So much for the good news. PerCP-labeled antibodies, available only from B-D, are, or at least were, on the pricey side. If you happen to be using a B-D FACScan or FACSCalibur, that may be the only bad news about PerCP; both Mandy et al[1027] and Nicholson, Jones, and Hubbard[1252] have reported excellent results with this label using that instrument. The other problem with PerCP, however, relates to its intolerance of high illumination power levels. The FACScan and FACSCalibur, which have very efficient light collection optics, use only 10-15 mW laser power for illumination, and the illuminating beam is on the order of 100 μm wide. If more power, or the same power in a narrower beam, is used for illumination, fluorescence signals diminish in intensity, instead of increasing in intensity. Many people found that their cytometers and PerCP didn't go well together, and favored PE-Cy5 as a third label.

Meanwhile, photon saturation and bleaching (pp. 115-8) had been rounded up as prime suspects in PerCP's "Case of the Disappearing Photons," but, as it turned out, that wasn't quite what was going on. A clue as to what might be happening came when Davis and Houck[2631], at B-D, described a **PerCP-Cy5.5** tandem conjugate, with maximum emission near 700 nm. The tandem exhibits even less fluorescence emission overlap with PE than does PerCP itself; PerCP-Cy5.5-labeled antibodies are now available from BD Biosciences. But what broke the case is that PerCP-Cy5.5 does not suffer from the same "saturation" problem as PerCP, even when laser powers as high as 200 mW are used.

Phycobiliproteins and Tandems: Dirty Little Secrets

PerCP is not completely unique in its response to high illumination levels; phycoerythrins and allophycocyanin exhibit similar behavior, to different extents. Two ISAC poster presentations from the Herzenberg lab[2632-3] described apparent saturation of phycoerythrin, with fluorescence emission intensities reaching a plateau when excitation energy (at 488 nm) was slightly less than 100 mW. An instrument with two 488 nm beams was set up, allowing fluorescence intensity to be measured in a 100 mW beam some 20 μs after a measurement was made in the first beam, which, during a series of runs, was operated at power levels ranging from a few mW to almost 1 W. It was noted that fluorescence intensity from cells bearing PE-labeled antibodies, measured in the second beam, decreased in a nonlinear manner as illumination intensity in the first beam increased. This appeared to indicate that PE had been bleached. Apparent bleaching was much less for PE-Cy5-labeled antibody, and still less for cells bearing fluoresceinated antibody.

However, the critical observation was that, if cells that had been saved after running through the instrument were reanalyzed after a few minutes, almost half of the PE fluorescence was recovered. It now seems clear that the culprit in the transient disappearance of fluorescence from PerCP and PE is accumulation of molecules in relatively long-lived (7 μs in the case of PerCP[2634]) triplet states. These molecules must return from the excited triplet state to the ground state before they can be excited again. Triplets (pp. 113-4) account for phosphorescence, a phenomenon in which emission may occur minutes, rather than nanoseconds, after excitation.

I once asked Alex Glazer whether phycoerythrin could be used to make a dye laser, and he explained that it couldn't, because, while one PE molecule is equipped with 34 chromophores, excitation of more than one of them at a time results in loss of energy by nonradiative mechanisms, rather than in fluorescence. For a dye laser to lase, photon saturation, i.e., the situation in which the number of chromophores in the excited state is equal to the number in the ground state (p. 116), must be achieved; the laser action is due to fluorescence emission from those excited molecules. This can't happen in PE, because multiple chromophore excitation doesn't lead to fluorescence, at least in part due to triplet conversion[2635].

Making a PE tandem creates a pathway whereby energy from one of the phycoerythrin chromophores can be transferred nonradiatively to an acceptor molecule such as Cy5, from which fluorescence emission will occur; this provides an alternative to triplet conversion and other means of depopulating the singlet excited state of the PE chromophore, decreasing the likelihood of triplet conversion. Thus, tandems should, and do, tolerate higher illumination power levels before beginning to manifest the saturation and bleaching syndrome noted in their parent macromolecules. This paradigm holds for PE and its tandems, and for PerCP and PerCP-Cy5.5, and I am sure that, if somebody goes looking, she or he will find that allophycocyanin and its tandems follow the same rules.

I don't mean for what I have just said to imply that phycobiliproteins and PerCP don't bleach; they do, and, if you don't believe me, look (by eye) at a small amount of phycoerythrin antibody solution before and after you leave it in sunlight for a few hours. The point is that triplet conversion, rather than bleaching, accounts for the relatively low illumination power tolerance of these materials.

For dyes for which triplet conversion is not a problem, e.g., DNA fluorochromes, maximum detection sensitivity is typically achieved by increasing illumination power to the point at which dye saturation occurs; in an observation period on the order of 1 μs, each dye molecule will go through one or two hundred excitation-emission cycles before being irreversibly bleached[1130]. However, optimizing detection of phycobiliproteins, which can allow detection at the single molecule level, typically requires much longer observation periods, on the order of a millisecond or more, during which time approximately 100 photons should be emitted by each phycobiliprotein molecule[660,885,2440].

I am indebted to Ken Davis of BD Biosciences and Dave Parks of the Herzenberg lab for providing me with details of the poster presentations for which abstracts appear in references 2631-4. I am assured that the details will be published at some time; for now, the manuscripts appear to be in long-lived triplet states.

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Speaking of tandems following rules, the probability that energy transfer between a donor and an acceptor species will occur varies with the extent to which the donor emission spectrum and the acceptor excitation spectrum overlap. Look back at Figure 7-9 (p. 296), and you will note that this overlap diminishes pretty drastically as we move from PE-Texas red to PE-Cy5 to PE-Cy5.5 to PE-Cy7. As a result, energy transfer between donor and acceptor in this series is progressively less efficient, with the result that the longer wavelength emitting tandems also exhibit more and more emission in the spectral range in which PE normally emits. There's just a speck of PE emission in the PE-Cy5 spectrum; there's quite a bit in the PE-Cy5.5 and PE-Cy7 spectra.

If there's a lot of PE emission in the PE-Cy7 spectrum, it means that a fair fraction of the PE chromophores have not donated energy to Cy7, and, therefore, that there isn't as much emission from Cy7 as there would be from Texas red in PE-Texas red or from Cy5 in PE-Cy5. In some instances, the number of photons or photoelectrons your instrument actually collects and generates from PE-Cy7 can be less than 1/100 the number it would get from PE. If you're designing a multicolor immunofluorescence experiment involving a bunch of tandem labels, you'll want to have an idea of the numbers of molecules of various antigens you're likely to find on your cells, so you don't end up using PE-Cy7 or other inefficient labels to attempt to discriminate cells bearing small amounts of surface antigen from unstained cells.

To make matters worse, while we more or less glibly refer to "PE-Cy5," "PE-Cy7," etc., what we are really talking about is a bunch of PE molecules with different numbers of Cy5 or Cy7 molecules covalently attached to them in different places. A monoclonal antibody is molecularly homogenous; a tandem conjugate is not. That means that the degree to which donor emission will bleed through into the tandem's emission spectrum varies from lot to lot of tandem conjugate or tandem conjugate-labeled antibody. If you're setting up fluorescence compensation for an experiment involving tandem conjugates, you need a single-label compensation control for each antibody. That is, if you're using PE-Cy5.5-antiCD4 in one set of tubes, and PE-Cy-5.5-antiCD8 in another, you need to make sure you compensate them differently, using the appropriate control for each.

If you look hard, you'll find that there are some phycobiliprotein labeling products out there other than PE, APC, and the tandems I have already mentioned. In the phycobiliprotein department, **Martek Biosciences** makes available both some offbeat phycobiliproteins derived from cryptomonad algae, and reactive **phycobilisomes**, which can provide at least an order of magnitude more fluorescence signal than individual phycobiliprotein molecules. Telford et al[2636a,b] examined several of the cryptomonad phycobiliproteins; some can be excited by yellow or red light, and others require green (520-550 nm) excitation.

Molecular Probes offers tandem conjugates of PE and APC with its **Alexa dyes**[2348], which will be discussed in a subsequent section. They have also (R. Haugland, personal communication) investigated tandems in which the phycobiliprotein is the acceptor and the low molecular weight compound is the donor, e.g. Alexa-488-APC. Alexa 488 has excitation and emission characteristics similar to fluorescein; the tandem conjugate excites at 488 nm and emits at the 660 nm emission wavelength of APC.

And Alex Glazer and his colleagues at Berkeley have managed to get *E. coli* to produce recombinant phycobiliproteins[2637-9], complete with biotin for attaching to streptavidin, antibodies, etc. and an affinity tag to facilitate purification. If we carried that over to our species, we could just program our lymphocyte subsets to fluoresce in different colors, and phenotype without benefit of antibodies. The antibody and phycobiliprotein vendors are probably not too worried just yet.

Future Tandems: Heterocycles Built for Two?

There should be some advantages to tandem labels that do not include a phycobiliprotein molecule, but which couple two lower molecular weight chromophores in a way that facilitates energy transfer between them. Dimeric nucleic acid stains designed in this way[1528-9], also from Alex Glazer et al, have been mentioned on p. 314. One such dye, TOTAB, incorporates a thiazole orange and a thiazole blue chromophore; when bound to nucleic acid, the thiazole orange donor dye absorbs maximally around 500 nm and transfers most of its energy to the thiazole blue acceptor, which emits at about 660 nm. It should be possible to synthesize similar molecules incorporating two or more low molecular weight chromophores and the reactive groups necessary to conjugate them to proteins. An oxacarbocyanine and an indocarbocyanine dye, for example, in such a tandem compound would yield good absorption at 488 nm and emission around 575 nm, providing the desirable spectral characteristics of phycoerythrin in a lower molecular weight label.

The lower molecular weight of a completely synthetic tandem label should make it easier to preserve activity in smaller ligands, such as hormones and growth factors, after labeling; the other principal advantage of synthetic ligands is the relative ease with which absorption and emission spectral characteristics can be tailored to enable several different labels to be used with a single source. This prospect is particularly appealing because it could allow labels to be designed to fit inexpensive sources such as red diode lasers.

Glazer et al have, in fact, continued work on other relatively low molecular weight materials in which energy transfer is exploited; they are not antibody labels, but a new generation of labels and primers for DNA sequencing and sizing[2640].

There's a nice kind of symmetry to the phycobiliprotein/tandem story. Phycobiliproteins first came into use as labels because nature had done an excellent job of designing molecules to transfer fluorescence excitation energy. The PE-APC tandem, however, which combines two natural products, isn't as good for some human purposes as some synthetic and semisynthetic molecules have turned and may

turn out to be. I expect that synthetic tandem labels will eventually come into wide use. I should also point out that nature is way ahead of us on the phycobilisomes in the photosynthetic apparatus, and that, if the dye designers turn their attention to an artificial photosynthetic molecule, they might accumulate considerably more wealth than can be realized by making slight improvements in cytometry.

Cyanine Dye Labels: From Cy-Fi to Hi5 for Cy5

During most of the 1980's, the phycobiliproteins were very much in the limelight, which was appropriate not only because lime is about the right excitation wavelength, but because they provided a means of attaching several efficient fluorescent labels, with spectra in regions in which cellular autofluorescence is minimal, to antibodies and other ligands. The fuss about the phycobiliproteins diverted many people's attention from the search for other, low molecular weight fluorescent labels with large Stokes' shifts and/or with excitation maxima in the green, red, and infrared spectral regions. In terms of the design of lower molecular weight labels, one of more successful efforts has been the development of **reactive cyanine dye labels**, largely due to the persistence of Alan Waggoner and his colleagues[1361-4].

Cyanines, like many of the compounds now in use as laser dyes, are not soluble to any appreciable extent in water, but are soluble in nonpolar solvents. This characteristic is essential to the use of cyanine dyes as probes of membrane potential, as it allows the dyes to pass freely through the lipid bilayer portion of cytoplasmic and organellar membranes, but is undesirable in labels because it increases the tendency of unconjugated label to stick to protein and the tendency of conjugates to bind nonspecifically by this mechanism. There is, in fact, considerable interest in the preparation of water-soluble analogues of existing laser dyes for use as laser dyes, since the substitution of aqueous dye solutions for the solutions in organic solvents now employed would make it easier to keep the dye medium cool.

The spectral characteristics of most fluorescent organic dyes are determined by the structure of the heterocyclic ring(s), and the conjugated double bonds connecting rings, if such connections are present (they are in cyanines), which form the backbone of the dye. Water solubility and reactivity can be modified by the addition of functional groups on the ring; many of the functional groups that can be added to increase water solubility may also be capable of being derivatized to produce dye molecules that can be covalently bound to proteins, etc. The art lies in adding the functional groups in ways that do not substantially alter spectra.

The first attempts of which I am aware to produce reactive derivatives of cyanines (A. Waggoner & H. Shapiro, unpublished), around 1981, failed to yield suitable antibody labels; the compounds synthesized shifted their absorption maxima and decreased their quantum efficiency on conjugation. Waggoner's group subsequently produced cyanines that could bind covalently to protein sulfhydryl groups[1361], and then isothiocyanate derivatives[1362]. The two newest series of labels[1363-4] use a succinimidyl ester group to link to proteins; the first of these[1363] employed carboxymethyl groups to increase solubility, while the second[1364] uses sulfonate substituents for the purpose.

The reactive **oxacarbocyanine** dye **Cy2** (see Figure 7-9) shares the spectrum of the $DiOC_N(3)$ series of dyes, which are well known as membrane potential probes. The absorption and emission spectral characteristics of these dyes are similar to those of fluorescein.

Cy3 and **Cy5** are, respectively, derived from **indocarbocyanine** and **indodicarbocyanine**; their spectral characteristics are shown in Figure 7-9. **Cy3** absorbs maximally at about 545 nm, and can be excited most effectively with a green He-Ne laser source at 543 nm or the mercury arc lamp line at 546 nm; however, the absorption of the material is high enough that it excites adequately at the 488 nm argon laser wavelength available in most cytometers. The 515 nm argon laser line is better, as are krypton lines at 520 and 530 nm and the emission from a frequency-doubled YAG laser at 532 nm. The emission peak of Cy3 is at about 565 nm; however, a substantial fraction of Cy3 emission is transmitted by the bandpass filters typically used for phycoerythrin detection. **Cy5** absorbs maximally near 640 nm; used by itself, it is very effectively excited by 633 nm He-Ne lasers, 635-650 nm diode lasers, or the 647 nm krypton laser line. For immunofluorescence work, Cy5 is now probably more widely used in the form of **PE-Cy5 tandem conjugates**[359], which can be excited at 488 nm, than as a primary label.

Cy5.5 is a reactive derivative of **dibenzoindodicarbocyanine**, with maximal absorption near 675 nm and maximal emission at 695-700 nm. This material is optimally excited by 670 nm diode lasers, which are dirt cheap (think red laser pointers); its absorption at 633 nm is sufficient to make it possible to use Cy5- and Cy5.5-labeled antibodies for two-color immunofluorescence analyses in an instrument with a 633 nm He-Ne or 635- 650 nm diode laser source. The **IMAGN 2000**, a clinical instrument which did lymphocyte subset analysis by a low-resolution scanning technique dubbed **volumetric capillary cytometry**[365,2484,2641], rather than flow cytometry, used Cy5 and Cy5.5 as antibody labels. **Biometric Imaging**, which produced the instrument, was acquired by Becton-Dickinson. **Cy3.5** , with maximum absorption near 580 nm and maximum emission near 600 nm, is to Cy3 as Cy5.5 is to Cy5.

Wessendorf and Brelje[1366], using microscopy and densitometry rather than flow cytometry, compared the brightness of immunofluorescent staining with the same antibody tagged with fluorescein, tetramethylrhodamine, Lissamine rhodamine, Texas Red, and Cy3.18, an early cyanine label; they concluded that the cyanine dye was brightest. This is not too surprising; cyanine dyes have higher extinction coefficients than most other dyes, and quantum efficiencies at least as high, and these authors used excitation wavelengths optimal for the individual dyes.

Cy7 is a reactive indotricarbocyanine dye; it absorbs in the near infrared (about 750 nm) and emits around 770 nm.

The indotricarbocyanine structure itself is not as stable chemically as are the indodicarbocyanine and -carbocyanine dyes fluorophores of Cy5 and Cy3; this makes it harder to prepare Cy7 labels and also results in a relatively short shelf life. Like Cy5, Cy7 is more widely used as an acceptor in PE- and APC-based tandems than as a primary immunofluorescent label; while Cy2, Cy3, and Cy5 are fairly popular as labels for nucleic acid probes used in **fluorescence in situ hybridization (FISH)**, Cy7 seems not to be.

Cy3, Cy5, and related dyes are available from **Amersham Biosciences** in kits that contain just about everything needed for antibody conjugation except the antibody.

There is a "sticking point" to cyanine dye labels; antibodies labeled with Cy3, Cy5, and/or PE-Cy5 and, possibly, other cyanines, seem to adhere to monocytes and, to a lesser extent, to granulocytes, resulting in low levels of nonspecific staining. I first heard about this from Carl Stewart and from Alan Waggoner, and the three of us (unpublished) demonstrated the effect using Cy3 and Cy5 antibodies in a Cytomutt with 543 and 633 nm He-Ne laser excitation. The effect came to general attention when PE-Cy5 tandem conjugate-labeled antibodies came into wider use and were found to bind to monocytes even when the antibody was not reactive with monocyte surface antigens. According to van Vugt et al[2642], this interaction involves the high-affinity IgG receptor CD64 present on the surface of monocytes. However, Stewart and Stewart[2643] report that some CD64-negative monocytic and myeloid leukemia cells also bind PE-Cy5 antibodies not reactive with their cell surface antigens. Tjioe et al[2628] noted that antibodies labeled with a PE-APC tandem conjugate bind to monocytes and granulocytes, although not to the same extent as do PE-Cy5-labeled antibodies. Antibody manufacturers have come up with various proprietary ways of minimizing such irrelevant binding.

Blue Notes: AMCA and Cascade Blue

A number of **UV-excited, blue fluorescent labels** have come into use for antibody labeling. After having been told at least twice that fluorescein was adopted as a label because the UV-excited blue autofluorescence present in most cells and tissues interfered with the UV-excited blue fluorescence of anthracene, which was the first label, you might well ask why. The simple answer is that people who had "maxed out" their instruments in terms of the number of detectors they could have looking at the 488 nm beam and the krypton, dye, or He-Ne laser beam wanted to measure still more antigens. If you had a UV laser available, you could use a UV-excited, blue fluorescent label; if you restricted its use to antibodies for which the cells of interest have relatively large numbers of binding sites, you wouldn't have a big problem with autofluorescence.

The **coumarins** are a family of compounds that exhibit UV-excited blue fluorescence and that, incidentally, were originally used therapeutically as anticoagulants and, in proportionally larger doses, as rodent poisons. Coumarin derivatives have been exploited as laser dyes, and may also act as pH indicators; the latter characteristic is usually an undesirable one for a label. The first popular coumarin label was **7-amino-4-methylcoumarin-3-acetic acid**, or **AMCA**. As shown in Figure 7-9, AMCA absorbs maximally at about 350 nm, and has an emission maximum near 455 nm; it can be excited optimally by argon or krypton UV lasers and by the mercury arc lamp line at 366 nm, and adequately by the 325 nm He-Cd laser line.

Aubry et al[1367] stated that, in addition to being usable for multicolor immunofluorescence analysis, AMCA was well suited for use in combination with acridine orange for simultaneous measurement of DNA and RNA content and cellular antigens, using UV and 488 nm excitation beams. My personal preference would be to use Hoechst 33342, pyronin Y, and fluorescein-antibodies[113] for the same purpose, in most cases; however, there are situations in which acridine orange may be more appropriate than pyronin for RNA content measurement, and also situations in which one would want to use acridine orange to measure something other than RNA content, e.g., DNA sensitivity to denaturation. In either of these cases, AMCA-labeled antibodies might be useful, provided the antigenic determinant of interest was preserved during the acridine orange staining procedure. Delia et al[1368] used AMCA as a third label in a flow cytometer with collinear UV and 488 nm illuminating beams. Molecular Probes' **Alexa 350**, a sulfonated aminocoumarin, has similar spectral characteristics, but a quantum yield almost twice as high as that of AMCA[2348].

