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Cytometry: Today's technology and tomorrow's horizons

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ABSTRACT

Flow cytometry has been the premier tool for single cell analysis since its invention in the 1960s. It has maintained this position through steady advances in technology and applications, becoming the main force behind interrogating the complexities of the immune system. Technology development was a three-pronged effort, including the hardware, reagents, and analysis algorithms to allow measurement of as many as 20 independent parameters on each cell, at tens of thousands of cells per second. In the coming years, cytometry technology will integrate with other techniques, such as transcriptomics, metabolomics, and so forth. Ongoing efforts are aimed at algorithms to analyse these aggregated datasaets over large numbers of samples. Here we review the development efforts heralding the next stage of flow cytometry.

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1. Introduction

Since the invention of flow cytometry in the 1960s, advances in the technology have come hand-in-hand with advances in the recognition and characterization of new leukocyte subsets. In the early years, with the advent of one- and two-color flow cytometers, major lymphocyte lineages comprising the cellular arm (T-cells) and the humoral arm (B-cells) were identified [1,2]. Through the 1980s, the ability to perform three- and four-color flow cytometry experiments enabled the enumeration of cells expressing combinations of CD3, CD4, and CD8 from a single tube; this was a necessity driven by the clinical demands of the emerging HIV epidemic [3]. The following decade saw continued development in multicolor technology and immunology, with the advent of polychromatic flow cytometry (detection of five or more markers simultaneously) enabling identification of naïve and memory T-cell subsets [4] and detailed functional characterization of antigen-specific lymphocytes (such as measurement of multiple cytokine production from individual cells [5]). Most recently, the new millennium brought 12–18 color technology [6,7] and an unprecedented resolution to immune analysis (including the identification of regulatory T-cells [8], follicular helper T-cells [9], TH17 cells [10], and the ability to combine functional and phenotypic analyses [11]; Fig. 1).The ongoing development of flow cytometry technology has left its mark on the analysis of hematopoetic development, cell signaling networks, and leukemia/lymphoma diagnoses.

Remarkable advances in hardware technology (e.g., high powered lasers emitting blue, red, green, violet, and yellow light), fluorochrome chemistry (e.g., tandem dyes, nanocrystals), and software tools have driven this co-evolution of flow cytometry and immune analysis [12]. However, data analysis strategies are still relatively underdeveloped; that is, the multitude of data available from a polychromatic flow cytometry experiment is rarely analyzed easily or in its entirety. To a lesser extent, improvements are possible in instrument calibration, experimental design, and reagents. In light of this, two questions can be posed: do we really need to measure this many parameters simultaneously, and, if so, what does the future hold in terms of advances in flow cytometry and the development of other single-cell technology platforms? This chapter will address these questions by examining the current and future states of cytometric analyses.

2. The need for multiparametric analysis

A basic advantage of polychromatic experiments (compared to two- or three-color analyses) is better specificity for desired cells, especially with the detection of low frequency populations. Such populations include antigen-specific T- and B-cells, which are commonly identified with fluorescent multimers of peptide + MHC [13] or antigen [14]. These multimeric complexes will often bind non-specifically to various leukocyte populations (particularly monocytes) present in the sample. Similarly, multimeric proteins or the antibody conjugates used for staining may bind dying cells non-specifically. Thus, the use of antibodies to negatively select for undesired populations (such as CD14 to identify and eliminate monocytes) as well as the use of viability dyes (to exclude dead cells, a common source of non-specific interactions) is critical. Even when these negative-selection reagents are combined in a single "dump" channel, simple experiments quickly become

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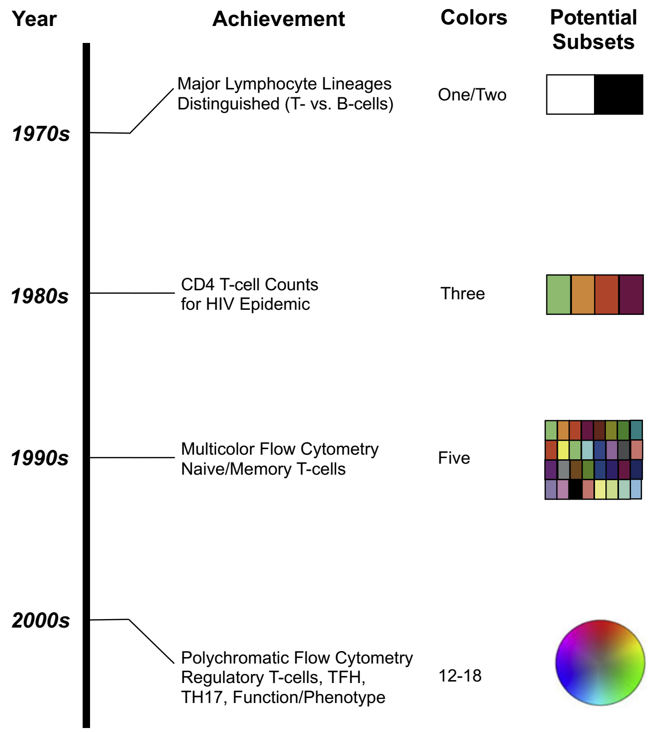

Fig. 1. A timeline illustrating coordinates advances in flow cytometry technology and understanding of the complexity of the T-cell compartment.

polychromatic. For example, basic phenotyping of an antigen-specific T-cell population requires one channel for exclusion markers, three channels to identify T-cell lineages, one channel for identification of the antigen-specific population (e.g., with a peptide-MHC Class I multimer), and ideally at least three channels for determination of T-cell differentiation (memory, effector, etc.) [3]. In total, this phenotyping experiment requires 8-color flow cytometry.

