Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine

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The FACS was born in our laboratory, in about 1968, after a long but natural labor. We formally christened the FACS as such in two 1972 publications1,2. When this name was trademarked by Becton-Dickinson (BD Biosciences, Milpitas, CA, USA) a decade or so later, it had already become part of the vernacular and had even become a verb in some laboratories. In this article we use it in the generic sense to refer to flow cytometry and cell-sorting instruments, regardless of their commercial origin. As an infant the FACS was cantankerous and slow and required intensive care and feeding. Nonetheless, it rapidly became productive and in 1969 we announced its birth in a publication demonstrating the automated separation of mammalian plasma cells as a function of intracellular fluorescence3. Over the next two years we introduced the automated separation of mammalian plasma cells as a function and in 1969 we announced its birth in a publication demonstrating its productivity and required intensive care and feeding. Nonetheless, it rapidly became productive and in 1969 we announced its birth in a publication demonstrating the automated separation of mammalian plasma cells as a function of intracellular fluorescence3. Over the next two years we introduced the automated separation of mammalian plasma cells as a function and in 1969 we announced its birth in a publication demonstrating its productivity and required intensive care and feeding. Nonetheless, it rapidly became productive and in 1969 we announced its birth in a publication demonstrating the automated separation of mammalian plasma cells as a function of intracellular fluorescence3. Over the next two years we introduced the automated separation of mammalian plasma cells as a function

The histories of monoclonal antibodies and the fluorescence activated cell sorter (FACS) are closely intertwined as their current uses in biology and medicine. Here, Leonore Herzenberg, Stephen De Rosa and Leonard Herzenberg recount the meeting and the mating of these two technologies, whose offspring now populate clinical and research laboratories throughout the world.

The FACS reagent problem

Despite its early promise, FACS technology still had major drawbacks. The instrumentation was fairly satisfactory. However, the fluorochrome-coupled reagents available in the early 1970s were prepared from conventional polyclonal antisera using methods developed originally for cytotoxicity and fluorescence-microscopy studies. In essence, mice, rats, rabbits or goats were immunized with cells or purified proteins. The sera collected (usually repeatedly) from the immunized animals were then screened to find those with high titers of antibodies of the desired specificity combined with low titers of contaminating antibodies. Although this goal was sometimes achieved, most sera contained some antibodies that reacted with broadly distributed determinants on cells or proteins. Thus, virtually all conventional sera had to be absorbed by incubation with cells or proteins that lacked the target determinant (if such could be found) in order to meet the demanding specificity requirements for FACS reagents.

Unfortunately, the absorption procedures frequently resulted in either dilution or loss of the desired antibodies and contamination with proteins or other material derived from the cells used for absorption. In addition, even when adequate titers of the desired antibodies remained and the preparation did not acquire significant amounts of cell-derived material, unsuspected or unabsorbable contaminant antibodies often persisted. Some of this contaminating material was removed by isolating the serum Ig fraction that contained the desired antibodies. However, as the isolation procedure resulted in additional loss of desired antibodies it was most often used for the preparation of indirect (second-step) fluorochrome-coupled staining reagents.

Fluorochrome-coupling procedures also took their toll during the preparation of FACS reagents. Although methods for chemically coupling fluorescent tags to antibodies were well established at the time, these methods were optimized for fluorescence microscopy and did not always yield reagents that were suitable for FACS studies. Fluorescence microscopy is more forgiving as the eye can be
trained to ignore low, background, fluorescence levels and to recognize ‘patched or capped’ fluorescence patterns that distinguish true-positive from false-positive cells. FACS, however, provides a quantitative rather than a qualitative measure that is based on cell-associated fluorescence and is independent of staining pattern. Therefore, FACS reagents cannot accommodate the levels of chemically denatured antibodies and ‘sticky’ aggregates of antibodies and other cell products that are not desirable but can be tolerated in fluorescence microscopy reagents.

