

Fluorescence Intensity Calibration for Immunophenotyping by Flow Cytometry¹

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Fluorescence intensity (FI) is the basis for classifying phenotypes by fluorescence-label flow cytometry. FI is customarily recorded as an arbitrary relative value, but with proper calibration it can be expressed in stoichiometric units called molecules of equivalent soluble fluorochrome (MESF) that reflect the concentrations of the fluorescent conjugates and the receptors they stain. Forthcoming availability of authoritative standards and consensus methods will alleviate many of the difficulties encountered in making valid MESF measurements. FI calibration establishes the true values for the critical parameters of the fluorescence measurement, a useful feature for quality control. It further allows the establishment of a comparable window of analysis across different times and laboratories, and it permits numeric assessment of antibody-binding capacity (ABC) values in selected cell populations. The relation between ABC values and receptor expression is complicated by several factors, but careful assessment of the binding chemistry can establish the actual number of receptors on cells stained by fluorescent conjugates.

Key Words: flow cytometry; fluorescence intensity; molecules of equivalent soluble fluorochrome; MESF; antibody-binding capacity; ABC; cell receptors; quality control; standardization.

Recent progress in calibrating fluorescence intensity (FI) has prompted a new phase of development

just as this issue goes to press (1). The National Institute for Standards and Technology (NIST, formerly the National Bureau of Standards) is currently developing the first standard reference material (SRM) for FI measurements. At the same time, the National Committee for Clinical Laboratory Standards (NCCLS) has convened a committee on fluorescence calibration to propose guidelines for biomedical applications of FI measurements. The fluorescence intensity SRM 1932 is scheduled for release in 2000, and the NCCLS proposed guideline I/LA24P will be composed by 2001. The reader can check Web sites to update the status of the fluorescein SRM (www.nist.gov, search on “fluorescence intensity”) and the NCCLS guidelines (www.nccls.org, select “Projects in Development”). This article anticipates the availability of the SRM and envisions the general principles expected to be addressed in the NCCLS guideline. We will concentrate on immunophenotyping by flow cytometry, as this application entails the special difficulties involved with FI measurements on particulates as well as the more general principles associated with all receptor–ligand assays.

Fluorescein was selected by NIST to be the first fluorochrome SRM because of its widespread use and longstanding experience with FI calibration (2, 3). The development of validated methods for FI calibration with fluorescein is expected to serve as a model that can be applied to other fluorochromes. In addition to the many other fluorescent dyes currently in common use, a new generation of fluoro-

¹ Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

chromes resistant to bleaching and customized for spectral properties has been under development (4, 5) and should soon impact biomedical technology. Each fluorochrome will likely present unique challenges to calibration and require continuing reassessment of the general approach.

An important component of standardized measurement is standardized nomenclature. Terminology for FI calibration (6) and for particulate FI standards (7) has been proposed, and we will recap and employ some of those definitions here. For general terms, we will use definitions from the NCCLS guideline (8). In particular, the term "receptor" will be taken to mean a molecule attached to a cell or particle which can bind complementary molecules ("ligands") such as hormones, neurotransmitters, antigens, or antibodies. A "conjugate" is a ligand labeled with tracer molecule such as a fluorochrome, radioisotope, or enzyme.

FLUORESCENCE CALIBRATION AND MESF UNITS

Fluorescence intensity is a form of luminous flux, a power measurement expressed in absolute terms as photons emitted per unit time. The fundamental constant associated with FI is the quantum yield: the ratio of photons absorbed to photons emitted (this is not a practical measurement in most laboratories). Maximum excitation wavelengths generally correspond to absorption peaks. The emitted photons will be red-shifted to higher wavelengths (lower energy) due to inefficiency of energy transfer; the difference between the peak excitation wavelength and the peak emission wavelength is the Stokes shift. Excitation and emission spectra tend to be broad in most commonly used fluorochromes. This can be an advantage, allowing efficient excitation of multiple fluorochromes with a single-wavelength laser. It also can be a disadvantage, causing overlap in the emission signals between different fluorochromes.