Cascade Blue[1075,2332,2644], a reactive derivative of **pyrene**, was introduced by Molecular Probes; its spectrum is shown in Figure 7-9. The absorption maximum is near 390 nm. While absorption at 325 nm is probably too low to rely on a UV He-Cd laser for excitation, adequate excitation should be available from the 366 nm arc lamp line or the UV lines from argon or krypton or mode-locked tripled YAG lasers. Anderson et al[2644] excited both Cascade Blue and Molecular Probes' **Cascade Yellow** (emission maximum near 550 nm) using the violet krypton lines at 407 and 413 nm, and a violet diode laser should be usable as well. The emission maximum of Cascade Blue is at about 415 nm; emission above 500 nm is considerably less than is the case with AMCA and Alexa 350, facilitating compensation in multicolor immunofluorescence experiments.

Hey, BODIPY!

Molecular Probes also offers the **BODIPY** dyes[1075,2332]; which are **boron dipyrromethane** derivatives with long enough formula names to justify one of the manufacturer's many catchy abbreviations. The original member of the series, **BODIPY FL**, has spectral properties similar to those of fluorescein, with a somewhat narrower emission peak, less sensitivity to pH changes, and greater resistance to bleaching, all desirable characteristics. The excitation maxima of the various BODIPY dyes cover the range from 500 to 646 nm; emission maxima range from 506 to 660 nm. Five of the most popular BODIPY dyes are available with water-

solubilizing spacers and amine-reactive ester groups attached to facilitate conjugation to antibodies or other proteins.

A variety of peptides, proteins, polysaccharides and other molecules with BODIPY dyes attached are also produced. **Bis-BODIPY-phosphatidylcholine**, localized in the inner leaflet of the plasma membrane, was used by Meshulam et al[1367] to measure phospholipase A activation in neutrophils by flow cytometry. Knaus et al[1370-1] and Martin et al[1372] have used BODIPY labeling with other small ligands; most recently, Haugland et al[1373] used phalloidin labeled with the new long-wavelength fluorophore **BODIPY 581/591** (581 nm excitation, 591 nm emission), which is spectrally similar to Texas Red, for fluorescence microscopic visualization of the intracellular distribution of F-actin. However, BODIPY dyes have still not moved as far up in the charts as might have been expected.

Alexa Dyes: Some Thoughts on Dyemographics

I write a new edition of *Practical Flow Cytometry* every few years and, if I'm lucky, sell a few thousand copies. Dick Haugland, who founded Molecular Probes, puts together a new edition of his *Handbook*[2332] every few years, with updates at intervals on CDs and or his web site, and gives away tens of thousands of copies. The rest of the time, he presides over a large and talented group of people who make dyes. They might be considered dye hackers, in the old, honorable sense of the word; they know the structures and the spectra, and how to tweak the structures to optimize the performance of the dyes. And the company isn't losing money.

The **Alexa dyes**[2332,2548] are a series of sulfonated coumarin- and rhodamine-based labels, with spectral characteristics similar to those of some of the more popular labels previously mentioned in this section: e.g., AMCA, fluorescein, Texas red, Cy3, Cy5, Cy5.5, and Cy7. However, the Alexa dyes have higher quantum yields, better photostability, and better charge characteristics (allowing more dye molecules to be put on a protein before the law of diminishing returns cuts in on fluorescence). So what happens? People who do their own dye conjugations have written testimonials for the Alexa dyes in the latest edition of the *Handbook*[2332], but the companies that make and sell antibodies in volume kept making conjugates with fluorescein, and tandems with Cy5, Cy5.5, and Cy7. The word on the street was that the antibody companies didn't want to pay the premium royalties Molecular Probes wanted to get paid for its premium dyes.

Just look at fluorescein, which is the Microsoft software equivalent in fluorescent labels. It has a lot of problems, e.g., its fluorescence yield varies markedly over the near-physiologic pH range, but, while everybody knows about, and many people complain about, the problems, the antibody manufacturers load fluorescein onto their antibodies, and almost everybody buys them and uses them. Molecular Probes has produced at least three green dyes – BODIPY FL, Oregon Green, and Alexa 488 – that are better labels than fluorescein, in most respects, including photostability, which keeps the dyes green longer – with fluorescein envy. The race is not always to the swift.

Luckily, Molecular Probes does well enough to keep the Alexa dyes and a few thousand other gourmet chemicals in the catalogue for its discerning customers. And, as of early 2003, at least a few antibody producers (BD/Pharmingen and Caltag, for sure) had started to offer Alexa dye labels.

Other Organic Fluorescent Labels: A Dye Named JOE, etc.

The DNA sequencing crowd uses fluorescent dyes, many of which are similar or identical to dyes used in cytometry, but is likely to know them by different names or nicknames. In the interest of nickname translation, I will mention that 6-JOE is 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein, 5-FAM is 5-carboxyfluorescein, TAMRA is carboxytetramethylrhodamine, ROX is carboxy-X-rhodamine, and TET is c-carboxy-2′,4,7,7′tetrachlorofluorescein. The halogenated fluoresceins have excitation and emission maxima at somewhat longer wavelengths than those of fluorescein itself. Molecular Probes sells them in reactive form as succinimidyl esters for sequencing and probe labeling; there doesn't seem to be any compelling reason to use them in cytometry.

In the golden oldies department, I should mention **nitrobenzoxadiazole (NBD)** as a label; this material is usually applied to proteins in the form of its reactive chloride derivative, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride), which reacts with amino and thiol groups. The adduct with amino groups has spectral properties similar to fluorescein; Wallace et al[622] used **NBD-phallacidin** for flow cytometric studies of cytoskeletal structure. NBD fluorescence is strongly environmentally dependent; for details, see the Molecular Probes *Handbook*[2332].

Most dyes suitable for use in CW tunable dye lasers emitting in the red and infrared have absorption maxima in the orange and red spectral regions; reactive derivatives of these dyes should, in principle, be suitable labels for use in instruments with He-Ne or diode laser excitation in the red. Joel Wright and I (unpublished) fooled around with a derivative of the oxazine dye **Nile blue** without much luck in the early 1980's; Monsigny et al[1374] have, more recently, had better luck, producing both a **reactive benzoxazinone dye** with red emission that can be excited at 488 nm and another that apparently stains nucleic acids.

Aluminum phthalocyanine dyes have been derivatized for use as labels[1375]; these dyes themselves have been used as sensitizers for photodynamic treatment of cancer, and their uptake has been monitored by flow cytometry[1376-8]. The derivatized versions had strong absorption in the UV and red spectral regions with emission at longer red wavelengths. I was able (D. Schindele and H. Shapiro, unpublished) to resolve populations of beads labeled with two different aluminum phthalocyanine dyes in a cytometer with 670 nm diode laser illumination; I didn't have great luck with cells bearing antibodies labeled with phthalocyanine derivatives,

but further work along this line would seem worthwhile, if a source of the dyes can be found.

Whitaker et al[1379] report that fluorescent **rhodol** derivatives can be made with fluorescence properties similar to fluorescein and rhodamine, but with better photostability and without the pH sensitivity of fluorescein.

In the development of laser dyes, attempts have been made to find and exploit chemical structures with fluorescence spectra showing large Stokes' shifts. Power levels of hundreds of milliwatts or more are required to pump CW dye lasers; at present, most dyes that emit in the red and infrared require a krypton ion laser (647/676 nm) for pumping. Since argon lasers are more tractable than their krypton counterparts, it would be desirable to use argon lasers as dye laser pumps for red- and IR-emitting dyes. Argon lasers, however, emit in the blue and green. Red and IR emission can be obtained with argon laser pumping when dyes with large Stokes' shifts, e.g., pyridyl and styryl dyes[307-9], are used as the lasing media. Derivatives of these dyes might make good labels; the pyridyl and styryl dyes might also be useful in low molecular weight tandem labels.

The IR diode lasers used in CD players emit at around 785 nm, which is above the emission maximum of the longest-wavelength label discussed up to this point (Cy7). Since relatively high power (tens of milliwatts) 785 nm diode lasers cost only a few dollars, the notion of using such a laser to excite at least one additional fluorescent label is attractive. One principal problem with this approach is that dyes with the large conjugated ring structures needed to produce IR absorption tend to be chemically unstable. Linda Lee, who synthesized thiazole orange while at B-D[769], made two relatively stable IR-excited dyes, **BHMP** and **BHDMAP**, during a brief stint at Biometric Imaging[2645]. The dyes, with emission maxima near 805 nm, were used successfully in a prototype volumetric capillary cytometer with dual (633 nm He-Ne and 785 nm diode) lasers. BHMP and BHDMAP are heptamethine cyanine dyes with a dialkylpyridinium ion at the central methine, which contributes to stability and makes the dyes water-soluble.

Quantum Dots

A fair amount of buzz has recently been devoted to the prospect of using **semiconductor nanocrystals**, better known as **quantum dots**, as labels for biological molecules or as tags for beads used in multiplex assays[2646-53].

In semiconductor light-emitting diodes (LEDs) and lasers, absorption of optical or electrical energy creates **electron-hole pairs**; one atom of the material temporarily loses an electron, while another somewhere in the vicinity temporarily gains one. When electron-hole pairs recombine, some of the absorbed energy is lost in the form of a photon. The emission wavelength is dependent on the **Bohr radius**, which is the average distance between electrons and holes in an excited-state pair. The Bohr radius is a function of the composition of the semiconductor material, but it normally on the order of tens of nanometers.

In semiconductor crystals with dimensions smaller than the Bohr radius, additional energy is required to create electron-hole pairs, and absorption and emission are thus shifted to shorter wavelengths. In such **nanocrystals**, typically no more than about 10 nm in diameter, the emission wavelength becomes more dependent on the size of the crystal than on its composition, since the energy required to keep an electron and a hole separated increases dramatically as the distance between them decreases. The emission wavelength of a CdSe crystal with a diameter of 2.1 nm is approximately 510 nm; that of a crystal with a 3.1 nm diameter crystal of the same material is approximately 560 nm.

The fluorescence spectrum of a nanocrystal is considerably different from that of an organic dye. Organic dyes (see Figure 4-7, p. 113, for the fluorescein spectrum) typically have small Stokes' shifts, i.e., their excitation maxima are within 20 nm of their emission maxima. The excitation spectrum and the emission spectrum of an organic dye often resemble mirror images of one another; both are substantially skewed, with a short-wavelength "shoulder" in the excitation spectrum and a long-wavelength "tail" in the emission spectrum.

The emission spectrum of a nanocrystal is typically symmetric, with a full width at half maximum of at most a few tens of nanometers, the emission bandwidth is thus substantially less than for a typical organic dye. This means that, given a pair of nanocrystals and a pair of dyes with the same emission peak wavelengths, a given combination of optical filters and dichroics will do a better job of separating fluorescence signals from the nanocrystals than of separating fluorescence signals from the dyes.

Moreover, the excitation spectra of nanocrystals are relatively independent of emission wavelength; progressively shorter wavelengths are increasingly effective for excitation. Excitation at 400 nm is typically at least twice that at 488 nm. This suggests that violet diode lasers will be economical and useful excitation sources for work with quantum dots, in either scanning or flow cytometers.

Being inorganic, nanocrystals are much less susceptible to photobleaching than are organic dyes; a nanocrystal is likely to be putting out over 75% of its original fluorescence output after an observation time sufficient to photobleach over 95% of the fluorescence emission from a dye. The relative chemical and photostabilities of nanocrystals should be particularly useful for work in the far red and infrared spectral regions (nanocrystals can be made with emission wavelengths above 1000 nm). Nanocrystals also have much higher absorption (and excitation) cross-sections than do dyes, meaning that, although the quantum efficiencies of nanocrystals and dyes are about the same, the fluorescence from a nanocrystal is typically equivalent to the fluorescence from a dozen or two dye molecules.

So why can't you buy 200 different monoclonal antibodies labeled with quantum dots? Well, as usual, there have been some practical problems, some of which appear to have been solved, and some of which remain.

Since both the emission wavelengths and the fluorescence intensities of nanocrystals depend on their size, preparative methods must yield crystals with highly homogenous size distributions in order to keep emission peaks confined to a small spectral range and maintain low emission bandwidths.

Earlier work with nanocrystals produced materials with fluorescence lifetimes in the range of 100 ns, which is definitely on the long side for flow cytometry, although acceptable for static cytometry and imaging. Newer materials have fluorescence lifetimes of approximately 15 ns, still a few times longer than the lifetimes of most commonly used dyes, but probably acceptable for flow cytometry.

Semiconductor materials don't much like hanging out in aqueous solutions. Practical quantum dot labels therefore must consist of a semiconductor core, a shell of another semiconductor material that confines excitation to the core, and an outer layer that allows the particle to remain dispersed in aqueous solution and that provides some means by which it may be attached to a biomolecule. CdSe crystals are typically made with ZnS or CdS shells; the hydrophilic coating may be silica or one of several sulfur-containing acids. A dihydrolipoic acid coating, in particular, readily binds avidin, allowing biotinylated antibodies to be bound to the coated crystals with very high affinity[2654].

However, once you've made a particle the size of a quantum dot and coated it with something hydrophilic that binds proteins, you've increased the likelihood that the particle will be more or less nonspecifically bound to cells, and/or scarfed up by phagocytes. While the effects of phagocytosis on any particular experiment may be difficult to predict, nonspecific binding is almost certain to decrease the ratio of fluorescence intensities you measure from cells that do and do not bear the material you are trying to detect with the aid of a quantum dot label.

Preliminary results on flow and image cytometry of cells labeled with quantum dot-tagged antibodies were presented by Bill Hyun of UCSF and collaborators from **Quantum Dot Corporation** at the 2000 ISAC meeting[2655], but I haven't yet seen a publication on flow cytometry using quantum dots. I guess people are still in the excited state over them, and need to relax and write a paper. You can now buy streptavidin-conjugated quantum dots with a 605 nm emission wavelength from Quantum Dot Corporation.

I put a brief writeup of green fluorescent protein in the last edition, guessing that while nobody had yet done flow cytometry of cells expressing GFP, it might be the next big thing; I have similar vibes about quantum dots. I won't tell you about all the predictions I made in earlier editions that didn't pan out, at least not here.

Getting Labels Onto Molecules of Interest

If there is one basic principle applicable to labeling, whether the label is a large or a small molecule, fluorescent or nonfluorescent, and whether what is being labeled is an antibody, drug, hormone, or other material, it is this: **It is absolutely critical to establish the effects of the fluorescent tagging procedure on the affinity and biological activity of the effector portion of the molecule being tagged.** Failure to do this can produce unpleasant surprises.

The more popular reactive forms of labels are those that bind to amino groups on proteins. Early in the game, fluorescein and tetramethylrhodamine isothiocyanates (FITC and TRITC) were among the most popular labeling reagents. Molecular Probes still sells isothiocyanate reagents, but recommends succinimidyl esters and sulfonyl halides because conjugates prepared using isothiocyanates reportedly deteriorate over time[2332]. **Research Organics** advocates dichlorotriazinylaminofluorescein (DTAF) as a replacement for fluorescein. Maleimide derivatives of labels react reasonably selectively with protein sulfhydryl groups.

The extremely high affinity of **biotin** for **avidin** or **streptavidin**[300] is widely exploited for labeling purposes. Labels may be covalently bound to avidin or streptavidin, and the labeled material used to detect biotinylated antibodies; alternatively, labels, especially larger molecules such as phycobiliproteins, may be biotinylated, and avidin or streptavidin then used to form a complex between the label and a biotinylated antibody. In some cases, at least, antibody-label complexes formed using a biotin-avidin reaction are stable enough to permit several complexes, each bearing a different antibody and a different fluorescent label, to be used simultaneously.

Maintaining activity of a labeled biomolecule may require the interposition of a third chemical structure, or **linker**, between the label and the molecule being labeled, particularly if the molecule itself is relatively small; I won't go into the details.

Having dwelt at length on the topic of labeling *per se*, we will now address the use of fluorescently labeled reagents for measurement of cell surface structures and properties.

7.6 IMPROVING SIGNALS FROM LABELS: AMPLIFICATION AND OTHER TECHNIQUES

In many cases, the cell- (or bead-) associated molecules we are trying to detect and quantify using a labeled reagent are abundant enough so that we can accomplish our task using **direct labeling**, incubating our cells or beads with a reagent bearing one or a few molecules of label, and then measuring the fluorescence intensity of the particles of interest in a cytometer, with or without a wash step to decrease the concentration of unbound reagent in the sample. When we are hunting rarer prey, we need to consider various ways of getting bigger signals from each molecule of interest than direct labeling can provide. In order to choose methodology appropriate to our measurement problems, we must appreciate several factors that potentially limit measurement sensitivity, and determine which of these are most relevant to our particular measurement problem.

The ultimate limit to sensitivity is set by the number of molecules of interest present in or on the particles we propose to analyze. Tomas Hirschfeld pointed out in the 1970's

that, once we are detecting and counting single molecules, or small numbers of molecules, we can expect fluctuations in the number of molecules in the observation volume to become a major source of statistical variance in quantitative measurements[2657]. Some 20 years later, when the sensitivity of their capillary electrophoresis detectors had reached the single-molecule level, Chen and Dovichi[2658] demonstrated the effect experimentally.

In the discussion of Q and B and their effects on measurement sensitivity (pp. 221-3), it was noted that a FACScan or FACSCalibur, which, as flow cytometers go, has very efficient collection optics, will typically generate only a single photoelectron at the PMT photocathode for each four molecules (strictly speaking, for each four MESF) of phycoerythrin passing through the observation point; when fluorescein is detected, an average of 80 MESF must pass through the observation point to generate a single photoelectron[2482]. In these instances, measurements are limited by photoelectron statistics.

In a slow flow system such as the apparatus at Los Alamos used for DNA sizing and for single molecule detection of phycoerythrin, the observation time is much longer than in a conventional flow cytometer, and each MESF of phycoerythrin may generate a burst of 100 photoelectrons during its passage through the measurement system. If there are an average of 100 phycoerythrin molecules in the measurement system at any given time, they will give rise to 10,000 photoelectrons; the expected standard deviation would be 100 photoelectrons, and, in the absence of other sources of variance, one would be able to achieve a measurement CV of 1 percent. However, Poisson statistics would dictate that the standard deviation of the number of molecules in the measurement volume would be 10 molecules; the lowest possible CV is then not 1 percent, but 10 percent. Thus, what Chen and Dovichi[2658] define as "**molecular shot noise**" becomes the limiting factor in precision; in order to achieve 1% precision, one must measure or count at least 10,000 molecules.

These days, it is not uncommon to be able to detect a few hundred molecules of cell- or bead-associated antibodies or other target molecules in a conventional flow cytometer; while there may be fluorescence amplification techniques that let us collect hundreds of photoelectrons from thousands of molecules of a label for each target molecule present, they will not liberate us from molecular shot noise; we will, instead, come to recognize it as a major source of variance. Of particular interest is a recent paper by Elowitz et al[2659], which considers molecular noise in the context of gene expression, noting that low intracellular copy numbers of molecules can limit the precision of gene regulation.

Limits to Sensitivity: Autofluorescence

The spectral characteristics of covalent labels exert a significant effect on the sensitivity of flow cytometric measurements of weak fluorescence signals such as are obtained from cells stained with fluorescently tagged antibodies, hormones, lectins, etc. This is so because it is frequently not instrument performance, but **cellular autofluorescence** that limits the number of labeled molecules detectable on or in cells.

As mentioned on pp. 216-7, the intensity of lymphocyte autofluorescence in the green spectral region used for fluorescein fluorescence measurement is the same as would be obtained from over 600 MESF of fluorescein. In most newer laser source instruments, this level of autofluorescence should be detectable above background.

There is, however, no way to use the green fluorescence signal alone to distinguish the proportions contributed by antibody or ligand fluorescence and autofluorescence. Consider two cells, one bearing no bound antibody and emitting autofluorescence equivalent to the emission from 1,000 fluorescein MESF, and the other bearing 300 MESF of fluorescein on cell-bound antibody or ligand and emitting autofluorescence equivalent to 700 fluorescein MESF. Both cells produce 1,000 fluorescein MESF at the detector. Good discrimination of stained and unstained cells will, therefore, require that several times as many fluorescein molecules as are detectable against zero background be present on the surface of the stained cells.