Ultimately, a battery of immunization, absorption, antibody isolation and fluorochrome-coupling strategies were devised to facilitate production of FACS staining reagents. However, even with this methodology, there were substantial barriers to obtaining reagents with sufficient specificity to be used in FACS studies. Many of the antibodies had to be produced in mice and so they were almost always in short supply and difficult to manipulate. Collectively, these problems made it extremely difficult to produce a high-titer and highly specific reagent for FACS staining and essentially impossible to duplicate the reagent once it was gone. However, the demand for reproducible FACS reagents was growing. By 1976, several commercial FACS instruments had been installed in the USA and Europe and laboratories were beginning to exploit FACS for mouse and human studies. Late that year, when the first International Flow Cytometry meeting was held in Cambridge, UK, reagent preparation stood high on the agenda and corridor discussions revolved around methods for fostering collegiality and sharing of reagents. Basically, it was clear that while FACS analysis could provide unambiguous answers to questions that bedeviled studies based on fluorescence microscopy, there was little hope of realizing the full potential of the FACS technology, while reagents prepared in a similar manner but from different pools of immune sera gave different results. Monoclonal antibodies (mAbs), thus, arrived on the scene none too soon. This FACS meeting marked the beginning of a sabbatical year that we (Len and Lee Herzenberg) were to spend in Cambridge. Len, seeking to recharge his somatic-cell genetics battery, had arranged to spend a year learning the nascent molecular biology techniques that César Milstein and colleagues at the Cambridge Molecular Biology Laboratory were developing. Between this arrangement and arriving in Cambridge, Kohler and Milstein published their landmark paper describing the fusion of spleen cells with an in vitro adapted myeloma to generate immortal cell lines producing mAbs originally produced by the individual spleen cells.

**mAbs as FACS reagents**

Work in Milstein’s laboratory on the methods for generating and using these antibody producing lines was progressing rapidly, particularly in the direction of using these lines to investigate the rules that governed pairing of Ig heavy chains and other issues central to basic immunology at the time. There was also a growing awareness, to which we contributed, of the value of selecting lines producing mAbs that could be used as reagents for immunological studies. On New Year’s Eve in 1976 we joined Milstein at the Cambridge University Club in a champagne toast christening these cell lines ‘hybridomas’. Shortly thereafter we coauthored, with Milstein, a prescient chapter (in the third edition of the *Handbook of Experimental Immunology*) that predicted most of the modern uses for the mAbs produced by these hybridomas.

In the spring of 1977, Vernon Oi, then a graduate student in our laboratory at Stanford, joined us in Cambridge. Using facilities provided by Arnold Feinstein and Jonathan Howard at the Babraham Animal Sciences Laboratories, Cambridge, Oi began generating hybridomas producing mAbs that detected genetically determined structural differences between the Ig isotypes produced by various mouse strains. We had previously developed conventional antisera that detected these differences, and had, in fact, shown that they were encoded by alleles at a series of linked loci in what we termed the mouse Ig heavy-chain chromosome region. Furthermore, as indicated above, we had completed a series of studies on B-cell development that relied on detection of these allotypic (i.e., allele encoded) differences within the same isotype produced by different mouse strains.

Being familiar, through these studies, with the limitations of working with conventional anti-allotype antibodies, we were immediately struck by the clarity with which mAbs revealed the allotypic determinants on IgH proteins, and with the simplicity of preparing these reagents for use. Thus, we were primed to return to Stanford, where we had access to the FACS and could screen for hybridomas that would produce limitless amounts of mAbs that could universally be used for FACS detection of cell-surface determinants.

The first set of mAbs produced at Stanford far outstripped expectations. We (the team now included Patricia Jones, James Goding, Barbara Osborne and Dick Goldsby) obtained mAbs to several cell-surface determinants, including major histocompatibility complex (MHC) class I and class II. As we were readily able to isolate large quantities of these antibodies we could prepare fluorochrome-coupled reagents for FACS studies and do immunoprecipitation and gel analyses to identify the reactive cell surface molecules without depleting our stores. Thus, for a time, we put our energy into producing mAbs to meet our needs for FACS reagents and other purposes. We found this highly rewarding because we were able to generate an excellent set of reagents and make these available to our colleagues, either by giving them the purified mAbs or, more importantly, by giving them clones that would produce limitless amounts of the mAbs.