The goal and *de facto* definition of fluorescence intensity calibration is to express fluorescence measurements on a scale that accurately reflects the mass quantities of each fluorochrome-labeled reactant and product (stoichiometry). Radioimmunoassays provide a convenient model for stoichiometry. Radiolabeled tracers can be accurately calibrated by

determining the specific activity of the conjugate in curies per unit mass, since this explicitly determines the radiative flux (disintegrations per minute). Gamma or beta counters can be calibrated to convert arbitrary counts per minute to the equivalent disintegrations per minute, leading to a direct measure of conjugate mass. On a molecular level, the specific activity of radioactive conjugates can be expressed as the average number of tracers labeling each conjugate molecule.

With fluorochrome tracers, the fluorochrome to protein ratio (F/P ratio) is analogous to the specific activity: it determines the number of fluorochromes per conjugate molecule. However, this molar F/P ratio cannot be used to predict fluorescence emission. Unlike the spontaneous predictable decay of radionuclides, fluorescence emission requires excitation energy and depends on the molecular environment of the emitter. Both excitation energy and emission environment will vary considerably across platforms and applications, and a stoichiometric system of FI calibration must account for such variances.

Stoichiometry becomes especially difficult when FI measurements in solution are compared with those on solid-phase materials such as gels, microbeads, and cells. The total intensity of the excitation energy (usually laser light) delivered to solid-phase materials is highly platform-dependent, and the solid phase permits microlocal differences in pH, hydrophobicity, and other factors that can influence FI. The preferred approach to FI calibration is analysis on the same platform of solid-phase standards that are comparable to the unknowns. To preserve stoichiometry, these solid-phase standards must be calibrated in units traceable to solution concentrations of fluorochrome. These units are called molecules of equivalent soluble fluorochrome, or MESF: they express the FI of fluorochrome-labeled particles as the number of fluorochrome molecules in solution required to produce the same FI as that measured on the labeled particle (6). MESF standards must be spectrally matched with the fluorochrome they represent, and they must respond to environmental changes in the same fashion as the free fluorochrome. As long as unknowns and MESF standards are read under the same conditions, a consistent value for the FI of the unknown may be interpolated from a calibration curve constructed with MESF standards.

The assignment of MESF values is an evolving process which so far has been conducted by individ-

ual suppliers of microbead standards (9, 10) without recourse to any universal authoritative standards. The FI of particulate standards must be compared with the FI of solutions containing known concentrations of the same fluorochrome in an identical environment. A number of technical details remain to be fully resolved, and SRM for solid-phase FI calibrated in MESF units are probably still a few years away. However, microbead standards with well-accepted MESF values for fluorescein have been studied extensively (9), and they currently provide the best example for general approaches to FI measurements. Careful studies have also been performed on cells and microbeads labeled with phycoerythrin (PE) (10), although the overall experience with these MESF values is less extensive.

MESF UNITS AND THE EFFECTIVE F/P RATIO

The use of MESF units provides the correct fluorescence analog to the specific activity of a radiolabeled molecule: the average number of MESF per conjugate molecule, called the "effective F/P ratio" (6). The effective F/P ratio can be used to calculate the mass of conjugate labeling a cell or particle if its MESF value is known:

Molecules of Conjugate

$$= (\text{MESF}) \times (\text{effective F/P ratio}).$$

The effective F/P ratio is influenced by the amount of fluorochrome used to label the conjugate and a number of other factors that diminish fluorescence, collectively referred to as "quenching." It is rather easily measured by cuvette fluorometry (Appendix). The problem lies in obtaining accurate standards for the fluorochromes and accurate values for the concentration of ligand molecules. Note that total ligand concentration (not just conjugate) must be known or error will be introduced by the presence of unlabeled ligand (this may still be a source of error if the unlabeled ligand binds preferably to the receptor). However, denatured ligand that will not bind the receptor and carrier proteins should be excluded. Also, since concentrations must be expressed in molarities, the molecular weight of the ligand (and fluorochrome, if significant) must be known. The result will be an average MESF per ligand molecule,

which will take into account any unconjugated ligand.