There is a potential problem with measurements of both sensitivity and autofluorescence, in that both are (or were) generally done using log amps, and both involve extrapolating a reference line calculated from measured values of fluorescence obtained from beads bearing known amounts of label. These known amounts typically range from 5,000 to 500,000 MESF, and PMT and instrument gains are generally adjusted to place peaks from the fluorescence distributions of labeled beads in the upper decades of a log scale. The blank beads used for sensitivity determination and the unstained cells used to determine autofluorescence levels fall at the low end of the scale, usually below 1,000 MESF.

When the regression line (see Figure 4-60 on p. 216) is calculated, a correlation coefficient and, in some cases, other measures of the distance from the line of the points representing labeled beads, are provided. If the correlation coefficient is low (low, in this instance, probably means less than 0.98, because we're working on a log scale), and/or the distances of the labeled beads from the line are large, it is fairly obvious that the log amp doesn't work all that well; the measured sensitivity and/or autofluorescence values are definitely suspect in such cases. However, all log amps become unreliable at some point in the lower region of their range, and good correlations and small distances of labeled beads from the regression line unfortunately provide no guarantee that the log amp is behaving at the level of the blank bead or the unstained cell. I have produced differences of several hundred MESF in measured sensitivity simply by interchanging log amps with DC offset levels differing by 1 or 2 mV. That's why I suggested (p. 217) that sensitivity and autofluorescence levels should be determined using a **linear fluorescence scale**, and that high-sensitivity quantitative fluorescence measurements also be done on a linear scale. I'll make the point again, because it's very important; if you're

trying to measure a few hundred molecules of bound antibody and you're not using a linear scale, you run the risk of discovering you've been kidding yourself in private by embarrassing yourself in public.

Improving Sensitivity: The New Wave(length)

In cases in which cellular autofluorescence is a factor limiting sensitivity, one obvious way of improving sensitivity is to work with excitation wavelengths at which cellular autofluorescence is lower than it is when 488 nm excitation is used.

The excitation and emission spectra of autofluorescence reported for several mammalian cell types[181,183] are close to the spectrum of riboflavin (Fig. 7-7, p.291). Relatively little cellular autofluorescence is excited by light at wavelengths above 515 nm[304], which are used for excitation of Cy3, tetramethylrhodamine, the rhodamine 101 derivatives XRITC and Texas red, Cy5, and the phycobiliproteins and tandem conjugates. Thus, the use of any of these labels can provide increased sensitivity for weak fluorescence measurements in mammalian cells.

The ratio of median emission from antibody-labeled cells to median emission from unstained cells, with both emission values expressed on a linear scale, can be taken as a measure of sensitivity; this figure was 10-15 times higher for cells stained with phycoerythrin- labeled antibodies and excited at 546 nm by the mercury arc lamp in an old B-D FACS Analyzer than for cells stained with fluorescein-antibodies and excited at 488 nm by an argon ion laser in a FACStar cell sorter (L. Lanier, M. Loken, N. Warner, personal communications and meeting presentations). While the FACScan has somewhat better PE fluorescence sensitivity than the FACStar, the analyzer, when using green excitation, remained superior[1380-1]. The 532 nm line from a doubled YAG laser and the 543 nm line from a green He-Ne laser are usable for high-sensitivity, low autofluorescence measurements, although the low power levels (1-2 mW) available from green He-Ne lasers may limit sensitivity in practice. Since many more current instruments have red He-Ne or diode lasers than have green YAG or He-Ne lasers, it will usually be most practical to use allophycocyanin as a label with a red excitation source to make sensitive fluorescence measurements in mammalian cells without interference from autofluorescence.

If, however, one were interested in measuring cell surface antigens on *Synechococcus* or other species of cyanobacteria, a phycobiliprotein would be an extremely poor choice of label; cyanobacteria contain phycobilisomes stuffed full of phycoerythrin, allophycocyanin, etc., with the result that their autofluorescence, which is considerable, would be likely to swamp the signal from a phycobiliprotein label.

Vesey et al[2660] provide a good example of how to select a label based on studies of the autofluorescence of particles of interest and of other particles and fluorescent solutes likely to be encountered in the sample; their target was *Cryptosporidium* oocysts in drinking water, and they found, using a Coulter EPICS Elite flow cytometer, that phycoerythrin-, Cy-3-, or tetramethylrhodamine-labeled antibodies excited by a low-power Green He-Ne laser provided the best signal-to-background ratio for detection.

Correcting and Quenching Autofluorescence

If your apparatus can discriminate between cellular autofluorescence and the fluorescence of your label, based on some difference in spectral characteristics, you may be able to pick out the label from the background.

If you happen to have a flow cytometer with two ion lasers, you can follow the lead of Steinkamp and Stewart[780], who, using the multilaser flow cytometer at Los Alamos, took advantage of the fact that autofluorescence is better excited in the violet than at 488 nm (Figure 7-7), while fluorescein is very poorly excited by violet light. They made dual-beam measurements of cells stained with fluorescein-antibodies using a 413 nm beam from a krypton laser and a 488 nm beam from an argon laser, and subtracted a portion of the violet-excited fluorescence signal from the fluorescence signal excited at 488 nm.

Roederer and Murphy[781] devised a method applicable to flow cytometers with only a 488 nm excitation beam; they noted that, while green (530 nm) fluorescence in cells stained with fluorescein-ligands represented the sum of autofluorescence and fluorescein fluorescence, red (625 nm) fluorescence resulted almost entirely from autofluorescence; thus, by subtracting a portion of the red signal from the green signal, they could compensate for autofluorescence. This was done by digital computer processing of list mode data; Alberti, Parks, and Herzenberg[782] used the fluorescence compensation electronics in the FACS to perform similar manipulations on-line.

The methods described in the preceding two paragraphs allow you to compensate for autofluorescence relatively well on a cell-by-cell basis. If the issue is detecting populations of weakly stained cells above autofluorescence background, it may be sufficient to do histogram subtraction, an approach described by Corsetti et al[1589] and Müller et al[1590] (also see pp. 245-6)

A direct chemical approach to autofluorescence on a cell-by-cell basis was taken by Hallden et al[1391] and Mosimann et al[2661], who, respectively used crystal violet and trypan blue to quench intracellular autofluorescence, allowing detection of surface antigens with fluorescein-labeled antibodies and of fluorescein-labeled nucleic acid probes.

The bottom line here, however, is that none of the methods just described deal as effectively with autofluorescence as does choosing a label and an excitation and emission wavelength at which autofluorescence is minimal.

Raman Scattering Effects on Sensitivity

Phycoerythrin offers an attractive alternative to fluorescein when extreme sensitivity is required, because it becomes possible to decrease the effect of cellular autofluorescence, as well as to place a large number of fluorescent

chromophores on each antibody molecule. When PE is excited at 488 nm, rather than above 515 nm, cellular autofluorescence is not decreased. Moreover, there is the potential for interference from **Raman scattering** (p. 118), which occurs when a molecule undergoes a vibrational transition while scattering light. Raman emission is most likely to be at a frequency that is the sum of the frequencies of the incident light and the vibrational transition. The frequencies are expressed in **wave numbers (cm^{-1})**, which are the number of wavelengths per centimeter; the relationship between wavelength λ in nm and frequency ν in cm^{-1} is $\nu = 10^7/\lambda$. In flow cytometry, the dominant transition involved in Raman scattering comes from stretching of O-H bonds in water, at around 3600 cm^{-1}. At 488 nm, or 20492 cm^{-1}, the Raman emission is at 16892 cm^{-1}, i.e., $10^7/(20492 - 3600)$ nm, or 592 nm, a wavelength that may be transmitted by filters used for PE fluorescence measurement. When excitation at 532 nm is used, Raman emission is at 658 nm, which should not interfere with measurement of PE fluorescence but which could interfere with measurements of a PE-Cy5 tandem conjugate. Water Raman frequencies for various excitation wavelengths appear in Table 7-4 below.

EXCITATION (nm)	H_2O RAMAN EMISSION (nm)
325	368
350	400
365	420
408	478
415	488
436	517
441	524
458	548
476	574
488	592
515	632
520	640
532	658
543	675
546	680
568	714
594	756
611	783
633	820
650	849
675	892

Table 7-4. Raman emission from water.

In extremely high-sensitivity fluorescence measurements using single photon counting, broadband Raman emission, at wavelengths other than those tabulated above, may be the limiting noise source[1144].

Increasing Sensitivity: Amplification Techniques

Since experimenters can't readily change the number of molecules of the molecules they're trying to find in or on cells, they often attempt to shift the odds in their favor by increasing the amount of label that will be hung on each target molecule.

Some flow cytometer users have the option of increasing illumination power; few have the option of increasing light collection efficiency, and, while you can always try to find a PMT with more sensitivity than the one you have, there is a limit to how much measurement sensitivity can be increased by going that route. When increasing excitation power or fluorescence collection or detection efficiency will not improve measurements, increasing the number of fluorescent molecules bound per molecule of antibody or ligand using an amplification technique may be the only option available.

Amplification by Indirect Staining

The simplest way to do this in the context of immunofluorescence measurements is by using **indirect staining**; approximately six molecules of a polyclonal secondary **developing** antibody can be bound to each molecule of primary antibody used, putting six times as many molecules of label at each antigenic site as would be there if a directly labeled antibody were used. There are a number of variations on the basic theme.

Cohen et al[778] approached the problem of detecting small numbers of cell surface receptor sites by devising what they termed the "**super avidin-biotin system**" **(SABS)**. Biotinylated antibodies bound to receptor sites are reacted, in succession, with phycoerythrin-streptavidin, with biotinylated anti-streptavidin antibody, and again with phycoerythrin-streptavidin. The SABS system reportedly allows quantification of antigenic sites within the range of 100-1300 sites per cell; as few as 50 sites/cell can be detected, a sensitivity approaching that of conventional radioimmunoassay.

Zola et al[1380-1] used a somewhat simpler technique, in which a primary mouse monoclonal antibody was developed with a horse anti-mouse antiserum that was then labeled with a phycoerythrin-streptavidin conjugate; they could reliably detect 100 primary binding sites, and applied their methodology to quantification of cytokine receptors.

Amplification Using Labeled Particles

A greater degree of amplification than is possible with indirect staining may be achieved by attaching antibodies (at other than their specific binding sites) to **larger particles**, among which are included fluorescently tagged **viruses**, **membrane microvesicles or liposomes**, **bacteria**, and **plastic beads** (typically coated with antibody, antigen, hormones, etc.) as cell surface labels. Quantum dots (pp. 339-40) also fit into the particle category. Problems with clumping, nonspecific binding, and endocytosis of labeled particulates can occur when this approach is taken, and caution is therefore advisable.

Truneh et al[777,1382] described the use of **unilamellar liposomes** containing **carboxyfluorescein** or sulforhodamine, and conjugated to **protein A**, for detection of small numbers of receptor sites on cell surfaces. Carboxyfluorescein diffuses

out of liposomes extremely slowly, and therefore provides a relatively stable internal label for these structures. Gray et al[383] also reported successful use of the carboxyfluorescein liposome technique. However, the sensitivities achieved are probably matched by modern instruments in analyses of samples stained only with directly labeled antibodies.

I have also already mentioned (p. 277) the use of a **nonfluorescent particle label, colloidal gold**[708], which can be conjugated to antibodies and other ligands and detected on cells by the change in their light scattering characteristics. Antibodies coupled to small (0.1-0.5 μm) nonfluorescent polystyrene beads can also be detected when bound to surfaces by strongly increased side scatter signals[2662]. Siiman et al[2663] were able to detect the binding of two different antibodies, one bound to a polystyrene bead coated with colloidal gold or silver and the other bound to an uncoated polystryene bead, to cells using measurements of light scattering at 10-20° and at 20-65°; different wavelengths were needed for maximum discrimination of populations depending on whether colloidal gold or silver was used.

While nonfluorescent particles and/or colloidal silver/gold labeling probably do not provide a substantial increase in sensitivity over what can routinely be obtained using direct fluorescence measurements, they do make it possible to detect cell- or bead-bound antibodies in instruments using less expensive light sources and detectors than are needed in most fluorescence flow cytometers.

Phycobilisomes (p. 335), with molecular weights in excess of 10,000,000, might be thought of as biological particles; when directly or closely linked to antibodies, they appear to sterically hinder binding to cell surface antigens. However, Telford et al[2664] found that a red-excited stabilized phycobilisome coupled to streptavidin via a spacer (PBXL-3L, Martek) provided substantial amplification when compared to APC-streptavidin in labeling cell-bound biotinylated antibodies.

Amplification Using Enzymes as Labels: Playing the Hole CARD

A classical amplification technique applicable (originally with some difficulty) to flow cytometry is that of **enzyme-linked assay**. A ligand that reacts specifically with the substance sought by the experimenter (the chemists call the substance sought the **analyte**, which has always sounded kinky to me) is conjugated to an enzyme; after excess ligand-enzyme is removed, the analyte-ligand-enzyme complex is detected by reaction with a **chromogenic** or **fluorogenic substrate**, a colorless material that is acted upon by the enzyme to form a colored or fluorescent product. Once this reaction is complete, thousands of molecules of detectable product are present in the vicinity of each molecule of analyte.

Because of its large amplification factor, enzyme-linked assay offers high sensitivity, and is particularly useful for detection of small amounts of antigens and small numbers of copies of specific genetic sequences in cells; the obstacle to the application of the technique in flow systems is the necessity to trap the reaction product of the enzyme reaction in the cells being analyzed. Technicon (now Bayer Diagnostics) did this successfully in the Hemalog[84-5] and H-1 series of hematology analyzers, which use absorption measurements to identify neutrophil and eosinophil leukocytes by the presence of peroxidase in their specific granules. Kim et al adapted **immunoperoxidase**[779] and **alkaline phosphatase**[1384] labeling techniques for use on the Technicon instruments to subtype lymphocytes; the results based on forward scatter and absorption measurements were comparable in speed, sensitivity, and accuracy to those obtained by fluorescence flow cytometry.

In 1989, Bobrow et al[2665-6] described the technique of **catalyzed reporter deposition**, now familiarly known as **CARD**, which has since facilitated enzyme-based amplification in flow cytometry. The analyte of interest (e.g., an antigen) is reacted with an appropriate reagent (e.g., an antibody) conjugated with horseradish peroxidase (HRP). The specimen is then reacted with a phenolic HRP substrate (e.g., tyramine). HRP action on tyramine yields a highly reactive species that binds covalently to proteins. The tyramine may be labeled with biotin, as done in the original publication, and its presence demonstrated by subsequent reaction with fluorescent labeled streptavidin[2667-70]. However, it is becoming more common in current practice to label the tyramine directly with a fluorescent dye[2332,2671]. In either case, many fluorescent labeled molecules of the tyramine reaction product will accumulate, and stay, in the region of each reagent-analyte complex. Multiple rounds of amplification are possible, using HRP-labeled antibodies against the label deposited in the first CARD stage. Tyramide amplification reagents and kits for flow cytometry, using biotin, as developed by Kaplan et al[2669-70], are available from **Flow-Amp, Ltd.**; Molecular Probes offers a line of reagents including both biotinylated and fluorescent dye-labeled tyramine[2332]. Molecular Probes also offers an enzyme amplification technology described as **Enzyme-Labeled Fluorescence (ELF)**; antibodies or other reagents are tagged with alkaline phosphatase, either directly or via a biotin-streptavidin reaction, and the **ELF 97** phosphatase substrate is added, forming a bright green fluorescent, insoluble precipitate in the vicinity of the antigen or analyte molecules of interest[2332].

Amplifying the Analyte: The Polymerase Chain Reaction (PCR)

An enzyme-based amplification method to which we will return in a later section is the **polymerase chain reaction (PCR)**, which can be used to produce multiple copies of a known genetic sequence *in situ* for detection using labeled probes, amplified or otherwise.

Amplification Techniques: Pros and Cons; Fluorescent vs. Nonfluorescent Labels

Since the fairly simple and straightforward modifications of the **indirect staining** technique described above have

permitted measurement of as few as 100 binding sites on cells, it is not clear that using larger labeled particles for amplification will further improve sensitivity.

Enzyme amplification is particularly useful in that it can allow qualitative analyses of small amounts of analytes in cells and tissues to be done with chromogenic substrates and a simple transmitted light microscope. As was mentioned on p. 77, the first approaches to antibody labeling involved conjugation of antisera with azo dyes; however, complexes of tissue antigens with such dyes, even in relatively high concentrations, exhibited only weak coloration under the light microscope. This led Coons, Creech, and Jones[44] to turn to fluorescent labels, which were more readily detectable at low concentrations against the darker background present in a fluorescence microscope. Enzyme-linked immunoassay achieves what simple labeling cannot.

Most fluorescence flow cytometers have neither absorption nor extinction measurement capabilities, and are therefore not well equipped for measurement of the reaction products of chromogenic substrates, although forward and right angle scatter measurements may be usable under some circumstances. Enzyme-linked methods have therefore, to date, been used principally with transmitted light microscopy and with static and image cytometry, and are probably in wider use than is fluorescence flow cytometry.

On one of my occasional descents from my ivory tower, I heard a real pathologist give a highly sophisticated talk on lymphomas, involving detection of multiple antigens, *in situ* hybridization with genetic probes, PCR, etc. Flow cytometers weren't involved; all the work was done using enzyme-linked reagents, and much of it was done on tissue slides, where the objective was to determine anatomical localization of various things, a task to which flow cytometers are, and will, for the most part, remain, unsuited. The semiquantitative analyses available from microscopic observation were all that was necessary to answer the biological questions at hand. I'm sure some of the work could have been done using fluorescence flow cytometry, but it wouldn't have been done any better, cheaper, or faster. Food for thought.

Coventry et al[2672] used video image analysis to compare the detection sensitivity of immunoperoxidase staining on slides with that of flow cytometry; they found that detection of 100-200 cell surface molecules in specimens on slides was possible only if heavy metal-enhanced immunoperoxidase methods were used, while high-sensitivity flow cytometric methods[1380-1] could, in some cases, detect as few as 50 molecules.

To some extent, amplification increases sensitivity at the expense of precision, because amplification factors aren't exact; you won't get exactly six molecules of developing antibody per molecule of primary antibody, or 100,000 molecules of product per molecule of enzyme-linked antibody. If you're looking for 100 copies of something, however, you have Poisson sampling statistics and molecular shot noise[2658] (p. 341) working against you, anyway. Even if you hung a 2 mW diode laser on each molecule, you'd still count only 100 molecules, and the measurement CV could be no less than 10 percent.

Improving Sensitivity: Time-Resolved Fluorescence

On p. 283, it was mentioned that phase-sensitive detection of a fluorescence signal in a flow cytometer using a modulated light source could discriminate fluorescence signals from different materials with different fluorescence lifetimes. The lifetimes of most of the materials responsible for autofluorescence are on the order of tens of nanoseconds; if one uses a label with a substantially longer lifetime, and a light source emitting brief (picosecond or shorter) pulses, and delays the fluorescence emission measurement for 50 nsec or so following the pulse, the contributions of autofluorescence and of other interferences, e.g., Raman scattering, stray scattered light, and filter fluorescence, are eliminated from this **time-resolved fluorescence** signal. Chelates of rare earth metals such as europium and terbium have fluorescence lifetimes on the order of microseconds (see p. 113), and Bob Leif suggested in the 1970's[1110], as did Tomas Hirschfeld (personal communication), that they might be useful as labels for time-resolved flow cytometry with pulsed sources. Condrau et al[1385-6] described measurements of cells bearing europium-chelate labeled antibodies in a modified flow cytometer in which acousto-optic modulation is used to deflect the illuminating beam away from the flow cell once a scatter signal is detected; their papers also include good bibliographies on time-resolved fluorescence immunoassays. Related work by Beverloo et al[1387-8] describes the use of **phosphor particles** as luminescent (in this case, phosphorescent rather than fluorescent) labels for time-resolved image cytometry. One drawback to both rare earth chelates and phosphors as labels in the context of flow cytometry is the long measurement time required to take advantage of long excited state lifetimes, which run to hundreds of microseconds; this would limit throughput to a few hundred cells/sec, but the tradeoff may be worthwhile if extreme sensitivity is required.