At the same time César Milstein, Alan Williams, Jonathan Howard, Tim Springer, Giovanni Galfre, Mel Greaves, Stuart Schlossman and their colleagues (and others) also began to make mAbs to cell-surface determinants. Despite this, many laboratories still did not have access to the technology or the reagents. Recognizing that this division between the ‘haves’ and ‘have-nots’ would only get greater with time, we urged Becton-Dickinson, the company that produced the initial FACS instrument, to develop a production center to supply fluorochrome-coupled mAbs for use as FACS reagents. This center, which began operation in about 1981, included the mAbs we produced in the first monoclonal reagents in its catalog. Ortho Diagnostic Systems (Raritan, NJ, USA; no longer in
mAbs and FACS in immunology: a new era

At the time mAbs were introduced, it was known that lymphocytes and other cells express surface H-2 antigens, including what are now known as MHC class I and class II molecules. B cells were known to express surface IgM and class II molecules and to give rise to antibody-producing cells. T cells, in contrast, were known to express the Thy-1 antigen and to perform functions ranging from cytotoxic killing to helping B cells make antibodies. In addition, evidence indicating that surface markers could distinguish subsets of T cells responsible for these activities had just begun to appear. However, the conventional reagents used in these and similar studies were hard to come by and even harder to work with, particularly for FACS studies. Thus, we and other immunologists were anxious to replace conventional antisera with monoclonal reagents that would allow us to identify, sort and characterize lymphocyte subsets responsible for various immune functions.

The methodology for doing this was deceptively simple: immunize animals using the cellular antigens and immunization procedures used to generate the conventional antibodies; identify animals making the desired antibodies; perform the cell fusions to generate clones making mAbs; and, finally, isolate stable clones producing the antibodies required. Basically, the procedures involved were straightforward up to the point of screening clones to identify those producing the desired antibodies. However, devising screening strategies to obtain clones producing mAbs to cell surface antigens was clearly a challenge, and compounded further by the need to find antibodies that detected determinants recognized by the conventional antisera with which cells responsible for various aspects of immune function had been identified.

FACS technology played a role in nearly all aspects of this effort. Cells from the spleen or other tissues were stained with hybridoma supernatants and FACS was used to identify hybridomas that produced reactive antibodies. FACS was also used in our laboratory (and eventually in many others) to clone the hybridoma cells, to identify productive clones and to reclone the hybridoma cells until stable clones were established. In addition, FACS was used to characterize the location and number of cells identified by mAbs produced by a newly isolated clone and, thus, to provide the earliest clues as to whether the antibodies detected a previously unknown determinant or reacted with a determinant that had already been identified.

Finding mAbs that corresponded to earlier, more conventional, reagents relied more on other technologies, rather than on FACS. The specificity of conventional antisera were defined mainly by their ability to deplete functional cells by complement-dependent cytotoxicity and the specificity of the principal antibodies in these antisera were identified by their ability to immunoprecipitate particular cell-surface molecules that could be visualized in gels. In some cases, conventional antisera had also been used with FACS to isolate and test (rather than deplete) functional lymphocyte subsets although, by and large, these studies did not provide substantial data for comparison. Thus, although the FACS identified candidates for replacing conventional reagents, the primary work necessary to move immunology from conventional to mAb-based studies involved immunoprecipitation and functional studies that mapped the emerging monoclonal reagent set onto the set of determinants identified with conventional reagents.

As this work progressed, the new monoclonal reagents were used to develop well-defined FACS staining patterns that characterized both the distribution of cells expressing the determinant and the level of the determinant expressed on various cells. Furthermore, monoclonal reagents were passed around rapidly, even before their specificity was established. Thus, the shift from an antisera-based to a monoclonal-based 'economy' occurred very rapidly and, in the process, FACS became a far more central technology in immunological studies.

By and large, the data obtained with mAbs corresponded well with the previous data. However, there were certain key exceptions that created somewhat of a short-term stir. For example, in the first studies demonstrating that distinct T-cell subsets help and suppress antibody production, the functional T-cell subsets were selectively depleted by complement-dependent cytotoxic treatment with conventional antibodies absorbed to detect either Ly-1 or Ly-2 (now known, respectively, as CD5 and CD8)6. However, although cytotoxic treatment with the conventional anti-CD5 removed helper but not suppressor T cells in these studies, FACS studies with monoclonal anti-CD5 (with the same specificity as the conventional reagent by immunoprecipitation criteria) demonstrated that CD5 is present on all T cells7. It was only later, when the monoclonal anti-CD4 reagent was produced, that the two primary functional T-cell subsets in the mouse were distinguished finally and unequivocally.
mAbs detecting functional subsets of human T cells

Several laboratories, including Stuart Schlossman’s group and Robbie Evans’ group at Sloan-Kettering produced mAbs to surface markers of human T cells (and other cells). Because some of these antibodies stained subsets of human T cells, Jeffrey Ledbetter in our laboratory collaborated with Evans’ group in a FACS study to compare the subsets detected by the monoclonal reagents in the mouse and the human. It was surprising to us at the time that the FACS-staining patterns obtained with mAbs to the human T-cell subsets were similar to the FACS-staining patterns obtained with mAbs to the mouse T-cell subsets\(^3\). In essence, these initial studies identified mAbs that distinguished the basic functional T-cell subsets (helper and cytotoxic/suppressor) in humans. Thus, within a matter of weeks, they opened the way to today’s complementary use of FACS and mAbs in clinical research and medicine.