A second, complementary method of determining the effective F/P ratio is to analyze conjugate bound to particles (cells or microbeads) on a cytometer calibrated with MESF microbead standards (see below). If the average mass of conjugate bound per particle is known, the effective F/P can be determined from the interpolated MESF value. The greatest difficulty with this method is the accurate measurement of bound conjugate by some independent method. Microbead standards with expected antibody-binding capacity (ABC) values are useful (11, 12), but the ABC values must be confirmed for each conjugate because different combinations of monoclonal antibodies and fluorochromes present unique epitopes. Also, since these microbeads use anti-immunoglobulin to bind the monoclonal antibody, the binding reactions do not reflect the antigen binding properties of the conjugate.

While the effective F/P ratio varies from conjugate to conjugate, it is possible to estimate a general value for most of the conjugates used in flow cytometry. Fluorescein conjugates have a rather narrow range of molar conjugation (2–4) and a predictable quenching factor (60–70%) that results in an effective F/P ratio close to 1. For PE conjugates, the effective F/P will generally be 1 or less. Since PE is twice as large as most antibody molecules, multiple substitutions are unlikely (they could also cause steric hindrance and interfere with binding). Fluorescence from the conjugated PE molecule is apparently not appreciably quenched compared to the free PE molecule in solution (13), so the effective F/P will be about the same as the molar F/P for PE conjugates. However, unconjugated antibody may comprise a significant proportion of PE conjugate reagents, lowering the average molar (and effective) F/P ratio. Special PE conjugates with molar F/P ratios of 1.0 have recently become available (10). With considerable oversimplification, the effective F/P ratio for most fluorescein and PE conjugates can be estimated as unity, making the MESF value approximately the same as the number of bound conjugate molecules.

We should mention that there is another approach to FI calibration on cytometers for the purpose of measuring binding capacities which does not require MESF or effective F/P values. In these systems, standards with presumed ABC values are used to construct a calibration curve directly relating the ABC value to the FI reading in arbitrary instrument

units. For direct staining (i.e., staining with labeled conjugates), the same conjugate-binding microbead standards used to determine the effective F/P ratio can be used to construct an ABC calibration curve (7). Again, the assigned ABC values of the microbeads must be confirmed for each particular conjugate. Other investigators have used CD4 expression on T-cells as a biological standard for measuring ABC of PE conjugates. By assuming a nominal value of 50,000 ABC for CD4 T-cells, a constant relating channel number to ABC is derived and applied to other PE conjugates with the same 1:1 molar F/P (14; see also the Web site <http://cyto.mednet.ucla.edu>, select "Protocols" then "CD38 Fluorescence Intensity Quantitation"). For measuring ABC by indirect staining (i.e., binding an unlabeled antibody to cell receptors then staining with a second anti-antibody conjugate), another system of calibration has been developed and applied extensively to hematopoietic cells (15; see also the Web site at www.biocytex.com, select "Product List" then "CellQuant Kits").

The independent determination of ABC values on cells or microbeads is simple in principle but often complicated in practice. Cuvette measurements alone can be used to determine the average ABC of a suspension of particles by measuring the depletion in FI of a conjugate solution after incubation and removal of the particles. Particle measurements alone can also be used in a two-dimensional "checkerboard" dilution technique called isoparametric titration (16). These methods generally work well with ABC values in the 100,000 range and above, but they are not sensitive enough for good measurements below that level. If credible ABC results can be documented for particles with moderate to high binding, the best approach is probably to use them as standards on a well-calibrated flow cytometer and read the lower ABC values from the MESF curve.