7.7 MEASURING CELL SURFACE AND INTRACELLULAR ANTIGENS

Almost all of the fluorescence flow cytometers now in use have been applied, at one time or another, to measurements of **cell surface immunofluorescence**. The desire to do immunofluorescence measurements motivated researchers and clinicians to learn about flow cytometry and buy flow cytometers; and this legitimized other flow cytometric techniques not based on immunofluorescence, such as DNA analysis and differential leukocyte counting.

The literature on flow cytometry of immunofluorescence is so vast that I will not even attempt to summarize the fraction of it that I have seen. Instead, I'll try to present the basics and to touch on some issues that I don't think have been well covered elsewhere.

History and Background

In the late 1960's, when most of the few people involved in flow cytometry were looking at DNA stains and trying to tell normal from abnormal cells in cervical cytology specimens, Len Herzenberg alone had the vision to appreciate that the combination of the apparatus and fluorescent antibody reagents could unravel the genealogy and developmental history of the lymphocytes.

Within a few years, flow cytometry, predominantly in the form of gated single-parameter immunofluorescence measurements, and cell sorting and subsequent functional analysis, had been used to define a taxonomy of lymphocytes, in mice and men, where none had existed previously. The methodology that Herzenberg and his associates and students had used to study murine cells was adapted and aggressively applied to human immunology by Stuart Schlossman in the United States, Melvyn Greaves in Great Britain and a rapidly growing crowd of others.

This first wave of activity was all the more remarkable because it occurred several years before the discovery of monoclonal antibodies; the identification and preparation of both reagents and samples were considerably more complicated than they are today. While immunologists, even in those days, seemed to have the opinion that fluorescent antibodies were the only specific reagents, and that neither cytochemistry nor multiparameter flow cytometry would have any use in future work on blood cell development, differentiation, and pathology, this viewpoint was not justified then; subsequent history has eroded any remaining basis for it.

Obviously, antibodies were exquisitely specific, but, until the advent of **monoclonal antibodies**[785], you could never be sure for what they were so specific, and never count on getting a serum with quite the same specificity next time you bled the same animal. For you physicists and electronikers out there (bear with me, immunologic sophisticates), I should amplify on this a bit.

Monoclonal Antibodies for the Uninitiated

For the past few hundred years, or since before anybody knew there were lymphocytes, we have been in the business of deliberately inducing **immunity** to various diseases in humans and animals, by injecting material from organisms that cause those diseases (or, sometimes from organisms closely related to the disease-causing organisms). The substance injected is called an **antigen**. If, a week or more after the antigen is injected, you extract some blood serum from the animal (or human) that received the antigen, you can (usually) demonstrate formation of a precipitate if antigen is added to the serum, due to the formation of complexes between molecules of antigen and molecules of serum proteins called **immunoglobulins (Igs)**, which are the **antibodies**.

Immunoglobulins are produced by **B lymphocytes**; in order for such production to occur, the concerted action and interaction of T **lymphocytes** and other blood cells called **macrophages** is also required. There are several different classes of Igs; B cells start out producing a very large molecule called IgM, and then typically switch to producing IgG. IgE, which is involved in allergic reactions, represents another class of Igs. The **gamma globulin** and **antitoxin** or **antiserum** that some of us remember being used in medicine and others have encountered in old novels are serum immunoglobulin fractions. All Igs of a given class have relatively similar "backbone" structures, but, at some points in these molecules, there exist **variable regions** that differ from molecule to molecule in amino acid composition and sequence; it is the variable regions that react with antigen. Also, Ig molecules are **multivalent**, i.e., each molecule contains at least two sites capable of binding antigen.

Antibodies react with antigens in a very specific fashion and, typically, with very high affinities, but a conventional antiserum contains not one type of Ig molecule with one unique structure, but a large number of antibodies, with different molecular structures, each reacting with different affinity with a different chemical group on the antigen. Lymphocytes, in the course of their development, undergo **gene rearrangement** of the genes that encode the variable regions of Igs; this "shuffling" of genetic material insures that there will be a great diversity of amino acid sequences, and therefore of reactivities, represented in the B cell population of any given individual, although any given B cell can produce only one specific molecular structure. B cells normally carry Igs around on their cell surfaces; when the **surface membrane immunoglobulin (SmIg)** on a B cell binds to an antigen, it stimulates the B cell to reproduce.

Thus, when an animal is **immunized**, or injected with antigen, B cells that produce, and bear on their surfaces, Igs that react with that antigen reproduce. Each B cell forms a **clone** of progeny, all of which produce Ig molecules with the same structure, which is different from the structures of the Igs produced by all the other clones. The antiserum is therefore described as **polyclonal**. Polyclonal antisera, by their biological nature, contain a small fraction of mixtures of Igs that react, with differing affinities, with different determinants on the same antigenic molecule, as well as a larger fraction of "irrelevant" antibodies which, while they may be very relevant to protecting the organism from infections, don't react at all with the antigen the experimenter injected to produce the antiserum, but may react with any of a tremendous variety of other proteins or macromolecules foreign to the animal in which the antiserum was produced.

If you extract the immunoglobulin fraction of an immunized animal, you end up with a number of Ig molecules that will react specifically with the antigen you're after and a much larger number of Ig molecules that will react with other things. **Adsorption**, or reaction with materials containing these irrelevant antigens, can be used to remove some of the irrelevant antibodies, and **affinity purification**, which traps desired antibodies on a column or other substrate containing immobilized antigen, can further increase the specificity of a polyclonal antiserum – to the point at which as many as a few percent of the Ig molecules will react with

antigen! Even at this rather shocking level of impurity, specificity may be sufficient to permit definition of the chemical structures on or in cells with which a polyclonal antiserum is reactive.

In the days before monoclonal antibodies, this was what had to be done before one could even think of using an antiserum for immunofluorescent staining; the whole process seemed more art – many would say black art – than science. For those of us who worked in industry, the difficulties of obtaining and/or standardizing immune reagents were a major obstacle to the development and dissemination of procedures involving immunofluorescence. Very few people envisioned a need for more than two immunofluorescent labels; it was hard enough to do single-color fluorescence, and two colors represented a *tour de force*.

Things changed dramatically for the better in the late 1970's, with the dissemination and refinement of methods for **monoclonal antibody production**[78,85]. If you have the opportunity to look at a copy of the First Edition of *Flow Cytometry and Sorting*[9], which was published in 1979, you can try to find the single appearance of the words "monoclonal antibodies" in that book's 716 pages. They are not, by the way, found in any of the chapters that deal with applications of flow cytometry to immunology.

Monoclonal antibodies are produced by forming hybrids between B cells from animals immunized with an antigen and cultured cells derived from B cell tumors. The tumor cells, unlike normal B cells, are **immortal**, i.e., they can be grown in culture for generations on end. The hybrid cells acquire the property of producing one molecular species of Ig with one specific reactivity from their normal B cell parents, and the ability to grow indefinitely from their malignant parents. Cultures can be started from a single hybrid cell; these will thereafter continue producing identical Ig molecules as long as the culture is maintained.

Monoclonal antibodies are, thus, very well defined, in terms of their own structure, and one can be pretty well sure that all of the Ig molecules in a monoclonal will react with the same site on the antigen with the same affinity. However, if the structure of a given cellular antigen is unknown, simply having a monoclonal antibody instead of a polyclonal antiserum against the antigen doesn't tell you any more about the structure, although it may make it operationally easier to elucidate that structure.

Cell Surface Antigens: Structure versus Function

It was obvious to some people long ago that antigens weren't present on cell surfaces simply for decoration, or as Nature's bar code, but, rather, that each antigen had a function, so that, in most cases, **one person's surface antigen is another person's receptor**. This is much better and more widely appreciated in 2003 than it was in 1978. Monoclonal antibodies have, in fact, greatly facilitated studies of receptor-ligand interactions, in which considerations of specificity formerly demanded use of labeled natural ligands; the antibodies can be made with higher affinity and the structure on the receptor to which they bind can be selected to a great extent.

The advent of monoclonals also eliminated most of the difficulties associated with reaction of cells with two or more antisera to demonstrate two or more discrete antigens on or in the cells; this, in turn, awakened interest among immunologists in increasing the number of available antibody labels. The 1977 publication by Loken, Parks and Herzenberg on two-color immunofluorescence using fluorescein and tetramethylrhodamine as labels with argon ion laser excitation[115] made it clear that improvements in labels and reagents, as well as in equipment, would be necessary to make multicolor work generally feasible.

Moving Toward Multicolor Immunofluorescence

The improvement in labels began with the development of the long-wavelength rhodamine 101 dyes XRITC and Texas red, and continued with the introduction of the phycobiliproteins and, later, of tandem conjugates. While XRITC and Texas red forced some improvement in equipment, by mandating the use of dual-wavelength excitation, the improvement increased the cost and complexity of apparatus. The phycobiliproteins and tandem conjugates have since made multicolor fluorescence easily accessible to immunologists and other workers without the need for much specialized training in instrumentation.

The expanding armamentarium of monoclonal antibody reagents and labels precipitated some changes in immunologists' attitudes; they had enough and good enough reagents to do multiparameter analysis and, now that they had more than two parameters to keep track of, they had adequate motivation to use computers for data analysis and storage. Without computerized data analysis systems, it is unlikely that two-color, let alone three- and four-color immunofluorescence measurements, would have become commonplace.

In retrospect, I should not have expected that immunologists would take kindly to my glib suggestions in the mid-1970's that they run out and do 3-color and 4-color immunofluorescence measurements using multiple excitation beam, multiparameter flow cytometry. Most immunologists were unaware, as I was aware, of the existence of the hardware and software technology required, and painfully aware, as I was unaware, of how difficult it was to produce even good two-color immunofluorescent staining using conventional antisera and the flow cytometers that were then generally available. We literally could not give away a multiple beam apparatus in the mid-1970's; times have certainly changed.

While the demonstration in 1979 of two-color immunofluorescence using dual-laser excitation of fluorescein and XRITC generated a substantial demand for dual-laser instruments, a look at the literature reveals that the number of papers involving fluorescein/XRITC or fluorescein/Texas red immunofluorescence published during the 5 years after the apparatus and labels became available was considerably smaller than the number of dual-laser flow cytometers pur-

chased with such studies in mind. Clearly, multicolor immunofluorescence was harder than it looked.

In the 1980's, history repeated itself, as people stampeded after flow cytometers with CW dye lasers, which could allow them to do 4-color immunofluorescence, using an argon laser at 488 nm to excite fluorescein and phycoerythrin, and a dye laser at 600 nm to excite Texas red and allophycocyanin, provided reagents were available. Some settled for 3-color immunofluorescence, which could be done with a single beam, using fluorescein, phycoerythrin, and phycocyanin[375] or a phycoerythrin-allophycocyanin[406] or phycoerythrin-Texas red tandem conjugate. The first couple of years' worth of publications and presentations actually demonstrating 3-[317-20] and 4-color (L. Herzenberg et al, personal communications and meeting presentations) immunofluorescence using the labels just mentioned came either from Herzenberg's laboratory at Stanford or from B-D's antibody production facility. This was strong evidence in favor of the view that **access to and skill in the use of reagents, and not access to hardware, were the most critical contributing factors to success with multicolor immunofluorescence measurements**.

Today, phycobiliprotein and tandem labels, an unbelievably wide selection of monoclonal antibodies, and instruments that can measure eight or more colors of fluorescence, are available and easy to use compared to what came before. Access is much less of a factor than skill.

Antibody Reagents and Staining Procedures

Most of us, myself included, don't make or conjugate our own **antibodies**; whatever isn't supplied by our collaborators has to be scrounged or bought. Once you get past the realization that there are few commodities you can legally buy that cost as much per small vial, you can appreciate the good points of commercial monoclonal antibodies. They are generally well-defined and well-controlled, you can get them with a variety of labels attached, and you generally can find out how much to use to stain how many cells by reading the label or package insert. Commercial polyclonal antisera, adsorbed and/or affinity-purified or otherwise enriched in antibodies for the antigens you're after, are also available and relatively convenient to use.

Susan Sharrow, who worked with both flow cytometers and monoclonals at NIH more or less since the inception of each, took me to task years ago for suggesting that it was easier to make flow cytometers than monoclonals. It's gotten easier to do both; hybridoma clones are available from places like the American Type Culture Collection, and you can buy kits for purifying the immunoglobulin fraction and labeling the antibodies. Given the range of monoclonals available, labeled, off the shelf, most people won't to have to roll their own. We did do it in my lab once, just for the experience.

Antibody Fragments versus Antibodies as Reagents

For some applications, it is preferable to use enzymatically cleaved antibody **fragments** instead of the whole immunoglobulin molecule. Certain cells, e.g., macrophages, monocytes, and granulocytes, have surface receptors that bind the **Fc** portion of antibodies more or less independently of the antigenic specificity of the antibodies; these cells may, by this mechanism, therefore be stained by labeled antibodies to antigens that are not present on the cell surface. Enzymes can be used to produce **monovalent (Fab)** or **bivalent (F(ab'$_2$) antigen binding fragments** that will not bind to Fc receptors, eliminating the problem of nonspecific staining. The Fab fragments are useful in situations in which it is necessary to avoid crosslinking of antigenic sites and/or agglutination, although their binding affinities are lower than those of the multivalent antibodies from which they are derived. Fragments of some antibodies are available off-the-shelf, with or without fluorescent labels.

Engineered Antibodies: Phage Display and scFvs

It is now possible to produce monoclonal antibodies, human as well as murine, without benefit of either immunization or hybridoma technology, using **phage display**. It was shown in the late 1980's that proteins coded for by gene sequences introduced into the genome of filamentous bacteriophages could be expressed on the surface of the virions[2673]; it was also demonstrated[2674-5] that a functional antibody variable region (**Fv**) could be expressed in the periplasmic region of *E. coli* into which genes coding for the variable regions of both light and heavy immunoglobulin chains had been introduced. In 1990, McCafferty et al[2676] described production of active antibody molecules in filamentous phage.

The technology has since come a long way; there now exists a phage display library of 2×10^9 members, about three-fifths of which code for functional human **single chain variable fragments (scFvs)**; it is possible, with a minimum of additional genetic manipulation, to derive Fabs and immunoglobulins with the specificity of the scFvs[2677]. Clonal selection has become the province of biotech companies; using appropriate gimmicks (including cell sorting), you can grow up a potful of monoclonal antibody to the antigen of your choice, starting, if you like, with a single B cell[2678]. Since you can hold the light and heavy chain portions of an scFv together with a synthetic linker, you can add things on to the linker, producing such goodies as antibodies tagged with alkaline phosphatase[2679] or GFP[2680], or high-avidity tetrameric antibodies[2681].

Much of the current work on engineered antibodies is focused on development of agents that can be used to treat human disease, rather than on expanding the repertoire of reagents for cytometry, but it's a safe bet that we'll get some interesting things to play with during the next few years.

Molecular Probes' Zenon Antibody Labeling

Some lower-tech, although not less interesting, antibody engineering is involved in a new methodology for antibody labeling recently developed by Molecular Probes[2352], which they call Zenon technology. The Zenon One Mouse IgG$_1$ labeling kits incorporate purified Fab fragments of a goat

antibody to the Fc portion of mouse IgG_1; the Fab fragments are available with any of a number of labels, including fluorescent dyes, phycobiliproteins and tandems, biotin, and enzymes, covalently attached. A Zenon One Fab reagent can be used to label a very small amount (1 µg or less) of a mouse monoclonal antibody; after 10 minutes incubation, any unbound labeling reagent is, in essence, inactivated by addition of an excess of indifferent mouse IgG_1. Multiple antibodies labeled with different dyes can be used together, and quantitative measurements are possible, although the excess indifferent antibody added to remove unreacted labeling reagent will, over a period of hours, remove some reagent from the antibodies of interest. The Zenon methodology should be particularly helpful to investigators developing their own monoclonal antibodies, as it should facilitate simultaneous analysis of two or more antibodies on cells.

Antibody Shelf Life and Quality Control

Antibodies keep pretty well, but they don't have an infinite shelf life. You need to keep an eye out for crud accumulating in your vials of antibody, spinning down aggregates and so on when bad things happen to good antibodies. Periodic measurement of the fluorescence intensity of antibody bound to beads with known binding capacities allows you to monitor reagent quality. Manufacturers usually tell you how many milligrams of antibody protein are in the vial, which may be helpful; they don't usually tell you how many molecules of label are on each antibody molecule, which would also be helpful.

Direct Staining Using Monoclonal Antibodies for 2, 3, and More Colors with 488 nm Excitation

To my way of thinking, the best way to proceed when doing immunofluorescence is the way that requires the least work. I use monoclonal antibodies whenever I can get them, and, unless I'm looking for an antigen that is scarce enough to force me to employ some kind of amplification procedure (pp. 343-5), I prefer to stain using antibodies with directly conjugated labels. Most people, myself included, have tended to use fluorescein on the first antibody and phycoerythrin on the second. Fluorescein is available on more antibodies than phycoerythrin, and fluorescein-labeled antibodies are usually cheaper; however, phycoerythrin is a better label in the sense that it is brighter on a per-molecule-of-antibody basis (see Figure 1-18, p. 37). The figure also indicates that, for 3-color work with 488 nm excitation, PE-Cy5 tandem conjugates are preferable as labels to PE-Texas red tandem conjugates, because there are fewer spectral overlap problems with phycoerythrin. Four-color immunofluorescence with a single 488 nm beam once pretty much forced you to use PE-Texas red conjugates, although phycocyanin conjugates were reported usable if the antigen density was high enough[775]. These days, you have PE-Cy5.5, PerCP-Cy5.5, and PE-Cy7 to play with, and you can do 7-color immunofluorescence using a 488 nm beam if you've got the right instrument.

Multicolor Work Using Multiple Lasers; Biotin-Avidin Labeling

If you have a second laser emitting at 590 nm or above, i.e., a CW dye laser, a red He-Ne laser, a krypton ion laser, or a visible diode laser, in addition to a 488 nm argon laser, it may make sense to use allophycocyanin even as a third label, because compensation should not be necessary. Lanier, Loken, and others at B-D[787-8] did a lot of serious, big-league three-color immunofluorescence in this fashion with fluorescein, phycoerythrin, and allophycocyanin, using an argon laser emitting about 100 mW at 488 nm to excite the first two labels and a He-Ne laser emitting under 50 mW at 633 nm to excite the third. Hoffman et al[646] did 5-color measurements using phycocyanin and allophycocyanin B as labels with 633 nm excitation, and fluorescein, phycoerythrin, and a PE-Texas red conjugate excited by a second 488 nm beam; these days, you'd probably choose APC, APC-Cy7, and APC-Cy5.5, in that order, as red-excited labels.

Three-laser instruments are more common than they used to be, and, in the future, we can expect the most common three-beam systems to use 488 nm argon or solid-state lasers, red He-Ne or diode lasers, and violet diode lasers; Cascade blue and cascade yellow, or materials with similar spectra, are probably the best bets as labels for immunofluorescence when using violet excitation.

Mixing Colors: Do's and Don'ts

It helps to consider in advance which labels you'll want on which antibodies. One of the more general rules with which you contend in flow cytometry is that, when you have equal intensities of fluorescence coming from two materials excited at the same wavelength, it's easier to eliminate interference in the shorter wavelength signal from the longer wavelength fluorescent material than to eliminate interference in the longer wavelength signal from the shorter wavelength fluorescent material. This is so because the emission spectra of most materials are skewed toward longer wavelengths; the "red tail" of fluorescein emission, for example, overlaps a substantial part of the phycoerythrin emission spectrum, while there is very little overlap between the shorter wavelength portion of the phycoerythrin emission spectrum and the peak region of the fluorescein emission spectrum. Thus, you should use fluorescein to label the antibody that will give the weaker signal; this will also minimize crosstalk between fluorescein and phycoerythrin immunofluorescence signals, so you will need less fluorescence compensation.

The same argument applies with even greater force when you are considering combining immunofluorescent stains with other dyes that produce very strong fluorescence signals, e.g., nucleic acid dyes. The weak fluorescence of fluorescein-labeled antibodies can be discriminated fairly effectively from propidium fluorescence, although some overlap compensation is advisable; it would be much more difficult to discriminate phycoerythrin fluorescence from that of a

nucleic acid dye such as TO-PRO-1. While phycoerythrin and propidium fluorescence emissions overlap considerably, Corver, Cornelisse, and Fleuren[392] have shown that phycoerythrin antibody fluorescence can be discriminated from propidium fluorescence at levels above about 50,000 antibody binding sites per cell, making measurement of DNA and two antigens possible on a 3-color instrument.