CD antigens: cutting the Tower of Babel down to size

The identification of corresponding cell-surface determinants in human and mouse created a notation crisis because independent groups used different notation philosophies. The crisis was further fueled by several cases in which mAbs that reacted with the same determinant in the same species were independently named by the research groups that developed them. In addition, commercial producers of monoclonal reagents (particularly to human-cell-surface determinants) wanted their chosen names to be the ones used that were used universally. The epitome of this notation nonsense is reflected in the naming by one of the leading research groups of the mAb to the determinant that distinguishes mouse helper T cells from cytotoxic/suppressor T cells. This determinant, now known as murine CD4, was originally named L3T4 as a mnemonic that reflected the two most popular names for the corresponding human determinant (Leu-3 and T-4).

The notation conference (First International Workshop and Conference on Human Leukocyte Differentiation Antigens, Paris, 1982) convened to resolve this situation dealt both with the naming question and with the more complex matter of determining whether two mAbs detected the same or different surface molecules. Identifying determinants detected by monoclonal reagents is not particularly difficult when the determinants can be visualized by immunoprecipitation and gel analysis. However, cell-surface determinants detected by mAbs are not necessarily amenable to study by these methods. Therefore, the conference set up a more general procedure for determining identity and made this a part of the naming convention.

In essence, mAbs were distributed to workshop investigators who used different technologies (FACS, cytotoxicity, immunoprecipitation, functional assays) to classify the reactivity of each antibody. FACS was used primarily to determine the distribution pattern of cells expressing the target determinant. Results were then compared and antibodies with the same (or approximately the same) reactivity were assigned the same CD number. As the term CD (cluster of differentiation) implies, the assignment of a CD number requires that two mAbs have similar reactivity.

When antibodies react with cell-surface determinants in the same species, this cumbersome methodology for identifying a pair of mAbs with similar reactivity can largely be avoided by labeling the mAbs with distinguishable fluorochromes, staining cells simultaneously with the two labeled antibodies and using two-color FACS analysis to determine whether their staining pattern is identical. When the mAbs detect determinants in different species, the staining patterns of target cells (such as lymphocytes) are similar in most cases, although there are exceptions to this rule. From a current perspective, therefore, it is somewhat difficult to understand what motivated development of the highly complex CD-workshop system. However, prior to the introduction of mAbs, FACS use was restricted to a small group of laboratories only a few of which were comfortable with two-color FACS work. Thus, there was far more support for the biological methods used to define CD determinants and the antibodies that detected them.

Two-color FACS analysis with a one-laser FACS

In our laboratory, the ability to directly label monoclonal FACS reagents with different fluorochromes provided the impetus to improving multicolor FACS analyses and making this technology more universally available. David Parks, together with Michael Loken, was responsible for developing the fluorescence compensation hardware that enables simultaneous measurement of two fluorochromes on individual cells (in today’s parlance, measurement of two colors off one laser). The compensation hardware corrects for overlap in the emission spectra of two (or more) fluorescent dyes excited simultaneously by light from a single laser. When implemented initially on the single-laser FACS in our laboratory, it enabled simultaneous measurement of the amounts of two FACS reagents bound to the same cell, one labeled with fluorescein and the other with rhodamine (the only red dye excited by the laser in use at the time).

Once we had fluorochrome-coupled mAbs to a variety of cell-surface determinants, we made significant use of single-laser, two-color FACS analysis. However, this technology was basically unsatisfactory, partly because the fluorescent dyes were poor (better ones would soon be developed) but mostly because the accuracy of the measurement was influenced by imbalances in the amounts of the determinant detected by each mAb. As the overlap corrections involve signal subtraction, a large amount of one determinant will seriously impair the ability to measure a small amount of the second. This problem is compounded by detection of the highly represented determinant with a fluorescein-coupled reagent, since fluorescein is a highly efficient fluorophore that, on a molar basis, generates more signal than most dyes. Therefore, the single-laser two-color system was most useful when the determinants being detected and the reagents used for detection could be properly organized but was highly problematic in situations where the balance between the two markers varied widely on different cell types as occurs with, for example, surface IgM and IgD on spleen cells.
The dual-laser FACS