A similar issue arises when trying to interpret the biology of cells with a particular phenotypic profile. This is particularly important when trying to distinguish biologically distinct cell types that share expression of some markers; our interpretation must be validated biologically. The identification of regulatory T-cells (Treg) is a notable example. Initial descriptions [15–17] of these cells relied solely on the level of CD25 expression (e.g., the top 2% of expressing cells were considered Treg); however, variability between reagents as well as samples led to considerable confusion with such identification. Suboptimal staining of CD25 had important consequences as expression of this marker is also observed on activated cells (a cell whose role is to amplify the immune response, as opposed to downregulate it!). Subsequent studies associated expression of the intracellular marker FoxP3 with regulatory T-cells [18]; however, again, stimulated cells also express this transcription factor. Most recently, the prevailing dogma requires multiple markers in combination to detect these cells (e.g., CD25/FoxP3/CD127/CD39) or the analysis of cytokine production following stimulation (TGFb, IL10) [19,20]. In either case, at a minimum, to ensure biologically valid identification of regulatory T-cells, six color flow cytometry experiments are needed.

The number of markers expressed by Treg, and the fact that many of these markers are also expressed by other cell types, provides a glimpse into the incredible diversity of memory T-cells. For example, with an 11-color flow cytometry experiment designed to phenotype memory T-cell subsets, expression of six markers (CD45RA, CCR7, CD27, CD127, CD57, CD28) can be investigated. If expression of each marker is considered to be either on or off (and ignoring variation in expression) 64 cell subsets within each of CD4 or CD8 T could theoretically be identified. However, if phenotypic diversity were limited, only a fraction of these T-cell subsets might be represented in the peripheral blood. In fact, this is not the case, as cells expressing each combination of these markers can be detected [21]. This suggests that there are at least 64 different subsets of circulating CD4 and CD8 T-cells – albeit with as-yet largely unknown functional correlates. As the number of markers used to characterize cells increases, the number of different cell populations detected in the peripheral blood rises as well, implying that the true diversity of memory T-cell subsets is even greater. Quantifying this diversity and determining which subsets of cells are functionally similar is fundamental to our understanding of the immune system.

More importantly, the ability to finely define T-cell subsets (based on multiple parameters) improves the likelihood of identifying cells important in disease pathogenesis or vaccination. The identification of critically-relevant cells is important from the standpoint of immune monitoring and optimizing vaccine efficacy. For example, in recent years, a body of literature has emerged that describes the importance of polyfunctional T-cells (cells simultaneously expressing a majority of the measured functional markers, including e.g. IFNγ, IL2, TNF, MIP1β, and CD107a) [22]; the frequency of these cells has been associated with less pathogenic disease (HIV in long-term non-progressors [23], HIV-2 [24]) or protective immunity (Leishmania [22], Vaccinia [25], Yellow Fever [26], SIV [27]). Notably, the relationship between these functional markers and disease was not apparent in earlier studies, measuring only one or two functional markers at a time, and can only be revealed with 9+ color flow cytometry.

The reason underlying the failure of single parameter approaches is illustrated in Fig. 2. In this hypothetical example, the measurement of four functions together defines a putative population of cells (IFNγ + IL2-TNF + CD107a+) whose frequency has a strong, inverse correlation with disease progression. When TNF is not measured in the experiment, then TNF-negative cells will also fall into the measured subset, thereby introducing noise into the assay and weakening the correlation. When two markers are absent, more noise is introduced into the assay, further reducing the power to predict disease progression. Finally, when only a single marker is measured (IFNγ alone, for example) predictive power could be completely lost. Thus, bulk measurements of only one or two cell types may reduce the power to detect cell populations important in disease. In this way, multiparametric technology (such as polychromatic flow cytometry) provides not only a better theoretical understanding of immunity, but can also have practical benefit.

3. Approaches to polychromatic experiments: Limitations and future directions

Today, commercial flow cytometers capable of measuring 10 parameters are common, and those capable of 12–20 parameters are widespread. This rapid dissemination of hardware technology has revealed pressing needs in other areas: (1) practical and efficient methods for instrument calibration and quality control (QC), (2) availability of a wider variety of fluorochromes and antibody conjugates, (3) assistance with experimental design, and (4) better strategies for data analysis.

3.1. Instrument calibration and QC

The success of polychromatic flow cytometry depends critically on instrument calibration; however, the old paradigm of analyzing unstained cells, and adjusting the gain (voltage) for all detectors