Since the emission spectrum of fluorescein and the absorption spectrum of phycoerythrin overlap, **energy transfer** from fluorescein to phycoerythrin will occur and, if fluorescein and phycoerythrin molecules are in close proximity, may be significant. This will result in lower fluorescein fluorescence intensity than would otherwise be expected. Chapple, Johnson, and Davidson[1494] showed, by dual labeling of cells with pairs of monoclonal antibodies, that R-phycoerythrin could quench fluorescein emission when both were attached to the same cell. If quantitative or semiquantitative information is important, it is advisable to compare intensities of one- and two-antibody control samples to determine the extent of quenching. It is also important to note that fluorescence compensation won't work properly when there is substantial energy transfer; this will interfere with quantitative measurements, and, in the most critical cases, it may be desirable to use multiple illumination beams for accurate quantification.

Cocktails for Five: Multiplex Immunofluorescence

In practice, there are numerous situations in which the identification of multiple, discrete cell subpopulations is of primary importance, while quantification is either secondary or not necessary.

The relative ease with which cell samples can be stained using mixtures of monoclonal antibodies has led to the independent development by several groups of immunofluorescence staining techniques involving **multiplex labeling**, which may allow demonstration of three or more antigenically distinct cell populations in a single sample using only two fluorescent labels, offering considerable savings of time and labor compared to conventional staining techniques.

Definition and counting of subpopulations in mixed populations, as in lymphocyte subset analysis, is most easily and most commonly done by using antibodies that react with only one of the cell types to be counted. If only a single label is used, a separate sample is required for each subset to be demonstrated. As a rule, two-color immunofluorescence labeling readily allows visualization of three stained subpopulations (one double positive and two single positives) and one unstained population. Multiplex labeling allows for more economical use of the two-parameter measurement space.

In the simplest form of multiplex labeling, a procedure described by Saunders and Chang[792] and proposed by Mack Fulwyler[793] and, independently, by Tomas Hirschfeld and Myron Block (personal communication, 1981), a mixed cell population is stained with mixtures of antibodies, each of which should, in principle, react with only one cell subpopulation. Any given antibody present in the mixture may bear one or both of the two labels used, but a unique ratio of labels must be chosen for each antibody present. If phycoerythrin and fluorescein are the two labels chosen, cells reacting with an antibody present only in the phycoerythrin-labeled form will appear along the phycoerythrin fluorescence axis, cells reacting with an antibody present exclusively as the fluorescein conjugate will appear along the fluorescein fluorescence axis, and cells reacting with an antibody present as a 1:1 mixture of fluorescein and phycoerythrin conjugates will appear along a line at 45° to both axes.

A refinement of multiplex staining, described by Horan et al[791], uses mixtures of fluorescein- and phycoerythrin-labeled and unlabeled antibodies to produce fluorescence intensity differences of several orders of magnitude among the various cell subsets, with data display on a logarithmic scale. Liu et al[1594] demonstrated that helper and suppressor/cytotoxic T cells, NK and B cells, and monocytes could be identified in this fashion in whole blood samples from both normal and HIV-infected individuals, as shown in Figure 7-18.

Multiplex labeling has the potential to fail ungracefully when applied to certain types of samples. For example, small numbers of T cells bearing both CD4 and CD8 antigens may be found in peripheral blood[791]; these might be counted as other cell types. This behavior can, however, be identified by comparisons between multiplex labeled and conventionally labeled samples, allowing the prospective user to determine when the method is and is not suitable. In this context, the results reported with multiplex labeling in HIV-infected samples by Liu et al[1594] were encouraging.

When the previous edition of this book was written, there was a great deal of interest in possible applications of multiplex immunofluorescence to analyses of cells. Most laboratories had only two- or three-color fluorescence measurement capability, and multiplex analysis would have provided them the only means for investigating many phenomena then (and, in many cases, now) of great interest. For example, it was felt that determining the state of activation of various cell subpopulations in HIV-infected patients might provide indicators of both clinical course and response to therapy. Semiquantitative or quantitative analysis of activation antigens such as CD25 and CD71 (the IL-2 and transferrin receptors), and/or of parameters such as RNA content, were known to provide reliable evidence of lymphocyte activation[597-606].

If conventional staining procedures were used, it would be necessary to use two antibodies, and, therefore, two fluorescence channels, simply to define a single subpopulation of interest. The analysis of an activation antigen or RNA in this population would require a third antibody, or a dye such as pyronin Y, and a third fluorescence channel. In a 3-color instrument, analysis of activation in T-helper and suppressor/cytotoxic cells, B cells, and NK cells would require three tubes using conventional staining. If multiplex staining were used, a cocktail of antibodies labeled with fluorescein and

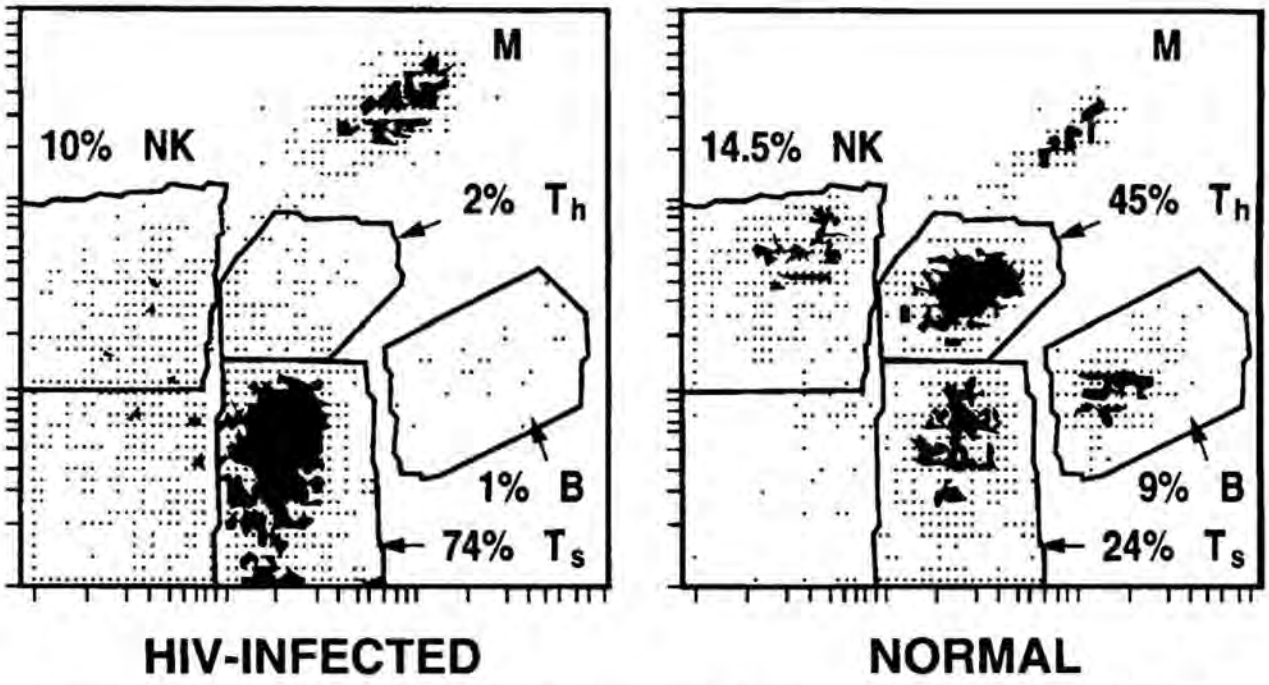


Figure 7-18. Multiplex immunofluorescent labeling to demonstrate helper (T_h) and suppressor/cytotoxic (T_s) T cells, B cells, NK cells, and monocytes (M) in peripheral blood from an HIV-infected and a normal individual[1394]. Erythrocytes were lysed after dextran sedimentation, and 2×10^6 washed leukocytes were stained with a cocktail containing anti-CD3 (T cells; B-D Leu-4: 10 µl unconjugated, 10 µl FITC-Ab), anti-CD4 (T_h; B-D Leu-3a: 20 µl PE-Ab), anti-CD8 (T_s; B-D Leu-2a: 19 µl unconjugated, 1 µl PE-Ab), anti-CD14 (monocytes; B-D Leu-M3: 20 µl FITC-Ab; Coulter My4: 5 µl PE-Ab), anti-CD16 (NK cells; B-D Leu-11c: 20 µl PE-Ab) anti-CD19 (B cells; Coulter B1: 5 µl FITC-Ab), and anti-CD20 (B cells; B-D Leu-12: 5 µl PE-Ab) antibodies at 4 C for 30 min. Cells were washed three times and fixed in 1.5% paraformaldehyde before analysis. Five leukocyte subclasses can be identified and counted in the two-dimensional measurement space. The figure was provided by Alan Landay of Rush-Presbyterian-St.Luke's Medical Center.

PE-Cy5 could be used to identify all four subclasses in one tube, with a third, spectrally distinct marker, e.g., PE-anti-CD25 or -CD71 or pyronin Y serving to define the activation state of all of the subclasses.

The multiplex labeling approach could be, and was, extended to analyses using cocktails containing more than two labels; Carayon, Bord, and Raymond did this to identify eight leukocyte subsets in a single tube[1395]. Buican and Purcell[329] reported that, by application of mathematical techniques similar to those used in computerized tomography, distributions of multiple antibody labeling could be uniquely reconstructed from two-color fluorescence analyses. Buican and Hoffmann[795] also explored the use of two-color measurements to identify multiple subpopulations based on analysis of multiple samples with different labeling ratios, and described apparatus for automated preparation of the required samples[796]. Multiplex labeling was also discussed by Wood[1396], Mansour et al[1397,1401], van Putten et al[1398,9], and Hunter et al[1400].

In a non-flow application, Ried et al[1402] used a 3-color multiplex fluorescence method to identify seven different DNA probes bound to chromosomes by digital imaging microscopy. However, a principal obstacle to the use of multiplex labeling with nucleic acid probes in flow cytometry lies in the low signal intensities expected due to low copy numbers of the nucleic acid sequences sought. Multiplex labeling cannot be expected to work well with very low intensity immunofluorescence signals, either; at levels at which one-color fluorescence is difficult to discriminate from noise, it is even more difficult to attempt to quantify intensity levels of two or more colors.

Where they were feasible, however, it was clear that multiplex labeling methods had great potential for saving time and money. Less sample and possibly less antibody is required, and only a single tube, instead of several, must be analyzed; this results in less preparation time, and in higher sample throughput for both flow cytometers and data analysis systems. However, interest in multiplex analysis of cells has waned as instruments capable of measuring four or more colors replaced two- and three-color systems in laboratories. Multicolor measurements offer most of the advantages of multiplex measurements; it is entirely feasible, for example, to derive counts of CD4+ and CD8+ T lymphocytes from a single tube of lysed whole blood using a mixture of anti-CD45, anti-CD3, anti-CD4, and anti-CD8 antibodies (see Figure 1-17, p.34), and this procedure, like a multiplex

staining procedure, uses relatively little reagent and sample, and requires relatively little analysis time.

What has kept interest in multiplex fluorescence measurement very much alive and well since the previous edition appeared is the application of the technique to chemical analyses of ligands bound to beads[2370-5,2682], this subject was introduced on p. 48, and will be discussed further in Chapters 8 and 10.

Cocktail Staining Helps Identify Rare Cells

Cocktail staining has proven useful for the identification of rare cells. Stained cells representing less than 0.1 percent of a population are almost impossible to discriminate from unstained cells on a single-parameter histogram or on a dot plot of fluorescence versus an indifferent parameter such as forward scatter. However, if most or all of the unwanted cells are labeled with a contrasting color, using a cocktail of antibodies, it becomes much easier to find very small subpopulations. This approach was used by Ryan et al[883,1010] for detection of small numbers of leukemic cells following therapy; cocktail staining was also used by Gross et al[1150,2331] to identify cells present at frequencies as low as 1:10,000,000.

Immunofluorescence Staining Procedures

Due to limitations on reagent specificity and to what was available in the way of labels, earlier immunofluorescence staining methods had to incorporate multiple staining and washing steps, which were time-consuming and labor-intensive. Staining was done either in the cold (4 °C) or with an inhibitor such as sodium azide added to the cells to prevent **capping** or **stripping** of antibody. Even today, when one is using conventional antisera and/or indirect labeling, washing is necessary to prevent unacceptable levels of nonspecific background fluorescence, and the low temperature and/or inhibitor become necessary because of the length of time required. With directly labeled antibodies, things get about as easy as they can get; you throw all the antibodies in at once.

It has been argued[175,176] (also R. Hoffman, personal communication) that, when one uses directly labeled monoclonal antibodies, most or all of the wash steps can be omitted with few deleterious effects on results. Hoffman and Hansen[176] described a method for T cell subset analysis in which 50 µl of whole blood were incubated with 100 ng of monoclonal antibody for 10 minutes at room temperature, following which 2 ml of a lysing reagent (8.29 g NH_4Cl, 37 mg disodium EDTA, and 1 g $KHCO_3$ per liter, pH 7.3-7.5) was added. Samples were introduced into the flow cytometer within 10 minutes after the lysing reagent was added; immunofluorescence measurements were gated using a combination of forward and side scatter measurements so that, in theory, only lymphocytes were analyzed. This basic methodology, which worked as well with multiple antibodies as with a single antibody, was widely adopted.

Hoffman and his colleagues, then at Ortho, also worked out some quick staining procedures for using **biotin-avidin** reagents almost as if they were direct labels; since the affinity of avidin for biotin is so high, if you know the amount of biotin-antibody in your staining mixture, you can figure out how much labeled avidin to add so as to end up with slight antibody excess.

Hoffman et al also described a technique[645] for immunofluorescence analysis of leukocytes in whole blood without lysis; a bright fluorescent counterstain, which stains leukocyte nuclei and/or cytoplasm but not erythrocytes, is used to detect and classify leukocytes as well as to gate the fluorescence measurements; Terstappen and Loken[1154] also described triggering on the fluorescence of nuclear stains for analysis in unlysed blood. I used the same trick when I used Hoechst dye staining of nuclei to gate immunofluorescence measurements, but I usually didn't go out of my way to work with unlysed whole blood, since, at one leukocyte per 1,000 erythrocytes, you have to run for several minutes to get immunofluorescence signals from a few thousand leukocytes. If you're willing to accept the longer analysis time, you can even trigger on immunofluorescence signals, which facilitates doing things like just looking at the T cells and ignoring everything else in the sample.

In my view there are few excuses for not using the simpler immunofluorescent staining methods whenever possible, especially since there never seem to be enough hands available to stain all the samples you want to stain. There are, however, situations in which fast, no-wash immunofluorescent staining methods don't work so well, e.g., in the case when the number of antigenic sites per cell is extremely low. Hoffman and Kuhlmann[2482] found that unbound fluorescein- and PE-labeled antibodies, respectively, contributed 4,000 and 1,600 MESF of background fluorescence to the signal in no-wash immunofluorescence analysis, meaning that, if you are set on detecting a few hundred molecules of cell- or bead-bound antibody, you need to wash your cells, and you may need to resort to one or another of the amplification techniques discussed on pp. 343-5.

The simplest amplification technique is, of course, **indirect staining**; Parks et al[674] have discussed alternative methods of indirect staining and their advantages and disadvantages. The most commonly used indirect staining technique uses a fluorescent polyvalent or monoclonal **second antibody** specific for the immunoglobulin type of the primary antibody. After cells are exposed to the primary antibody and washed, they are incubated with the second antibody and washed again before being analyzed. Polyvalent antisera contain antibodies reactive with multiple sites on the primary antibody, and therefore can provide greater amplification than can monoclonal second antibodies; however, unless polyvalent sera are subjected to adsorption and/or affinity chromatography to enrich their content of anti-immunoglobulin antibodies, they are apt to produce high background fluorescence due to binding of labeled irrelevant antibodies to cells not bearing any primary antibody. Monoclonal second antibodies should not produce high background fluorescence; it should therefore be possible, albeit

expensive, to use a mixture of monoclonal antibodies reactive with different sites on the primary antibody to achieve the amplification factor associated with polyclonal antisera and avoid the background fluorescence. I'm not sure whether or not anybody actually does this.

Protein A, which is produced by bacteria of the genus *Staphylococcus*, binds strongly to some IgG antibodies and, when fluorescently labeled, can be used as a second reagent for indirect staining. The principal disadvantage of using protein A results from its lack of reactivity with some primary antibodies and its predictable reactivity with host immunoglobulin already bound to cells.

Use of a **biotin-avidin** reaction[300], in which fluorescently labeled avidin or streptavidin is reacted with biotinylated primary antibody, typically produces brighter staining than would be obtained if the fluorochrome were directly bound to the antibody; this is especially likely to be the case when the fluorochrome is a large molecule such as a phycobiliprotein.

The combination of **hapten-conjugated antibodies** and **fluorescently labeled anti-hapten antibodies**[301] for indirect staining offers advantages in that this approach provides one of the more straightforward ways of indirectly labeling two or more antigens. For example, one could use primary antibodies conjugated with arsanilate and trinitrophenyl radicals, and fluorescein-anti-arsanilate and phycoerythrin-anti-trinitrophenyl secondary antibodies, to obtain amplified signals while minimizing interference between the two staining systems.

Putting antibodies and labels together seems to hold the same fascination for some of us as does playing with Legos and Erector Sets. Along this line, Wognum et al[789] described labeling cells with phycobiliproteins using a tetrameric complex of a mouse monoclonal antibody to a cell surface antigen and a mouse monoclonal antibody to a phycobiliprotein cross-linked with two molecules of a rat monoclonal antibody to mouse immunoglobulin.

Automated Sample Preparation

Several manufacturers offer devices for rapid lysis, fixation, and immunofluorescent staining of whole blood. You add the blood and antibodies; it does the rest, and, minutes later, you run the sample. Automation has been welcomed, especially by clinical users; more versatile preparation apparatus to meet the more varied needs of the research community is at an earlier evolutionary stage.

Fluorescence Measurements: Lurching Toward Quantitation

Calibration and Controls: Round One

Calibration and control procedures for immunofluorescence or ligand binding work differ substantially from those used for an application such as DNA analysis. In DNA analysis, the cells are typically brightly stained, and one calibrates the apparatus with beads and/or (preferably and) nuclei stained with the DNA stain being used, with precision being the primary concern and linearity next on the list. In measurements of weaker fluorescence, the small number of photons coming from stained cells may itself be the limit to precision; the emphasis is on **sensitivity and specificity**. We have to have some idea of how many fluorescent molecules we are detecting and of how much of the fluorescence is due to label specifically bound to the cells of interest.

I have already made the point that most of the argon ion laser source flow cytometers now in use have enough radiometric sensitivity to detect fewer than 1,000 molecules of antibody-bound fluorescein, and that the practical limit of sensitivity is set by the autofluorescence background. Instrumental approaches to discriminating fluorescein immunofluorescence or ligand fluorescence from autofluorescence were discussed on p. 342; in most cases, however, we don't have to carry things quite so far, because we can control for both autofluorescence and nonspecific fluorescence in properly designed **negative control samples**. These, ideally, should be cells that are known not to bear the antigen or ligand-binding entity being measured, to which labeled antibody or ligand has been added.

Isotype controls, containing cells and a labeled "irrelevant" antibody of the same isotype class as the reagent, were once almost universally used, and are still widely used; although some have deemed them unnecessary for many common clinical measurements[2683], others have not[2684]. If you are trying to do quantitative measurements, it's a good idea to have isotype controls, especially if the antigen you are looking for is not present in abundance. When an indirect staining procedure is employed, negative controls should also include samples in which the labeled second reagent is added to cells in the absence of primary antibody.

The ideal **positive control** is composed of cells that are known to bear approximately the same amount of antigen as the cells being analyzed; the objective in instrument setup is to use negative and positive controls to keep both negative and positive cells on scale. This is rarely a problem when you use log amplifiers, but it can be tricky when you're working on a linear scale. Through the years, people have come up with a number of nonfluorescent and not-very-fluorescent test objects to make this stage of the game easier. To answer an old and common question, in this instance, at least, the chicken came first.