The development of the dual-laser FACS, stimulated by the broad availability of monoclonal FACS reagents, opened the way to the development of modern single and dual-laser instruments by encouraging the development of additional dyes that can be used in FACS and, hence, increasing the number of markers that could be simultaneously detected on individual cells. This instrument, which went into operation ca. 1982 (Ref. 39), was initially used in two-color studies to explore B-cell subsets expressing different amounts of IgM, IgD and other markers. However, the availability of increasing numbers of mAbs to detect B-cell determinants meant a return to using fluorescence compensation, this time to enable the detection of three and, soon, four fluorescence colors with the dual-laser instrument (i.e., two off each laser).

Today, single-laser FACS instruments are commonly used to simultaneously measure the binding of three mAbs identified by fluorescence colors that are resolved by current fluorescence-compensation methods. Dual-laser FACS instruments are found in many laboratories and are used to measure up to five fluorescence colors simultaneously. In addition, at Stanford we have recently developed a three-laser instrument, and dyes to match (Fig. 1), that allows simultaneous measurement of up to 11 fluorescence colors.

Fluorescence compensation in these modern FACS systems is still problematic. Even though modern optics and optical filters have decreased overlaps, it is still necessary to match mAbs and fluorescence dyes carefully in order to prevent highly represented markers from overwhelming the signals generated by markers that are less abundant on cells of interest. Nevertheless, effective reagent and dye combinations have been developed and much productive work done with these systems (for examples, see Refs 46–54).

**Why use so many fluorescence colors?**

As the number of surface markers detectable with mAbs increases, the number of functional subsets of cells defined by qualitative or quantitative differences in marker expression also increases. Single-color analyses using reagents that detect one of a set of markers expressed in a given population reveals the number of cells that express the individual markers but provides no information about joint marker expression, which is central to the identification of subsets. The same principle holds when more reagents are used: less information is obtained when reagents are used alone or combined in small sets (i.e., two two-color stains are not equivalent to one four-color stain).
Basically, the more reagents that can be used together in a single stain, the less ambiguity there is in defining a subpopulation of cells with a common phenotype. Subpopulations with phenotypes that overlap when two sets of two reagents are used can often be resolved when the four reagents are used together. Thus, even though the gating strategies are more complex with multiple colors (Fig. 2), the assignment of cells to individual subsets is more straightforward because a given cell will fall into one and only one set of gates (gating tree).
Because counting and sorting functional subsets of cells that express several markers is central to many basic and clinical studies, the use of two to four color FACS analysis has become relatively common in recent years. Five and six color analysis and sorting are still state-of-the-art capabilities, but are already available in many laboratories. In addition, as mentioned above, we routinely use a Stanford instrument capable of analyzing and sorting with combinations of up to 11 FACS colors and two light scatter measurements46–48,53–57. Similar instruments are also now being introduced commercially, as is software for handling multicolor analyses.

We find that the use of this instrument has several benefits, in addition to providing greater resolution for subset characterization. For example, by combining several reagents in a single staining combination, fewer tubes are analyzed per sample and, hence, the overall number of cells required for analysis is lower. This is significant when handling samples of pediatric peripheral blood mononuclear cells or charting changes in gene expression in cells in culture. Thus, the introduction of mAbs as FACS reagents is, undeniably, the single most important breakthrough underlying the universal use of FACS in biology and medicine today.

The converse is also true. FACS technology is undeniably the single most important factor underlying the widespread use of mAbs in modern medical practice. FACS analysis and sorting studies using mAbs to define the surface markers on normal and neoplastic cell populations created the basis for routine clinical diagnostic assays that now range from leukemia classification to monitoring CD4 T-cell loss as HIV disease progresses. FACS has also played a central role in the development and use of mAbs in therapeutic strategies and remains crucial to the commercial production of mAbs for all purposes. Thus, mAbs and FACS emerge as truly complementary tools whose remarkable synergy continues to fuel major advances in immunology and medicine.

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References
5 Jones, P.P. et al. (1973) Immunoglobulin (Ig) allotype markers on rabbit lymphocytes: separation of cells bearing different allotypes and demonstration of the binding of Ig to lymphoid cell membranes. J. Immunol. 111, 1334–1348
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