.

Indeed, Hicks et al. has demonstrated that variable detection rate and other technical effects account for much of the "biological" variation that has been presented in some of the early single cell transcriptomic studies [88••]. Careful experimental design can partially alleviate the influence of batch effects in single cell profiling studies; however, we note that Tung et al. have shown that common normalization methods for scRNA-seq like spike-in controls and the use of unique molecular identifiers (UMIs) are insufficient for fully removing technical variation [89]. For single cell transcriptomic studies, critical steps include applying quality control methods to remove poorly captured cells and quantifying transcripts to determine cell expression levels. In single cell cytometry studies, quality control is effectively performed by selecting cells for analysis based upon forward and side scatter parameters (flow cytometry) or DNA content (mass cytometry) and inclusion of a live/dead marker, while marker expression quantification is normally provided by onboard software.

However, since batch variability is difficult to completely eliminate *post hoc*, careful experimental design is essential. First, the importance of minimizing variation in experimental procedure cannot be overstated. Best practices include ensuring that samples are collected from the same source, handled in the same fashion, and assayed using the same protocols to the extent that it is possible. Ideally, samples would be prepared using the same lot of reagents; however, this can be difficult to achieve, and steps such as RNA preparation

or antibody staining should be performed in a limited number of batches. Second, as large-scale studies typically require performing assays in batches, sample randomization is crucial. Interspersing cases and controls within each batch guards against the possibility of discovering biological associations that are perfectly confounded with batch. Finally, ensuring that sample processing is done in a short window of time and that samples are assayed using the same equipment also minimizes technical variation. For example, the AMP RA/SLE network significantly reduced batch effects by processing and assaying samples in a single location, as opposed to trying to analyze data obtained at different sites.

The choice of tools for computational analysis of high-dimensional data is another important consideration in conducting single cell immunoprofiling studies. Although produced using very different technologies, both transcriptomic and cytometric single cell data can be analyzed similarly by treating the data as matrices where rows represent single cells and columns represent expression measurements for transcripts or proteins. In the context of studying disease association, analysis of single cell immunoprofiling data can be split into two steps: clustering, where the goal is to identify groups of cells that are related by similarity of expression, and association testing, where the goal is to determine significant changes in the abundance or character of immune cell populations in disease.

While many different algorithms have been applied to the analysis of single cell data, we believe that the following methods represent some of the best tools for use with single cell immunophenotyping. Seurat is an R package that contains multiple methods for clustering and visualizing single cell sequencing data, as well as performing differential expression testing between groups and finding associations [51]. One particularly intriguing application of Seurat is to use single cell transcriptomic data to reconstruct the spatial organization of cells, which has been demonstrated in zebrafish embryos [90••]. Multiple clustering methods have been developed for the analysis of flow cytometry [91,92] and mass cytometry [46,93–97]; a recent comparison of these methods identified FlowSOM [96] and PhenoGraph [97] as the best performers [98].

While the set of algorithms available for clustering single cell data is rapidly expanding, there is a relative paucity of methods designed to perform association testing with cytometry data [99,100]. We have recently presented MASC (mixed-effect Modeling of Associations of Single Cells), which accepts user-identified populations regardless of clustering method, directly reports the significance of case-control associations for each cluster, provides an estimate of the effect size of the association itself, and incorporates both technical covariates (e.g. batch) and clinical covariates when modeling associations, a key feature when analyzing high-dimensional datasets of large disease cohorts[113••]. In comparison, the association testing method Citrus uses nested hierarchical clustering and penalized regression models to identify features (defined here as clusters of single cells or median expression levels of markers within a cluster) that are predictive of clinical endpoints; however, Citrus requires down-sampling cells from each sample and does not retain single cell resolution, which impedes the interpretation of clusters found to be predictive. Tools such as these now empower investigators to efficiently identify novel cell phenotypes associated with a disease state.

Given the high levels of inter-individual variability in the human immune system, the ability to aggregate data across multiple studies is an attractive goal for conducting well-powered analyses. Currently, data aggregation is challenging due to the high dimensionality of single cell data and the difficulty of overcoming different datasets for analysis which include differences in the use of specific sequencing protocols, technical batch effects, and differences in sample handling. Standardization of normalization and quality control methods will be key, as small differences in data processing can overpower biological signals in the noisy context of immunoprofiling; for example, the use of different software pipelines for processing single cell RNA-seq data will impede combined analysis. One important question is whether the use of imputation-based techniques will be effective to fill in missing data and meta-analyze across multiple studies in single cell analyses. These approaches have been critical in allowing human genetic studies to scale rapidly, and have supported meta-analysis of different data sets obtained on different platforms. While methods for single cell RNA-seq data have been recently described, the effectiveness of imputation is an active question in the field [101–103]. Finally, given the identifiable nature of single-cell transcriptomic sequencing data in particular, a framework to support data sharing while protecting patient privacy is essential.

For immunological applications, a key initial step should be to better characterize human lymphocytes using single cell data. Building a reference map of the human immune system is a difficult and complicated task; however, the dendritic cell atlas or the work of Wong et al. characterizing T cells across tissues provide examples of the power of this approach [57••, 104•]. Incorporating data on from multiple assays to define lymphocyte profiles will be essential for understanding their functional impact, as shown by multiple studies that utilize repertoire sequences or expression data in combination with single cell cytometry to identify disease-relevant populations [71••,105•,106,107]. The development of new peptide-MHC multimeric complexes supports the detection and isolation of antigen-specific lymphocytes at much lower frequencies [108] than was previously feasible. New methods have been recently developed to provide high-throughput single cell repertoire sequencing of B and T lymphocytes [109•,110].