Glutaraldehyde-fixed chicken erythrocytes become weakly fluorescent due to the presence of compounds formed by the aldehyde binding to amino groups in proteins, and were used since the early 1970's as controls approximating the fluorescence intensity of cells stained with fluorescent antibodies. **Osmium tetroxide-fixed cells**, which are essentially nonfluorescent, have been used to demonstrate spurious fluorescence signals due to fluorescence induced in color glass filters by scattered light.

As hard as it may be for some of you "younger" readers to believe, back in the 1970's, cell sorter operators in many prestigious immunology labs didn't bother with bead con-

trols at all. By the mid-1980's, Coulter, Flow Cytometry Standards Corp., and Polysciences sold lightly labeled fluorescent beads with fluorescence in the same range as that of antibody-stained cells. Ortho distributed Fluorotrol, a mixture of fixed unstained thymocytes and thymocytes covalently labeled at two different intensities with fluorescein; Coulter supplied a preparation of fluorescent fixed cells under the name ImmunoSure. Flow Cytometry Standards and Ortho gave estimates of the number of fluorescein molecules bound to their materials. Caldwell et al[790] proposed the use of cellular fluorescence intensity itself as a quality control parameter in clinical flow cytometry, describing procedures for utilizing the relatively constant stainability of normal human peripheral lymphocytes to control for alterations in reagents as well as for instrumental sources of variability. However, while microscope photometry[1487] demonstrated consistency and reproducibility in fluorescence measurements of beads from Coulter, Covalent Technology, and Polysciences, these beads incorporated dyes other than fluorescein, and therefore could not readily be used to standardize measurements of fluorescein immunofluorescence.

In the late 1980's and 1990's, manufacturers shifted toward the production of controls bearing known amounts of common antibody labels, or known numbers of antibody binding sites, attempting to simplify the process of quantifying flow cytometric measurements of ligand binding or immunofluorescence. These materials were evaluated by relatively large groups of investigators in the United States, Canada, and Europe, and a series of meetings held in 1997 and 1998 brought most of the interested parties together and generated a special issue of *Cytometry*[2379] in which progress was summarized and critical issues were addressed.

Quantitative Fluorescence Cytometry: Definitions

Henderson et al[2685] defined **quantitative fluorescence cytometry (QFCM)** as "the calibrated measurement of fluorescence intensity from labeled particles...so as to determine the actual number of fluorescent ligands...labeling each particle." A cytometer measures **fluorescence intensity (FI)** of particles, but the measurements are initially reported as unitless histogram channel numbers. **Relative fluorescence intensities (RFIs)** express the relationship between the FIs of two measured particles; they can also be used to convert from histogram channel numbers on a logarithmic scale to linear units. The objective of QFCM as applied to immunofluorescence is determining **antibody-binding capacity (ABC)**, i.e., "the number of antibody molecules bound by a particle when specific binding sites are saturated." To convert from **MESF (Molecules of Equivalent Soluble Fluorochrome) Units** (see pp. 216-7) to ABC, it is necessary to know the labeled antibody's **effective fluorescence/protein ratio, F/P_{eff}**, where F/P_{eff} is defined as "the average number of MESF per functional ligand molecule in a fluorochrome-ligand conjugate." F/P_{eff} is analogous to, but more precise than, the older fluorochrome/protein, or F/P, ratio.

Schwartz et al[2686] established a taxonomy of **fluorescence standards** used for flow cytometry. A **Type 0 standard**, or **certified blank**, is a particle approximately the size of a cell (a lymphocyte is the typical cell in most discussions of QFCM). Certified blanks have a single, very low fluorescence intensity. **Type I standards** are better known as **alignment particles**; they are typically highly labeled and highly uniform in size, producing a very bright fluorescence signal with a low CV. Type Ia standards are smaller than cells (think of the 1-2 μm beads typically used to align an instrument and check its precision); Type Ib standards are cell-sized.

Type II standards, or **reference particles**, are the size of cells, and yield bright fluorescence signals; they need not be uniform in size. Type IIA particles are not spectrally matched to fluorochromes in the sample, and their fluorescence is not environmentally sensitive; Type IIB particles are environmentally sensitive and spectrally matched. Type IIC particles are not themselves labeled, but bind a relatively large amount of labeled antibody, obviously thereby sharing its spectrum and environmental sensitivity.

Type III standards, or **calibration particles**, are cell-sized, and not necessarily uniform in size. They come in sets including particles with several fluorescence intensity levels, ranging from dim to bright. Type IIIA, IIIB, and IIIC standards, respectively, share the properties of Type IIA, IIB, and IIC standards in terms of labeling, spectra and environmental sensitivity, and antibody binding. Type IIIC standards may be used to determine appropriate settings for **fluorescence compensation**; since they provide a known, wide range of fluorescence intensities using the same antibodies as are used to stain cell samples, they may be superior to single antibody-stained cell samples for this purpose.

Calibration Particles for QFCM

Successful quantitative fluorescence cytometry requires a well-aligned instrument; there has been some discussion of measurements of instrument performance on pp. 214-7, and additional coverage will be found in Chapter 10. I will deal here with the particles and methods needed to do QFCM with an apparatus in good working order.

"**Quantum Beads**" bearing known numbers of **Molecule Equivalents of Soluble Fluorochrome (MESF)** of fluorescein and phycoerythrin are Type IIIB standards. They were developed and produced by Flow Cytometry Standards Corporation[1161], which also developed **Quantum Simply Cellular®** (**QSC**) beads, Type IIIC standards capable of binding known numbers of antibody molecules (see pp. 48-9 and 199-201). The surface of a QSC bead is coated with a mixture of polyclonal goat antibodies to the Fc portions of various mouse Ig subtypes; the mechanism of binding of mouse monoclonal antibodies (MoAbs) to QSC beads is therefore quite unlike the mechanism by which the monoclonal antibodies bind to their targets. Fluorescent MoAbs may need to be incubated with QSC beads for a week or more in order for the fluorescence intensity of the beads to

reach a plateau; the beads can then be used for some time thereafter[2687]. QSC beads, used with directly labeled antibodies, provide a standard curve spanning a range from fewer than 10,000 to over 100,000 molecules of bound antibody, allowing direct comparison with labeled cells (Figure 1-20, p. 49). Both Quantum Beads (PE-Cy5- and APC-labeled beads are now offered in addition to the fluorescein- and PE-labeled products) and Quantum Simply Cellular Beads (now available to bind human antibodies as well as mouse antibodies) may be purchased from **Bangs Laboratories**.

In 1985, Poncelet and Carayon[1488] developed a method they called **quantitative indirect immunofluorescence assay (QIFI)** to determine the absolute number of antibody binding sites on lymphoid cells by indirect immunofluorescence flow cytometry after saturation with monoclonal antibody, when binding is likely to be monovalent. The p67 T cell-associated antigen was quantified on cell lines by the binding of radiolabeled antibody. Then, saturating doses of unlabeled antibody followed by fluorescent anti-mouse antibody were applied to the cell lines to build a standard curve relating the mean fluorescence intensity of cells to the mean number of cell-bound primary antibody molecules. This curve (a straight line) was used to assess the absolute number of antibody molecules bound to other lymphoid cells. The technique was shown to be applicable to other IgG antibodies even when they were used in unpurified form.

The QIFI assay as originally described required the use of cell lines and radiolabeled antibodies to establish a standard curve; this limited its appeal, although, as Dux et al[1489] reported, the method allowed quantitative analysis even of antigens expressed at low densities. Poncelet et al[1490,1493] subsequently developed beads, now available as the **QIFIKIT™** (Biocytex, Marseille, France; available from **DakoCytomation**), which can be used as secondary standards, making quantitative indirect immunofluorescence assay generally accessible. The QIFIKIT beads are Type IIIC standards, containing known numbers of mouse monoclonal antibodies bound to an antigen (CD5) on the bead surface. A labeled secondary antibody therefore binds to a QIFIKIT bead by the same mechanism as if it were binding to a mouse monoclonal antibody in or on a cell. Using the QIFI methodology, Poncelet et al[1491] examined normal human lymphocytes, and found that expression of CD4 and CD45 antigens was relatively stable, at 48,000 ± 6,000 and 180,000 ± 17,000 binding sites/cell, respectively, while other antigens were more variable. In HIV-infected individuals, CD4 expression remained at 46,000 sites/cell, even with disease progression[1492]. Bikoue et al[2688], using QIFI technology, reported finding a mean CD4 expression level of 47,000 sites/cell on CD4+ lymphocytes.

BD Biosciences, which was just B-D at the time, took a serious plunge into quantitative immunofluorescence in the mid-1990's, developing the **QuantiBRITE** family of phycoerythrin-labeled beads and antibodies.[2481-2,2689]. QuantiBRITE beads are Type IIIB particles with four intensity levels of phycoerythrin labeling, ranging from approximately 1,500 PE molecules/bead to approximately 130,000 PE molecules/bead. QuantiBRITE kits include one antibody that is a 1:1 PE:IgG conjugate; antibodies to CD20, CD38, CD64, and HLA-DR are available, as well as custom 1:1 PE conjugates. Using the QuantiBRITE system, Davis et al[2481] reported the mean level of expression of CD4 on CD4+ lymphocytes to be 49,000 binding sites/cell; they found binding of their (B-D's) monoclonal antibody to be bivalent, indicating that there should be approximately 98,000 CD4 binding epitopes on the cell surface. Pannu et al, also at B-D, found a mean of 51,000 CD4 sites/CD4+ lymphocyte[2482].

Although it does not seem to be in the current catalogue, B-D made a 1:1 PE:CD4 antibody that was used in the CD4 studies they did and also in several studies of CD38 levels on CD8+ T cells in patients with HIV infection. Janis Giorgi had noted that elevated RFI of CD38 expression was an indicator of poor prognosis in HIV infection[2690], and initially developed a method for quantitation of cell surface CD38 on CD8+ T cells using the fluorescence intensity of CD4 on CD4+ T cells as a standard[2691]. While an initial study by Iyer et al[2689] found that measured CD38 fluorescence intensity values obtained using QuantiBRITE beads as intensity standards were essentially the same as those obtained using CD4 as a standard, a later multicenter study[2692] detected small but significant differences, and recommended that laboratories consistently use one standard or the other.

I will generalize from this; while, in principle, it would be desirable for flow cytometry labs everywhere to get the same numbers from the same samples, experience with quality assurance surveys over the years tells us that a great deal of effort is required simply to insure that critical clinical data such as CD4+ T cell counts remain reliable from lab to lab. Quantitative fluorescence measurements, and immunofluorescence measurements in particular, present more of a challenge. Recall that, while cellular DNA content can be measured with very high precision, the agreed upon definition of DNA aneuploidy is not based on a numerical value for DNA content, but, instead, on the DNA index, i.e., the ratio of DNA contents in tumor and stromal cells in the same sample (p. 317). DNA content measurement is based on analyses of strong signals, measured on linear scales, with minimal contributions to variance from photoelectron statistics, and the biological variance of DNA content, at least in G_0/G_1 phase stromal cells, is extremely small. Fluorescence compensation is not, as a general rule, used for DNA content measurement.

Immunofluorescence measurement is based on analyses of relatively weak signals, with fluorescence intensities typically no more than 1/100 of those typically encountered in DNA content measurement. Measurements are almost always made on logarithmic scales, and often represent the outputs of logarithmic amplifiers, which are likely to deviate substantially from true logarithmic response. Photoelectron statistics may contribute substantially to measurement variance, particularly at low signal levels, and the biological variance of the amounts of most antigens in or on most cell

types is relatively large. Fluorescence compensation is likely to be applied to measurements used for quantitative immunofluorescence flow cytometry, and may, particularly if done in hardware, introduce significant inaccuracy into results. All of this makes it unlikely that a large number of labs will be able to get fluorescence intensity measurements that agree within one or two percent.

It is not unlikely, however, that we will be able to get agreement within ten percent, or even five percent, and that may be good enough. I mentioned above that Quantum Simply Cellular Beads incorporate antibodies to several classes of mouse immunoglobulins; they are therefore unlikely to have exactly the same number of binding sites for antibodies of each class, and might also bind differently to antibodies bearing different labels. This could make it possible to get different values for numbers of binding sites per cell when the beads are used with different antibodies to the same cell surface antigen, although the results would remain consistent for individual antibodies. However, as can be seen from Figure 7-19, the number of CD4 epitopes on CD4+ T lymphocytes determined using antibody-binding beads (R. Vogt, personal communication), about 47,000 sites/cell, is very close to those reported by Poncelet et al[1491-2] and Bikoue et al[2688] using the QIFI method, and by others using QuantiBRITE beads[2481-2].

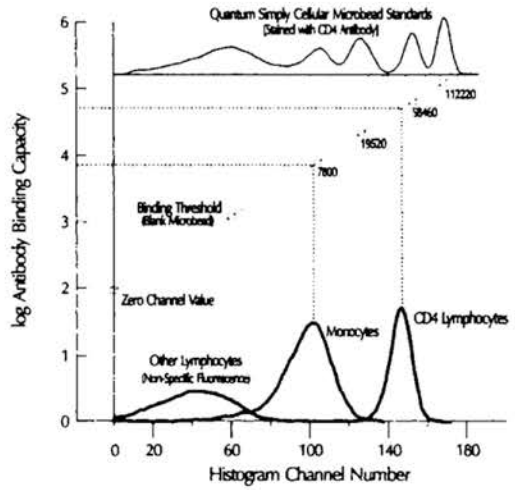


Figure 7-19. Quantitative determination of CD4 epitopes on peripheral blood lymphocytes and monocytes using QSC antibody-binding beads as intensity standards (provided by Robert Vogt, Centers for Disease Control).

In 1989 and 1991, Vogt et al[1505-6] examined intra- and interlaboratory variation in quantitative fluorescence measurement using both fluorescein-labeled beads and Fluorotrol as standards; while reproducibility was very good in a single laboratory, a study of 43 cytometers in 34 laboratories showed CVs in the 20-30% range for measurements of CD4 intensity of lymphocytes, although CVs dropped to 11% when a corrective factor based on Fluorotrol intensity values was applied. Those early results were encouraging. However, Lenkei et al[2693] reported in 1998 that ABCs determined using three different instruments in one laboratory, with different calibrators and with different fluorochrome conjugates of monoclonal antibodies, varied by 20-40 percent. Serke et al[2687], while noting that ABCs determined using the QIFIKIT and QuantiBRITE beads were similar, found large differences in titration curves between cells and QSC beads for a large number of MoAbs to different CD antigens, and differences in binding of fluorescein- and PE-labeled conjugates of the same MoAb to QSC beads. It is likely that the behavior reported for QSC beads relates to the fact that these beads bind antibodies via the Fc portion, while relevant cellular antigens bind the variable regions of the same antibodies.

Defining a Window of Analysis

Relating a measurement channel number to ABCs or MESF units requires the definition of a **Window of Analysis**, according to a procedure specified by Schwartz et al[2694]. While Figure 7-19 illustrates the results of such a procedure, assigning ABC values for anti-CD4 antibody to lymphocytes and monocytes based on fluorescence values obtained from QSC beads to which the antibody had been bound, I will work from Figure 4-60 (p. 216), which deals in MESF units, because it includes more of the "nuts and bolts."

The first item of business is measurement of a Type IIIB or IIIC standard; the former will give a scale calibrated in MESF units, and the latter a scale calibrated in ABCs. The Type IIIB standard used in Figure 4-60 was a set of Quantum beads labeled with fluorescein. The values we need are the histogram channel numbers for the peaks representing the four labeled beads and the blank bead in the bead kit. In this instance, measurements were recorded on a 256-channel (8-bit) logarithmic scale; Schwartz et al recommend converting measurements on higher resolution scales to a 256-channel scale, for reasons that will shortly become apparent.

Working with the known values for MESF units (or ABCs) assigned to the labeled beads by the vendor, we perform a linear regression analysis to get an equation of the form:

$$[\text{channel \#}] = a\,[\log \text{MESF}] + b.$$

In the example shown in Figure 4-60, I happen to have used the log of [MESF/100] which is [log MESF] - 2, so the right regression equation is actually:

$$[\text{channel \#}] = 43 \log \text{MESF} - 101,$$

rather than the equation shown at the top of the figure.

The slope, a, of the regression line defines the **coefficient of response**, indicating, at least in theory, the number of decades of dynamic range available in the measurement. If the 256-channel scale includes exactly four decades, the value of a should be 64 (i.e., 256/4); if it is a three-decade scale, a should be 85.33 (i.e., 256/3). Note that the value of a associated with Figure 4-60 is 43; this suggests that the

measurement scale encompasses 5.9 decades. Don't believe it. What it actually indicates is that the maximum voltage output from the log amp used to process the signals was less than the full-scale input voltage of the analog-to-digital converter used to collect the data. That happened in my home-built Cytomutt; you are unlikely to have the same problem in your instrument, and, if you have what is nominally a four-decade logarithmic scale and end up with a value of a that is less than 59 or more than 69 (these values correspond to 4.3 and 3.7 decade ranges), you should probably have your system looked at. In case you haven't already guessed, we convert everything to a 256-channel scale so we can always use 64 as the ideal value of a for a four-decade logarithmic scale and 85.33 as the ideal value for a three-decade scale.

The points representing the measured values of the beads are pretty close to the regression line in both Figure 7-19 and Figure 4-60. Part of that is due to the fact that we are working on a semi-log scale; the deviations would be larger on a linear scale. However, we really can't rely on our eyeballs to give us a robust measure of how close our measured points are to the calculated line. The regression line is calculated using the method of least squares, meaning that the chosen line minimizes the sum of the squares of the differences between observed and calculated values of channel numbers. While I am on the subject of the regression line, I should point out something else that might otherwise confuse you.

Under most circumstances, we plot the value of a dependent variable in the vertical (Y) direction, and the value of an independent variable in the horizontal (X) direction, and calculate a regression equation: $Y = a X + b$. In the game we're playing here, we have the dependent variable, channel number, plotted on the X axis, and the independent variable, MESF units (or ABCs) plotted on the Y axis, so our regression equation is really: $X = a Y + b$. Just so you know.

In order to determine the extent to which our logarithmic scale deviates from ideal response, we go back to our regression equation, and, taking the actual channel numbers we used to derive it, work backwards to calculate the MESF values that would fall on the line. For example, in Figure 4-60, the data point representing the bead with 70,000 MESF is at channel 108. The equation tells us that

$$108 = 43 \log [\text{MESF}] - 101,$$

so log [MESF] = (209/43), or 4.860, and the value of MESF that would fall on the line at channel 108 is $10^{4.860}$, or 72,521 MESF.

Having calculated all of the MESF (or ABC) values as just described, we find the percentage differences between them and the known MESF (or ABC) values supplied by the bead vendor. For example, 72,521/70,000 = 1.036; the known data value therefore deviates from the value calculated to lie on the regression line by 3.6 percent. Our quality measure is the **average residual percent**, which we get by doing the calculations for all the data points corresponding to known MESF or ABC values (we leave the blank out), adding them up, and dividing by the number of data points. The average residual percent should be less than 3 for MESF and less than 5 for ABCs; the line in Figure 4.60, which looks so close to the data points, actually has an unacceptably high value.

Getting back to the window of analysis, we next obtain the MESF numbers corresponding to channels 0 and 255, in the same fashion as we calculated values on the line to get the average residual percentage. For the data in Figure 4-60, the **zero channel value (ZCV)** is 223 MESF; the **maximum channel value** would be 190,138,370 MESF, but, as I noted a few paragraphs back, that's not a believable value because of a peculiarity of the electronics. The **window of analysis** runs from the zero channel value to the maximum channel value; it is generally suggested that instrument gain settings be adjusted to make the window of analysis run from 100-300 MESF or ABC (an acceptable range for ZCV) to 1,000,000-3,000,000 MESF or ABC.

Note that if the MESF or ABC value for the blank bead is higher than the ZCV, which it will be if some or all of the blank beads appear on scale, the instrument cannot actually detect a signal at the level of the ZCV. It used to be commonplace to define the **detection threshold** as the median value of the peak representing blank beads or unstained cells; it is advisable to use the median rather than the mean because low values tend to pile up in channel 0, which would skew the value of the mean, but not the value of the median. It makes more sense to define a threshold value to include no more than the upper few percent of events in the blank peak; that this number should be no higher than 1000 MESF or ABC, and, if it is that high, the bottom decade of the four-decade range (from 100 to 999 MESF/ABC) is essentially totally occupied by noise, making measurement values in this range meaningless.