CALIBRATING FLOW CYTOMETER IN MESF UNITS

The steps in calibrating a flow cytometer for MESF units have been described in detail (17), and software is available that automates most of the process (for example, the QuickCal Analysis Software described at www.fcstd.com; select "Products" from the home page). The cytometer must first be properly aligned, for which a different type of mi-

crobead standard is preferred (7). After alignment, the use of proper MESF standards is essential to FI calibration, as the standards must be spectrally matched to the fluorochrome and responsive to environmental changes. The photomultiplier tube (PMT) for the fluorescence channel to be calibrated should be adjusted to achieve a typical "window of analysis" in which the population of blank microbeads is just resolved from the lowest channel of the fluorescence histogram. Target calibrators for setting a typical window of analysis are available (see www.fcstd.com; select "Products" then "QC Windows"). The automated software and calibrators provided for immunophenotyping by cytometer manufacturers yield approximately the same window if the cytometer is functioning properly. Higher or lower PMT settings may be appropriate for analyzing especially dim or bright particles (see the instructional program at www.fcstd.com; select "animations" on the left side of the home page and then "PMT" to see the window of analysis move as a function of PMT voltage). In addition to the PMT voltage, electronic spectral compensation may be adjusted to cancel overlap between the emission spectra of different fluorochromes.

Once the cytometer settings are in place, analysis of MESF microbead standards allows construction of a calibration curve that relates histogram channel number to MESF value (Fig. 1). This curve may be used to read MESF values from the fluorescence histograms of unknowns on a channel-by-channel basis or to characterize the overall FI of a population using the mean or median channel of the distribution (18). Of course, the same cytometer settings must be used in analyzing the MESF standards and the unknowns. Any change in fluorescence amplifier gain, PMT voltage, or compensation settings requires a new calibration curve for MESF values.

The MESF calibration curve is the basis of FI calibration in cytometry. Not only does it preserve stoichiometry for receptor-ligand binding calculations, it also provides information about the dynamic range, linearity, sensitivity, and the theoretical upper and lower boundaries of the fluorescence measurement. Nominal ranges for these parameters are fairly consistent across different flow cytometers unless they are malfunctioning (17). Levy-Jennings plots (a general format which shows the variation over time in parameters or measurements) provide complete quality assessment for the instrument component of the fluorescence measurement. A consistent window of analysis should show only small,

random variances in the parameters of the calibration curve.

CHARACTERIZING ABC AND RECEPTOR EXPRESSION

The term antibody-binding capacity is often used to denote the number of monoclonal antibody conjugate molecules bound to a stained cell when virtually all receptors are occupied (6). ABC is thus an indirect indicator of receptor expression. If the effective F/P ratio of the conjugate is known, the ABC value of a particle stained to saturation can be determined directly from its MESF reading:

$$(ABC) = (MESF) \times (\text{Effective F/P Ratio}).$$

However, several factors must be evaluated before a molar relationship between the ABC and number of receptors can be established.

Saturation should be demonstrated by titration of

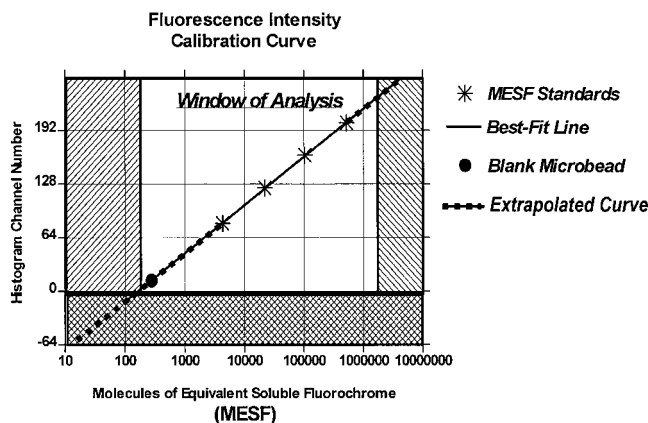


FIG. 1. A typical calibration curve from a flow cytometer relating the fluorescence intensity in MESF units (X axis) to the fluorescence histogram channel number (Y axis). The curve is constructed by simple linear regression in log space of the four data points from the MESF standards to obtain the best-fit line. The calibration curve is extrapolated below and above the highest standards to obtain hypothetical MESF values corresponding to the zero channel and to the maximum channel respectively. In this example, the zero channel value is about 200 MESF and the maximum channel value is about two million MESF. The window of analysis is the region between the zero and maximum channels. The MESF value of the blank microbead, called the "fluorescence threshold," is an indicator of sensitivity and should be below 1000 MESF in properly functioning cytometers.