Beyond integrating data across studies and across assays, the next stage of advancement for single cell technologies will be the simultaneous acquisition of transcriptomic and proteomic data from a single cell. Multiple methods for conducting such analyses have been described [111, ••112] but have yet to be applied in any large-scale immunoprofiling efforts. The ability to obtain this type of data would allow research into the temporal dynamics of transcription and protein expression as well as provide higher-resolution definition of single cells.

Conclusions

The advent of single cell technologies has the potential to revolutionize the study of RA by offering an unbiased approach to detecting and characterizing cell heterogeneity in blood and tissue. High-dimensional single cell analyses of RA synovium have revealed novel lymphocyte and stromal cell populations that are pathologically expanded in the joints of RA patients. These cell populations may now be evaluated as potential therapeutic targets.

Single cell transcriptomics and TCR repertoire sequencing have enabled detailed characterization of the specific clones of CD4+ T cells that are expanded in RA and may highlight new cell phenotypes to pursue as therapeutic targets or biomarkers.

However, the current absence of rigorous standards for experimental design and analysis significantly limits the value of single cell assays. The increased resolution of single cell analyses will be wasted without defining a set of standards for experiments that enable combining experimental data across batches, assays, and studies. This will be particularly important for studying CD4+ T cells in RA, where heterogeneity among both cell types and patients has yielded conflicting and contradictory results. As the magnitude of data that is produced by single cell immunoprofiling increases and reveals unprecedented levels of diversity among immune cell, methodological rigor will be critical for deciphering mechanisms of disease.

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Highlights

- Single cell immunoprofiling reveals extensive heterogeneity among CD4+ T cells.
- Multidimensional analyses identify novel CD4+ T cell populations associated with rheumatoid arthritis.
- Single cell disease association studies require careful attention to study design to avoid confounding technical effects.
- New analysis methods are emerging to take full advantage of complex single cell datasets.

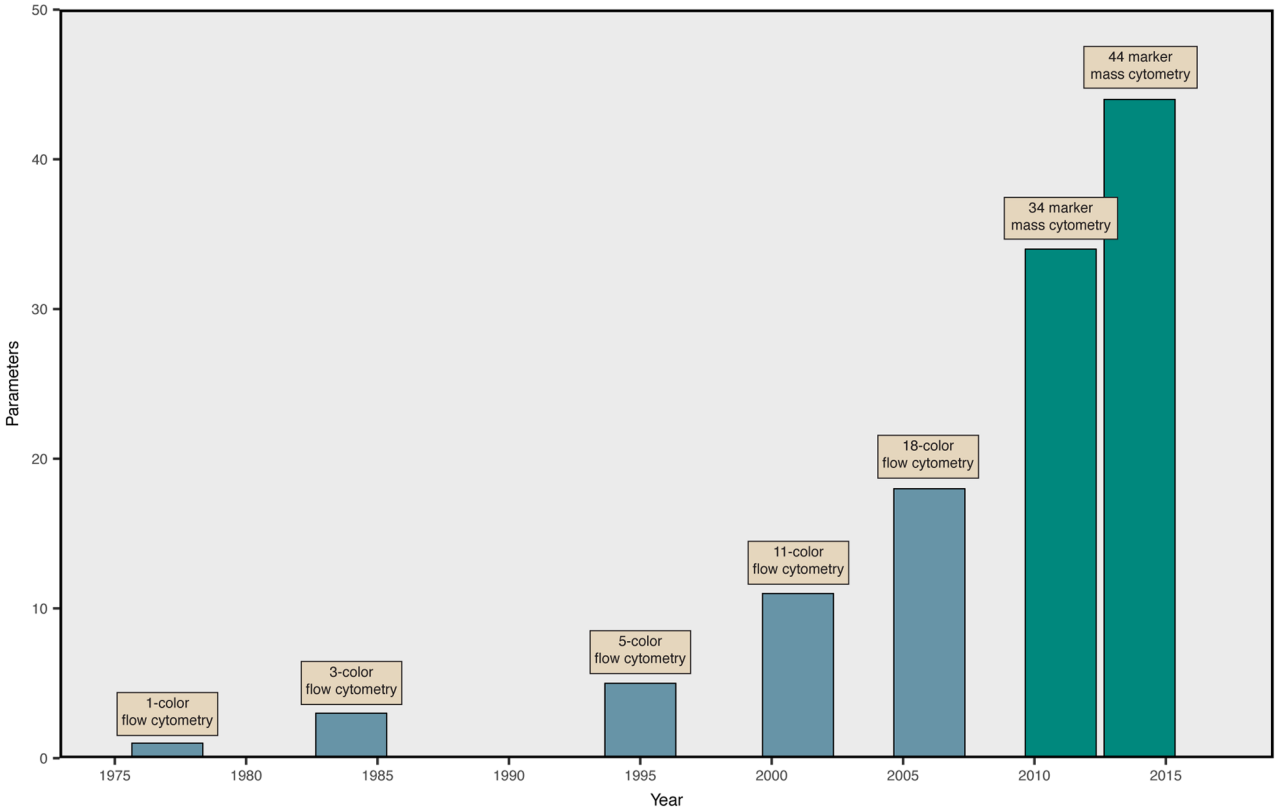

Figure 1. Advances in Single Cell Cytometry

The number of unique molecules that can be simultaneously characterized for a single cell has progressively increased. The introduction of new fluorchromes has improved polychromatic flow cytometry and enabled the development of 18-color assays. Mass cytometry, which uses stable isotopes of non-biological rare earth metals linked to antibodies to detect protein epitopes, is currently capable of acquiring 44 markers simultaneously. Current equipment for mass cytometry supports the acquisition of over 100 markers, but experiments are limited by the availability of isotopically pure reagents.