The bottom line is that you can't expect to get accurate quantitation of values in peaks that overlap substantially with the blank; that concludes this discussion, which has gotten long enough so that it might be called "Window of Analysis Restaurant." You can get the programs to do the calculations just described from the instrument manufacturers and from third parties (see Software Sources in Chapter 11); just remember that you can't get anything you want from QFCM.

Type IIIA versus Type IIIB and IIIC Standards

While Type IIIB and IIIC standard beads provide accurate calibration of a cytometer's fluorescence intensity scale, and are spectrally matched to the labels used for QFCM, these types of beads, and particularly those bearing phycobiliproteins or antibodies, are somewhat less stable than Type IIIA beads. Type IIIA beads, which are hard-dyed, emit fluorescence in one or more of the same spectral regions in which common labels emit, but are not spectrally matched to the labels and are not environmentally sensitive. The relative fluorescence intensities of a Type IIIA and a

Type IIIB or IIIC bead may be influenced by the excitation wavelength and power used, and the detector bandwidth and sensitivity.

However, once a Type IIIB or Type IIIC bead has been used to establish a window of analysis, running a sample of Type IIIA beads with appropriate emission characteristics, using the same instrument gain settings, can provide a "Rosetta Stone" that will allow conversion between fluorescence values obtained from the Type IIIA beads and MESF or ABC values. As long as no significant changes are made in instrument configuration, setting up the instrument by adjusting gains to keep the Type IIIA beads at or very near the same channels in which they appeared in the original run will restore the window of analysis. Type IIIA beads are available from **Beckman Coulter**, **DakoCytomation**, **Molecular Probes**, **Polysciences**, and **Spherotech**.

Other Aspects of Fluorescence Quantitation

What is "Positive"? What is "Negative"?

Some of the harder-to-answer questions in analysis of immunofluorescence and ligand binding relate to how one defines **what is "negative" and what is "positive,"** this could be considered to be quantitation at the 1-bit level. Even at this level, there aren't always unequivocal answers. Generally speaking, if the cells in your sample have been taken directly from places in which they acquire or lose a particular antigen, you'll see a **continuous distribution** of immunofluorescence intensities going from some positive value all the way down to zero. This type of distribution is typically observed when cells such as stimulated peripheral blood lymphocytes are stained for activation antigens such as the IL-2 and transferrin receptors (CD25 and CD71, respectively), which emerge following exposure of cells to antigens or mitogens.

People typically consider cells from such continuous immunofluorescence distributions as "positive" for the antigen in question when their fluorescence intensity exceeds that of all but a small (usually 1-5%) fraction of a control population. It is not always clear what constitutes an appropriate control population. When stimulated lymphocytes are analyzed, unstimulated lymphocytes are generally used as a control. However, unstimulated lymphocytes may express low levels of activation antigens, and an unstimulated cell population may contain some cells that were activated before being drawn from the donor, which can be expected to bear the antigen being sought. Things get even more complicated when the antigen in question is one that is expressed on other cell types, such as monocytes, which may contaminate a lymphocyte gate.

When cells acquire or lose an antigen outside the compartment in which they are sampled, it is possible to obtain distributions containing clearly positive and clearly negative cells. T lymphocytes, for example, acquire both CD4 and CD8 antigens in the thymus, and generally lose one or the other before leaving the thymus. Thus, the distributions of these antigens in T cells from peripheral blood are typically bimodal, with few, if any, cells lying between the positive and negative peaks, while the distributions of the same antigens in thymocytes are continuous[78] (also see Figure 5-11, p. 241).

Chapter 5 devotes a fair amount of space to discussion of mathematical and statistical techniques that attempt to resolve weakly stained from unstained cells. Sladek and Jacobberger[1497] considered the problem of analysis of data from cells expressing low levels of intracellular antigen and, in a comparison of several methods, found mathematical modeling preferable to histogram subtraction (p. 245).

I am of the opinion that is advisable to rephrase unanswerable questions rather than to torture the data to extract the answers you want; when dealing with continuous distributions, it makes more sense to me to get accurate numerical values for medians and quartiles (p. 235-6) than to assign more arbitrary numbers to represent fractions of "positive" and "negative" cells. However, there may be a way of testing mathematical methods that purport to discriminate dim positives from negatives.

Making Weakly Fluorescent Beads and Cells: Do Try This Trick at Home!

The precision with which you can put only a few hundred molecules of a label or antibody on a cell or bead is limited by Poisson statistics (see the discussion of molecular shot noise on p. 341). You can't buy antibody-binding beads with a binding capacity of 100 antibodies. However, you can buy beads with a binding capacity of 5,000 antibodies, and, by incubating them with a 1:49 mixture of labeled and unlabeled antibodies, get an average of 100 labeled antibodies on each bead[2695]. The standard deviation, thanks to Poisson statistics, will, of course, be at least 10, meaning that the CV of the fluorescence distribution can be no lower than 10 percent, but you may not even be able to detect the fluorescence on your flow cytometer, and, if you can, it's a pretty good bet that photoelectron statistics will contribute more than molecular shot noise to the variance. You can play the same game with cells; a CD4+ T cell has about 50,000 binding sites for CD4 antibody, and, if you stain peripheral blood cells with a 1:99 mixture of labeled and unlabeled antibody, you'll get an average of 500 molecules of label on each CD4+ T cell[2695].

The stoichiometry described above only applies if the labeled and unlabeled antibody have equal shots at binding to cells. An unlabeled IgG antibody has a molecular weight of about 160,000, and, if you put 3 molecules of fluorescein on it, the molecular weight goes up to about 161,200. The difference in molecular weights between labeled and unlabeled antibody won't affect the binding characteristics much, so the ratio of labeled to unlabeled antibodies on beads, or cells, will be the same as the ratio in solution.

An IgG antibody labeled with 1 molecule of phycoerythrin has a molecular weight of about 400,000, and the much lighter unlabeled antibody will have an advantage in

binding to beads or cells, with the result that the ratio of labeled to unlabeled bead- or cell-bound antibody will be lower than the ratio of labeled to unlabeled antibody in solution. You can fix this, and get nearly the same ratios for bead- or cell-bound antibodies as you start with in solution, by using phycoerythrin that has been bleached by exposure to strong light (an hour or two of sunlight will do) as the "unlabeled" antibody when you work with a phycoerythrin-labeled antibody.

A slight variation on this theme should provide a control for testing various computational methods of discriminating weakly labeled and unlabeled cells. It requires an instrument with 488 nm and red (633 or 635 nm) excitation beams. Stain beads or cells with a near-normal concentration of APC-labeled antibody to which you add a very small amount of fluorescein-labeled antibody. You should then be readily able to discriminate stained and unstained beads or cells on the basis of APC fluorescence, and to titrate the fluorescein fluorescence down so you have difficulty discriminating stained and unstained objects. If the computational method gives you the same numbers you get from gating and counting the APC-stained and unstained beads or cells, it's a winner. You don't need to know the exact stoichiometry of stained and unstained cells here, so it doesn't matter that conjugated APC adds at least 33,000 to the molecular weight of an antibody. You also don't have to worry about energy transfer from the fluorescein to the APC, because you can raise the ratio of fluorescein- and APC-labeled antibodies to get a weak, but detectable fluorescein signal.

Correlating Cytometry and Biochemistry: Studies of Antibody Binding Chemistry

Binding of antibodies, or any other ligands, to chemical structures on or in cells follows the rules of chemical kinetics and thermodynamics. Most of the antibodies used for cytometry are bivalent, but whether binding to their targets is bivalent, monovalent, or mixed depends on a number of factors, with the affinity or avidity of the antibody and the abundance and availability of the targets all exerting strong influences.

We generally attempt to saturate binding sites when performing immunofluorescent staining procedures, particularly when the objective is quantitative fluorescence measurement. However, Eric Martz pointed out a long time ago (personal communication, 1993) that if antibody and cell concentrations and incubation time are arranged so that binding is limited by the diffusion rate of antibody, rather than by the amount of antibody present in the sample, the fluorescence intensities of cells and beads should remain proportional to the number of binding sites on each, even at subsaturating antibody concentrations. In theory, we should be able to get away with using less antibody. In practice, most people will continue to titrate antibodies, adding more until the cells of interest don't get any brighter, and only take comfort from the theory when they find, as we all do from time to time, that an antibody straight out of the bottle is too dilute to saturate binding sites.

Flow cytometry can be, and has been, used to determine the binding parameters of antibodies. Bardsley et al[1496] developed a model and computer program for determining association constants of antibodies binding to cell surface antigens, which takes into account variations in antibody concentration as well as variations in antigen expression. Benedict et al[2697] described a flow cytometric assay and its use to determine the binding affinity of an anti-CD34 scFv antibody. Siiman and Burshteyn[2698] used competitive binding to determine binding constants for a variety of labeled and unlabeled MoAbs to cell surface markers, and to enumerate the numbers of target binding sites present on cells. A provocative paper by Lamvik et al[2699] reports that binding of unlabeled secondary antibody may increase binding of primary monoclonal antibodies (labeled in this instance, to allow the determination to be done) to cell surface antigens on fixed, permeabilized cells. Both equilibrium and kinetic measurements (about which more will be said in a later section) have been used in flow cytometric studies of antibody binding[2700].

Quantitative measurement of cellular constituents using immunofluorescence flow cytometry benefits from comparison and correlation of results obtained using flow cytometry and other analytical techniques. Sarin and Saxena[1500], for example, established a correlation between flow cytometric measurements of histocompatibility antigens and measurements by ELISA.

Correlating Cytometry and Biochemistry: Intracellular Antigen Measurements

Establishing correlations between flow cytometry results and results of more classical methods is of particular importance in analysis of intracellular antigens. The usual procedures for analysis of intracellular antigens require fixation and permeabilization of cells, in order to achieve the conflicting ends of allowing molecules as big as labeled antibodies to get into cells and retaining molecular targets which may be smaller in size.

Jacobberger and his colleagues[2701-2] have studied quantitative aspects of antibody binding to cellular constituents, with particular emphasis on intracellular antigens. A comprehensive review[2701] discusses basic chemistry and immunochemistry, fixation and sample preparation, and both equilibrium and kinetic measurement methods for flow cytometric analysis. Another paper[2702] describes correlation of quantitative measurements of SV40 T antigen with quantitative Western blots.

There are at least a few tricks that allow flow cytometry to be used to demonstrate and quantitate intracellular antigens in living cells, allowing cells bearing desired antigens in appropriate amounts to be sorted for culture and further analysis.

Detection of cytokine production by T cells (reviewed by Maino and Picker[2703]) has become a favored means of identi-

fying cells participating in an immune reaction against a defined antigen. The usual procedure involves incubation of cells with the antigen, addition of brefeldin A to prevent cytokines from being secreted, staining of relevant surface antigens, fixation and permeabilization, and staining of intracellular cytokines. An alternative technology developed by Manz et al[2704] creates a **cell-surface affinity matrix** on the cell surface, first biotinylating cell surface proteins, and subsequently attaching streptavidin-tagged anti-cytokine antibodies to the biotin molecules. Cytokine secretion is not inhibited; instead, cytokines are secreted, and captured by the antibodies attached to the cell surface, on which they can be demonstrated following addition of fluorescent-labeled anti-cytokine antibodies. Pittet et al[2705] combined an anti-cytokine cell-surface affinity matrix with tetramer staining (introduced on pp. 47-8); this permits sorting of live, antigen-specific, cytokine-secreting cells.

Berglund and Starkey[1234] used **electroporation** to permit the introduction of labeled anti-oncogene antibodies into cells, at least some of which survived the procedure. Morris et al[2706] may have developed a kinder, gentler method; they made a 21-residue peptide carrier, pep-1, incorporating sequences known to be able to promote transport of proteins across membranes, and succeeded in introducing a number of proteins, including two different fluoresceinated antibodies, into living mammalian cells following the formation of complexes between the proteins and pep-1. The antibodies localized correctly to intracellular actin and to a lysosome-associated membrane protein. It remains to be seen whether carrier peptides can make it possible to do quantitative or semiquantitative immunofluorescence analyses of intracellular antigens in living cells.

Analyzing Immunofluorescence Data

We now turn our attention to what we can and can't learn from immunofluorescence measurements. Some aspects of this have come up in Chapter 5, others will come up in Chapter 10.

Some quantitative questions may actually be easier to answer than some qualitative ones. Like most histograms obtained from flow cytometry, immunofluorescence histograms typically contain data from 10,000 or more cells, and thus represent huge sample sizes when compared to most of the data sets with which statisticians normally have to contend. As a result, you can pretty much assume that a visible difference between two smoothed histograms is statistically significant; you cannot, however, assume that the significant difference is due to a significant biological difference between the cell populations from which the distributions were obtained, because instrumental variation and differences in reagents and sample preparation can also produce statistically significant differences. Intensity calibration makes histogram comparison easier than it might otherwise be.

If you are trying to demonstrate **differences between populations**, it is often useful to construct "envelopes" in which the area between the high and low values for each channel, taken from two or three replicates of control and experimental histograms done at different times, is shaded. Gaps between the control and experimental envelopes provide strong support for the hypothesis that control and experimental populations differ. Alternative methods for averaging and comparing histograms have been described by Marti et al[798] and Traill et al[799].

Mathematical models, while not applied to immunofluorescence to nearly the extent to which they have been used for DNA analysis, had been used by some authors. Takase et al[1494] used nonlinear least squares curve fitting to calculate the mean and standard deviation of immunofluorescence distributions, and Shabtai et al[1495] used a Euclidean distance vector to describe fluorescence intensity changes in two-color measurements.

When the previous edition of this book appeared, there were a few papers in the literature[1501-4] reporting fluorescence intensity measurements in terms of fluorescein MESF units. Others had used simpler, more empirical approaches; Terstappen et al[1498] represented amounts of surface antigens on myeloid cells by their mean fluorescence intensities in comparison with the background fluorescence of each cell type. Christopoulos et al[1499] used the same measure of intensity to quantify platelet-bound immunoglobulin.

Estimating Antigen or Receptor Surface Density

Surface density of antigenic or receptor sites can be estimated by dividing the number of bound ligand molecules by the cell surface area. An approximation to an absolute value for cell surface area can be obtained from an electronic measurement of cell volume by analog or digital computation of the 2/3 power of volume[311]. Use of this technique is obviously feasible only with flow cytometers capable of making volume measurements. However, since both cell surface area and the "cell size" measurement obtained from forward light scattering are (allegedly) approximately proportional to the square of cell diameter, the ratio of immunofluorescence and scatter signals can serve acceptably as a parameter representative of antigen surface density for making comparisons.

If we actually know how many antigenic or receptor sites there are on the cell, you might think we should aim for surface density in sites per square micrometer. However, while flow cytometry, with appropriate calibrators, can fairly readily give us cell volumes in femtoliters, we'd have to make some pretty rash assumptions to get a surface area from that. It's acceptable to assume the cell is roughly spherical, and calculate an approximate radius from the volume. In reality, we know that no cell surface is a completely smooth sphere, and that real cell surfaces differ in their degree of apparent roughness, as seen, for example, by scanning electron microscopy; this means that cells of roughly the same size can have substantially different true surface areas. Assuming cells are smooth and spherical so you can report "exact" values of surface antigen density doesn't make much sense.

Quantitative Fluorescence: Problems and Prospects

Shortly before the publication of the October 1998 *Cytometry* special issue on Quantitative Fluorescence Cytometry[2579], Nicholson and Stetler-Stevenson opined: "Although quantitative flow cytometry is currently drawing a lot of attention, much of the attention is focused in the wrong area. The greatest value that quantitation has provided to the clinical laboratory has been on focusing attention to properly setting up and calibrating flow cytometers. Being able to consistently perform semiquantitative measurements has the most benefit in most clinical settings." They noted that quantitation of CD38 expression on CD8+ T cells in HIV infection[2689-92] represented the only case in which measurements of antibody binding capacity might be important for clinical management.

I won't argue. I am also of the opinion that paying attention to quantitative aspects of immunofluorescence measurement is at least as important for quality assurance as for any other clinical purpose. However, had quantitative fluorescence cytometry not developed to the level at which it was by 1998, it would not have been possible to establish the clinical relevance of CD38 measurements, and we can only find additional instances in which quantitation may be necessary by making more quantitative measurements.

At the present time, we have more problems than solutions. The number of antibody binding sites on cells is known to be influenced by preparative methods, including lysis and fixation, and we will need to standardize the preparative techniques in order to permit widespread application of any quantitative fluorescence measurement procedure. Antibody affinity and binding valency can exert profound effects on ABC determinations, and antibody labels may influence these characteristics. Ideally, there should be little or no spectral overlap between the antibody label used for quantitative measurements and those used to define the gates in which the cells of interest lie, because spectral crosstalk into the channel used for quantitation will increase the background noise level and reduce measurement sensitivity. The label best established for quantitation is probably phycoerythrin; however, the temptation to attempt to do quantitative measurements using phycoerythrin, with a 488 nm laser also exciting antibodies labeled with fluorescein, PE-Texas red, PE-Cy5, PE-Cy5.5, and PE-Cy7, must be strongly resisted. We can, after all, now use three red-excited labels (APC, APC-Cy5.5, and APC-Cy7) for gating, minimizing interference with quantitative measurements using PE. And we still worry about calibrating the calibrators and standardizing the standards.

As I mentioned on pp. 294-5, there are some heavy hitters on the case. The National Institute of Standards and Technology (NIST) is getting into the business of standardizing fluorescent materials[2582-4], and will be producing and certifying standard solutions and particles, and an NCCLS (NCCLS used to be the National Committee for Clinical Laboratory Standards; having gone international, it is now simply NCCLS, usually pronounced "nickels") subcommittee is developing a guideline for Fluorescence Calibration and Quantitative Measurements of Fluorescence Intensity, which should help implement standardization not only in cytometry, but in other fields in which fluorescence quantitation is becoming of interest, e.g., microarray analysis. A brief prospectus was provided in 2000 by three key players in the NIST and NCCLS projects[2581].

7.8 NUCLEIC ACID SEQUENCE DETECTION

Figure 7-20 provides a relatively dramatic illustration of the combination of molecular biologic techniques with flow cytometry for detection of specific nucleic acid sequences. The figure shows a small population of CD4-positive lymphocytes containing HIV-1 viral nucleic acid, as detected by Patterson et al[1403] using fluorescein-labeled oligonucleotide probes after *in situ* PCR amplification.

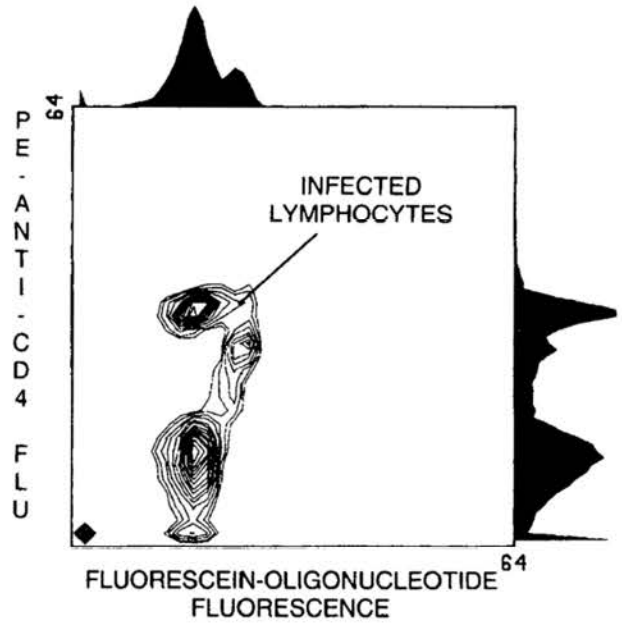


Figure 7-20. Two-dimensional contour plot of log PE-anti-CD4 fluorescence vs. log of fluorescence of fluorescein-labeled oligonucleotide probes specific for HIV-1 nucleic acids, showing oligonucleotide probe fluorescence in some of the CD4-positive lymphocytes after in situ PCR of viral sequences[1403]. The figure was provided by Charles Goolsby of Northwestern University.