the conjugate used for staining the cell or microbead. Twofold serial dilutions across a sufficient range to obtain the typical sigmoid curve (Fig. 2) are used to stain the cells or microbeads of interest at pertinent concentrations (generally 100,000 to 10,000,000 particles per milliliter). The highest concentration of conjugate should show an upper plateau and the lowest concentration should approximate unstained cells. The working dilution used to stain cells or microbeads should represent one or two serial dilutions above the inflection point of the plateau. In evaluating prepackaged conjugates with recommended concentrations for staining, we begin the titration at twice the recommended concentration and continue the dilution until a clear decrease is seen (Fig. 3). Some second-order increase in FI may continue on the plateau, but the inflection point should be clear, and the working dilution should be at least a twofold increase beyond it. Failure to observe a plateau on titration is a good indicator that quantitative ABC values using that particular conjugate will be fallacious.

The particular staining method may have a profound artifactual influence on FI, especially when red cell lysis is part of the preparation (19). For whole blood staining, we have found consistent results for FI measurements using a saponin-based stain-and-lyse procedure (Immuno-Lyse, Part 6602764, Beckman-Coulter, Hialeah, FL; see <http://coulter.com>; select "Products," then "flow cytometry," under "Cellular Analysis," then "Reagents,"

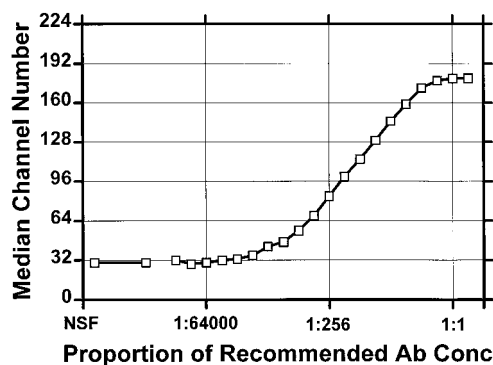


FIG. 2. A titration curve of CD45 FITC on human peripheral blood lymphocytes showing the FI (expressed as the channel number from the log-fluorescence histogram) as a function of conjugate dilution (expressed as the dilution relative to the recommended amount). The upper plateau comprises the twice-recommended, recommended, and half-recommended concentrations. The curve declines linearly until nonspecific fluorescence (NSF, mostly due to autofluorescence) causes the lower plateau.

then “QC and Support Reagents,” then “Lysing Reagents” and look for “Lysing Kit”) (20). Some preparative systems may give acceptable results for phenotype percentages but are still not suitable for ABC measurements (21). Therefore, the conjugate titration should always be performed using the same preparative method as the analytes of interest will undergo.

Nonspecific binding (NSB) due to low-affinity high-capacity interactions or to spurious cross-reactivities may give the mistaken impression of receptor expression. Isotype or subclass controls (conjugates of the same fluorochrome and antibody isotype but having no specificity for cell receptors) are often used to detect or rule out NSB. These controls are useful but not ideal, since, even with the same isotype, different monoclonal antibody conjugates may differ in their extent of NSB to cells, depending on both the antibody and the fluorochrome. The best control for NSB is a duplicate reaction with large excess of soluble receptor. Unlabeled soluble antibody, which is more readily available than soluble receptor, also provides antigen-specific competition (Fig. 4). However, cold antibody cannot rule out competition for binding by other cell receptors (such as Fc receptors) to alternate sites on the antibody conjugate molecule.

Valency is a more complex determination. In one system evaluating a CD4–PE conjugate, bivalent antibody binding was nicely demonstrated by comparing the FI of cells stained with intact monoclonal antibody to cells stained with Fab fragments con-

taining only one antigen-binding site (13). Cells stained with the intact antibody conjugate showed average values around 45,000 ABC, while cells stained with the Fab fragment showed values around 90,000 ABC. Evidently the equilibrium constant is high enough and free diffusion of CD4 molecules on the cell surface rapid enough for most conjugate molecules to bind two CD4 molecules simultaneously. On microbeads, where receptor diffusion cannot occur, binding appears to be an unpredictable mix of univalent and multivalent interactions. The same may be true for certain cell receptors that have limited mobility or that present steric hindrance to the unbound site on the antibody, and these properties may vary depending on the metabolic state of the cell. Valency will likely remain a tangled issue, and the best approach at this time may be to use well-characterized reagents and seek consistent ABC values on cells and microbeads between lots. In general, the term ABC should not be taken to imply a given valency (and therefore an exact receptor quantification).

Steric hindrance and epitope masking both lead to underestimates of receptor expression, and very few systems have been carefully evaluated for these effects. In one example, the failure of one particular CD4 monoclonal antibody conjugate to stain CD4 lymphocytes in a small proportion of persons was traced to a masked epitope in the region where the receptor penetrated the cell membrane. The mask-

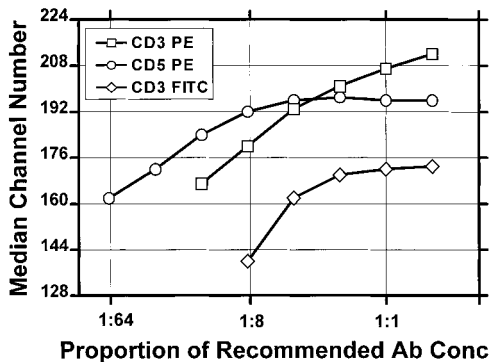


FIG. 3. The upper portion of titration curves from three different conjugates. The CD5–PE curve shows a long plateau from twice to one-fourth the recommended concentration. The CD3–FITC curve shows a plateau that extends just to the half-recommended concentration, but the CD3–PE conjugate (from the same clone) shows no clear plateau and would be unsuitable for ABC measurements at any of the concentrations tested.

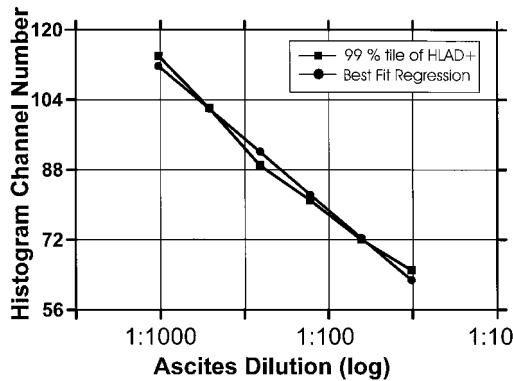


FIG. 4. A competition binding curve of cells stained with a FITC conjugate staining HLA-DR receptors on peripheral blood lymphocytes. The curve shows that the decreasing FI as the concentration of ascites increases is nearly linear over the range tested. At higher ascites concentrations, staining was nearly completely abrogated so that the FI was indistinguishable from that of unstained cells. The ascites was used for competition without further purification as a measure of nonspecific binding.

ing was due to an autosomal recessive trait unrelated to the primary structure of the CD4 protein (22). Less obvious effects may be hard to ascertain. One clue may be a failure to achieve saturation in the titration curve (Fig. 3). Some larger fluorochromes such as phycoerythrin may be more prone to cause steric hindrance, perhaps causing different ABC values from the same monoclonal antibody conjugated to different fluorochromes. Again, the term ABC should not be taken to imply known saturation of all receptor molecules, but rather saturation of available sites for that particular conjugate–receptor interaction (in that sense, antibody-binding capacity is a misnomer, since the actual measurement is conjugate-binding capacity).

The several factors that complicate the relationship between ABC and receptor expression are generally small by comparison with total binding, and the ABC value may be taken as a reasonable estimate of receptor expression for most staining reactions. For example, differences in valency would produce at most a 2-fold error, a relatively small difference when considering the 1000-fold range of common receptor expression. Although variations may be expected between different reagents, any given system of reagents using the same conjugates, particles, and staining methods should show consistent ABC values. In particular, only small variances should be seen between lots of the same conjugate. However, for exact molar relationships between labeled ligand and receptor, each system must be evaluated independently.

INTEGRATED FI CALIBRATION, CYTOMETER OPERATION, AND REAGENT EVALUATION

Purvis and Stelzer have described a comprehensive system based on FI calibration that includes cytometer operation and reagent evaluation (23). This system, used to qualify instruments and reagents for multisite drug trials and other studies, ensures consistent results across different laboratories and provides clear documentation of good laboratory practice. The system integrates all the components described above: target calibrators for a consistent window of analysis, MESF microbead standards for quantitative fluorescence calibration, and titration with measurement of effective F/P ratios for the conjugate characterization. Given the vagaries of both fluorescence measurements and

receptor–ligand assays, this type of comprehensive approach is recommended for any laboratory that uses flow cytometry to measure receptor expression by immunophenotyping.

SUMMARY AND CONCLUSIONS

Standardized reagents and techniques for making quantitative FI measurements on cytometers are just now emerging into practical use. FI calibration establishes the true values for the critical parameters of the fluorescence measurement, thus providing an essential element of quality control. It further allows the establishment of comparable windows of analysis across different times and laboratories, promoting pattern recognition for atypical phenotypes associated with immune suppression, activation, infection, or malignancy. Finally, it permits numeric assessment of ABC values in populations and subpopulations defined by appropriate gating strategies. This latter feature should see expanding application in both basic science and medical applications as the development of therapeutics increasingly targets specific cell receptors.

APPENDIX

Determining Effective F/P Ratios

1. The exact molarity of the ligand protein must be known. For typical fluorescein-labeled monoclonal antibodies used in flow cytometry, working concentrations of antibody are around 10 $\mu\text{g/ml}$. For fluorescein conjugates with molecular weights of 150,000 Da, this is equivalent to 67 nM. (The low mass of fluorescein (280 Da) allows us to ignore the difference in molecular weight between free antibody and conjugate.)

2. For fluorometers with monochromators, set the excitation and emission wavelengths to the maximum values for the fluorochrome. For fluorometers using optical filters, choose a bandpass filter that is comparable to the filter on the cytometer used to analyze stained cells (typical for fluorescein would be a 525 nm bandpass with a bandwidth of 10 nm). Adjust all other settings (slitwidths, PMT voltages, amplifier gains) to nominal values. Note any adjustments that are required for future setup.

3. Measure the relative fluorescence intensity (RFI) in a series of known dilutions of the fluorochrome standard. A typical range would be from 1 μM down to 1 nM in 10 twofold decrements. For fluorometers with monochromators, scan the whole range of emission wavelengths and use the integral (area) of the emission spectra from about 10 nm below the peak to 10 nm above it as the RFI reading. For nonscanning fluorometers, a single reading with the proper optical filters in place should yield the same results.

4. Adjust RFI measurements for any factors (e.g., amplifier gain) required for common scaling. If RFI values are reported on a logarithmic scale, use the values as reported. If RFI values are reported on a linear scale, they should be converted to logarithmic. Construct a calibration curve with log of the fluorochrome concentration on the X axis and log RFI on the Y axis. This should be a simple straight line with the regression equation

$$\log \text{RFI} = \{\text{Slope}\} * \log[\text{fluorochrome}] + \{\text{Y intercept}\}.$$

5. Measure the RFI value of the conjugate dilution. Determine its MESF concentration from the RFI reading:

$$[\text{MESF}] = 10^{((\log \text{RFI} - \{\text{Y intercept}\})/\{\text{slope}\})}.$$

6. The effective F/P ratio is given by:

$$\text{Eff F/P} = [\text{MESF}]/[\text{Ligand}].$$

If concentrations are in the same units, the ratio is reduced to molecules of equivalent soluble fluorochrome per molecule of ligand.

7. Once the approximate range of relevant concentrations is known, a more precise determination can be done in linear space with a smaller range of fluorochrome dilutions.

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