Detection of sequences by flow cytometry after **Fluorescent *In Situ* Hybridization (FISH)** was described by Trask et al[902] in 1985. Between then and the time the previous edition of this book appeared, a variety of methods to attach fluorescent labels to probes were tried and described. Bauman, Bayer, and van Dekken[1404-5] detected poly-(A)+ RNA using a poly-biotin-d(U)-tailed oligo-d(T) probe and streptavidin-fluorescein; nuclei were stained with DAPI to provide a trigger signal. Amann et al[1406] used detection of

specific ribosomal RNA sequences, present at the level of several thousand copies per cell, for bacterial identification, initially employing probes end-labeled with a single molecule of tetramethylrhodamine. Some signal amplification was obtained by substituting digoxigenin-labeled probes, which were rendered fluorescent by attaching fluoresceinated anti-digoxigenin antibodies[1407]. However, this made it somewhat more difficult to get probe and label through bacterial cell walls. Oligonucleotide probes end-labeled with single molecules of fluorescein and tetramethylrhodamine could be used for two-color analysis; the addition of more molecules of label per probe molecule increased signal, but decreased signal-to-noise due to increased nonspecific binding[1408].

Timm and Stewart[1409] discussed procedural aspects of "Fluorescent *In Situ* Hybridization En Suspension" (FISHES), including preparation of digoxigenin-labeled probes and staining and washing steps; they found that washing with a solution containing formamide and bovine serum albumin reduced cell loss. Lalli et al[1410] also discussed optimization of the preparative procedures. Bardin et al[1411] noted that biotin could be attached to the 5′ as well as to the 3′ end of oligonucleotide probes, offering some potential for increasing signal when fluorescent streptavidin is attached.

Bains et al[1412] described PRINS (Primed IN Situ labeling) to quantify poly-A and histone messenger RNA in cells. Poly(A) mRNA was labeled by incorporation of fluoresceinated nucleotides into cDNA chains generated by reverse transcription from the site of oligo(dT) primer-specific annealing on the poly(A) template.

While the sea of literature threatened to become filled with TOADFISH (Totally Outlandish Acronyms Describing Fluorescent *In Situ* Hybridization), this didn't stopped a lot of people for going after the big game, which certainly includes detection of low copy numbers of viral gene sequences in infected cells. When the previous issue of this book appeared, this required amplification of those sequences by *in situ* PCR[2708-9], as was done in the cells used to generate Figure 7-20. Surpassing even this feat of genetic engineering, Embleton et al[1413] reported that it was possible to separately produce and assemble amplified heavy and light chain DNA by PCR and subsequent primer additions in cells in suspension. Long et al[1414] examined *in situ* PCR on slides and in cell suspensions and found that results were comparable; nonspecific results were less frequent with cell suspensions.

Mirsky et al[1415], using conventional methods of detecting DNA after sorting single cells into microtiter plates, reported that the operational sensitivity of *in situ* PCR for detection of bovine leukemia virus was 90% when testing single infected cells. In addition, they were able to reliably amplify DNA from a single infected cell among as many as 10^5 uninfected cells and established that the sensitivity for detecting a single infected cell among 20, 100, or 1000 uninfected cells was at least 90%. This work put the concept of detecting small numbers of virus-infected cells directly by fluorescence flow cytometry following *in situ* PCR on a firm footing.

In a more mundane but possibly more practical application of genetic sequence detection by flow cytometry, van Dekken et al[1416] used labeled chromosomal probes to discriminate bladder cancer cells with monosomy-9 from stromal cells, enabling determination of the tumor cell kinetics by gated analysis of BrUdR incorporation.

If immunofluorescent staining has pretty much been reduced to cookbook procedures, nucleic acid probe staining may be said to still require both technical skill and FISHerman's luck (FISHerperson's luck, if you insist). Andreeff and Pinkel have edited a big book on basic principles and clinical applications of FISH[2710].

In recent years, it has become possible to use tyramide signal amplification (p. 344) in FISH; new probe labeling techniques[2671,2711-2] can reduce or eliminate nonspecific background fluorescence, making it possible to detect a few dozen copies of a sequence in cells.

Peptide Nucleic Acid (PNA) Probes

A new class of probes, **peptide nucleic acid (PNA)** probes[2713-4], appear to offer some significant advantages for cytometry, but clearly have a potential range of applications that extends far beyond this field.

A PNA molecule is comprised of the same purine and pyrimidine bases as are found in nucleic acids, attached to a pseudopeptide with amide or peptide bonds forming the backbone of the polymer, rather than sugars linked by phosphates. PNA molecules are insensitive to digestion by proteases and nucleases, and bind to DNA and RNA; a strand of PNA complementary to a region of double helical DNA will disrupt the double helix and bind to it, displacing its complementary strand. PNAs have been used in experiments on antisense therapy; some short sequences can enter intact cells, while others can be transported inside cells by carrier peptides. PNA probes tagged with thiazole orange or a homologue are nonfluorescent, but become fluorescent on hybridization with nucleic acid; PNA probes incorporating a fluorescent label and a quencher also become fluorescent on hybridization. Less stringent conditions are required for PNA probe hybridization than for hybridization with oligonucleotide probes.

I can leap into fantasy and envision using PNA probes to do FISH on living cells, which can subsequently be sorted. In the real world, PNA probes have been applied to identification of microorganisms based on rRNA sequences[2714] and to determination of telomere length[2715-6], both of which will be discussed in Chapter 10.

7.9 PROBES FOR VARIOUS CELL CONSTITUENTS

Surface Sugars (Lectin Binding Sites)

The specificity of binding of various **lectins** to different carbohydrate moieties of cell surface and other glycoproteins was appreciated well before the first fluorescence measurements were made in a flow cytometer. In the early 1970's, as flow cytometry began to become popular, the use of lectins

as reagents for characterization of cell surfaces was logical and attractive; before the advent of monoclonal antibodies, no immune reagents were nearly as well defined, chemically or functionally, as were lectins. In addition, the mitogenic activity of lectins such as **concanavalin A (con A)**, **phytohemagglutinin (PHA)**, and **pokeweed mitogen (PWM)**, and reports of different patterns of lectin binding in normal and transformed cells, suggested that quantitative analysis of lectin binding to cells might provide valuable information in research and clinical laboratories.

The first reported flow cytometric analyses of lectin binding to cells were done by Kraemer et al[310-2] at Los Alamos, who examined binding of fluorescein-con A conjugates. In the initial study, fluorescence was the only cellular parameter measured, and it was difficult to resolve the weak fluorescence of fluorescein-con A above background noise. Later work was done with a multiparameter instrument, in which cell volume measurements were used to gate fluorescence measurements, improving discrimination of cell-associated fluorescence from noise. Analog electronics were used to compute the 2/3 power of cell volume, a quantity proportional to cell surface area, and to derive a measure of the cell surface density of lectin binding sites from the ratio of the fluorescence signal to the derived surface area.

Another relatively early paper[313], published by Bohn in 1976, described the use of a dye exclusion technique with two-color fluorescence measurement to discriminate between intact cells, to the surfaces of which lectin binding was measured, and cells with damaged membranes. In this paper, Bohn also pointed out that the capability of flow cytometry to precisely measure cell-associated ligand in cell suspensions from which unbound ligand had not been removed by washing could be useful in the analysis of many types of **ligand-receptor interactions**. Both Bohn's work and that of Kraemer et al were considerably more sophisticated than most of what was in print when they were published, and were probably not widely appreciated on that account.

While the present emphasis on analysis of cell surface structures with antibodies has decreased interest in the use of labeled lectins, the latter can still be used effectively as aids in enumeration and separation of cell subpopulations. Measurements of lectin binding have typically been used with measurements of other parameters for this purpose. For example, Nicola et al[179] used measurements of forward light scattering and of the fluorescence of fluorescein-PWM and rhodamine-labeled antineutrophil serum to separate hematopoietic progenitor cells from murine fetal liver, while Bauman et al[1417] purified murine stem cells and committed progenitors using a combination of wheat germ agglutinin and monoclonal antibodies. The anti-H lectin identifies nucleated and non-nucleated erythrocytes[1418]; other lectins have been described as markers for eosinophils[1419], and so on.

Lectin binding has also been applied to flow cytometric analysis of parasites[1420-1] and to subcellular organelles; Guasch, Guerri, and O'Connor[1422,2717] have examined lectin binding to Golgi fractions from rat liver. Others have examined changes in lectin binding before and after physiologic changes induced in cells, e.g., by cell-cell and cell-cytokine interactions[1423-6].

Lectin binding patterns change during immortalization and neoplastic transformation of cell lines[1427] and, conversely, during induced differentiation of tumor cells[1428]; heterogeneity of tumor cells[1429] may reflect different propensities of different cells for metastasis, which has been shown to be related to expression of cell surface glycoproteins that are themselves lectins[1430-2].

Because lectins are polyvalent, they have a somewhat greater tendency to agglutinate cells than do antibodies. McCoy et al [784] describe a method for blocking all but one active site of a fluoresceinated lectin to produce a monovalent label. The extent to which this technique may be generalized is not completely clear; however, monovalent, monomeric derivatives of lectins may be produced by other chemical means[1433]. Some formal chemistry can be done with lectins on cell surfaces; sugar competition assays can be used to define binding affinities[1434] and sites[1435].

Reagents for anyone interested in future work along these lines, in the form of fluorescent conjugates of a good selection of lectins, are available from a number of sources (e.g., Molecular Probes and Polysciences). I haven't found recent general review on the use of lectins in flow cytometry; there is a 1987 paper by McCoy[1436].

Analysis of Total Cellular Carbohydrate Content

While lectins bind specifically to particular carbohydrates, they cannot effectively be used as reagents for determination of total cellular carbohydrate content. Instead, Duijndam and van Duijn[1437] employed a technique well known in classical histochemistry, the **periodic acid-Schiff reaction**, to determine the carbohydrate content of erythrocytes. Periodic acid oxidation was followed by pararosaniline staining, providing a strong enough fluorescence signal to allow measurement by flow cytometry. Dimethylsuberimidate fixation was used and yielded low autofluorescence and only faint staining of unoxidized cells. This methodology should be generally applicable.

Specific Detection of Cellulose

Taylor et al[2718] have shown that a cellulase and an isolated bacterial cellulose binding domain (CBD) conjugated to fluorescent dyes can be used for specific detection of cellulose by flow cytometry or laser scanning cytometry.

A Probe for Cell Surface Aldehydes

In conjunction with studies on the mechanism of lymphocyte mitogenesis by compounds that produce oxidation at sites in the cell membrane, Roffman and Wilchek[906] synthesized a **fluorescein diaminobutyryl hydrazide** that could be used for quantitative determination of **aldehydes** in the membrane. They found, incidentally, that the mitogenic activity of different oxidizing agents was not correlated with the degree of aldehyde formation.

Probes for Lipids and Cholesterol

Nile Red

Greenspan et al[802,3] described **Nile red**, an oxazone prepared from the oxazine dye Nile blue, as a fluorescent stain for **neutral lipids** in cells and tissues. The dye fluoresces yellow when dissolved in neutral lipids, and red when in more polar lipids, e.g., phospholipids; its fluorescence is quenched in aqueous solution. Nile red can be excited at 488 nm; fluorescence of the lipid-bound dye is typically measured at about 550 nm. Dive et al[1458] used Fourier transform flow cytometry[1140] to detect emission spectral changes in Nile red in differentiating ovarian granulosa cells; the fluorescence component related to lipid droplets increased with maturation. While the Fourier transform technique is not accessible to most investigators, Smyth and Wharton[1459], examining differentiating adipocytes, found that the ratio of gold to red fluorescence from Nile red also reflects the accumulation of cytoplasmic lipid droplets, and can be used to define cells as being differentiated or undifferentiated. Brown, Sullivan, and Greenspan[1440] reported that flow cytometry of Nile red fluorescence could distinguish macrophages bearing orange-colored phospholipid inclusions from control alveolar macrophages, in which yellow-gold fluorescence from the neutral lipid droplets predominates. Nile red has lately been used to quantitate polyhydroxyalkanoic acids in bacteria[2719,20].

Filipin

Muller et al[804] reported in 1984 that the polyene antibiotic **filipin**, which was known to form fluorescent complexes with **membrane-associated cholesterol**, could be used for quantitative flow cytometric analysis of unesterified cholesterol in formaldehyde-fixed cells. Kruth et al[805] used filipin staining to detect and isolate cholesteryl ester-containing "foam" cells from atherosclerotic aortas by flow cytometry. Filipin is excited in the UV; fluorescence is measured between 510 and 540 nm. Hassall[1441] used a combination of cyanine dye-labeled low-density lipoprotein (LDL) to measure lipoprotein uptake, Nile Red to measure cholesteryl ester accumulation, and filipin to study free cholesterol homeostasis in a multiparameter analysis of human foam cell-forming macrophages. Hassall and Graham[2721] found that changes in filipin fluorescence correlated with changes in cholesterol biosynthesis in these cells.

Lipid Droplet Detection Using Scatter Signals

Suzuki et al[1442], studying triacylglycerol accumulation in cytoplasmic lipid droplets in the U937 macrophage-derived cell line, found that the extent of lipid droplet formation in each cell could be assessed in the absence of any staining by changes in the intensity of 90° light scatter; using the scatter measurement might save you a fluorescence channel if you're running short.

Probes for Cytoskeletal Organization / Actins

Wallace et al[622] used the fluorescent probe **NBD-phallacidin** for flow cytometric analysis of changes in **cytoskeletal organization** (in particular, changes in actin conformation) in blood neutrophil granulocytes following exposure to chemotactic peptides. This material is available from Molecular Probes. Phalloidin binds to F-actin; deoxyribonuclease I (DNAse I) binds to G-actin, and Haugland et al[1475] have shown that the two types of actin can be analyzed simultaneously in cells using **BODIPY-phalloidin** and **fluorescein-DNAse I**, both available from Molecular Probes.

7.10 TIME AS A PARAMETER: KINETIC MEASUREMENTS

Flow cytometry can be used for quantitative analyses of the degree to which almost any fluorescent substance associates with cells, whether the material is bound to the cell surface or is taken into the cell; it is often useful to examine the processes of binding, uptake, and efflux over time.

When the material is taken up over a period of hours, it is customary and sensible to examine cell samples at intervals. When uptake occurs over a period of seconds to minutes, the speed and throughput of flow cytometry can be utilized to best advantage if a single sample is analyzed continuously, and the time at which each cell arrived at the observation point is recorded with the values of scatter, fluorescence, and other more conventional cytometric parameters. I have alluded to the existence of **kinetic measurements** on p. 2, and, on pp. 177-8, mentioned the fact that the more sophisticated varieties may require nonstandard hardware.

I will stress here, as I did on p. 2, that, while kinetic measurements in flow cytometry are based upon measurements of single cells, the temporal patterns obtained represent data from successive cells in the same sample. We measure cell 1 at time 1, cell 2 at time 2, ... , cell n at time n, and make the assumption that cell 1, had we been able to bring it back into the flow cytometer and remeasure it at time n, would have looked more or less the same as cell n did. If that assumption holds, we can expect a flow cytometric kinetic measurement to provide more or less the same results as might be obtained from multiple sequential microphotometric measurements of the same cell. If not, we will have to resort to a technique such as scanning laser cytometry, which will allow us to observe the same cell repeatedly over time, to get the kinetic data we need.

Measurements of fluorescence versus time can, as should be obvious, be used to analyze the kinetics of efflux or dissociation of fluorescent material from cells[347], as well as the kinetics of binding and/or uptake. The time measurement technique is also applicable to studies of enzyme kinetics, in which development of color or fluorescence in cells as a result of enzyme action on chromogenic[348] or fluorogenic substrates is followed over time, and is essential in analyses of other rapidly changing functional parameters such as membrane potential and cytoplasmic calcium ion concentration.

The first publication on time measurement in flow cytometry was by Martin and Swartzendruber; it appeared in 1980[346].

Figure 7-21 is a typical display of data from a timed measurement; it shows changes in cytoplasmic [Ca^{++}] over a period of about 200 seconds following stimulation of T-lymphoblastoid cells with a mitogenic monoclonal antibody.

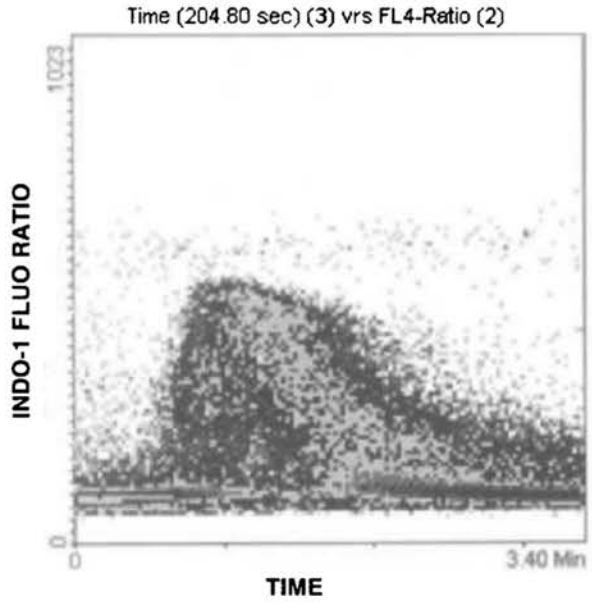


Figure 7-21. Plot of cytoplasmic [Ca^{++}], as indicated by indo-1 fluorescence ratio (pp. 47, 403-4), versus time in T lymphoblastoid cells following stimulation with OKT3 antibody to the T cell receptor. Data were provided by Keith Kelley (Miles Research Center).

Note that while most of the cells represented in Figure 7-21 appear to change their cytoplasmic calcium concentration in response to the applied stimulus, there is considerable heterogeneity in the degree of response. This pattern is not uncommon in kinetic measurements of functional parameters; data from fluorescent ligand binding to ostensibly homogeneous cell populations usually produces nicer looking curves.

The time measurement implemented by Martin and Swartzendruber[346] relied on a linear ramp generator to produce a voltage that increased as a linear function of time; this voltage was used in lieu of a detector signal as an input parameter for the data analysis system. It is now more common to use digital timers, the outputs of which can be read by the data acquisition hardware, for time measurements. Kachel et al[811] and Beumer et al[812] have described digital timing circuitry for collecting kinetic data; such circuits, some of which make use of the real-time clock incorporated in the computer system[813], are now available in most commercial flow cytometers.

A crude estimate of cells' arrival times in systems that lack a time parameter can be obtained by simply plotting events in the order in which they occurred; this makes the erroneous assumption that cells arrive at evenly spaced intervals, and Lindmo and Fundingsrud[351] showed that this was very much not the case (see pp. 144-5). Nonetheless, if your instrument hasn't got a time parameter, erroneous assumptions may be better than nothing at all. Some third-party data acquisition systems and add-ons for older instruments add time measurement capability.

Sample Handling for Kinetic Measurements

When the events being monitored in flow cytometric kinetic studies occur over very short time periods, it is desirable to be able to minimize the delay between the start of sample incubation and the passage of the first cells through the observation point(s) of the flow cytometer. Sample handling systems to permit rapid kinetic analysis by flow cytometry have evolved over the years[349,50,810,1532,4]. A commercial version of Kelley's system[1533], available from **Cytek Development**, provides mixing and temperature regulation in a unit that mounts close to the nozzle holder for reduced transit times, and allows stimuli to be added to a sample with observation of results within 1 second. An electronic circuit activated at the time of injection generates a data time stamp for direct correlation of injection and cellular response.

Dunne[1534] emphasized the importance of **time window analysis**, also called **fixed-time flow cytometry**, in which each cell reaches the observation point in a controllable, fixed time after a stimulus is added, in studies of rapid cellular responses; this can be achieved by allowing the cell sample and the solution containing the stimulus to mix in a T-junction upstream from the nozzle; the time between stimulation and observation is varied by adjusting the pressure and the length of tubing between the mixing junction and the observation point. Tárnok[2722-3] describes a relatively simple mixing device for time window analysis that he has used to study calcium transients in neuronal cells, and to sort cells with unusual patterns of calcium response. The constant pressure maintained by his mixing arrangement is essential for neuronal cells, because they undergo calcium shifts in response to pressure changes that may occur in syringe pump-driven mixing apparatus.

John Nolan, Larry Sklar, and their collaborators at Los Alamos and the University of New Mexico have been involved for some years in efforts to increase both the time resolution of kinetic measurements and the sample throughput of flow cytometers. They have optimized nozzle design parameters for stream-in-air instruments to stabilize flow in the shortest possible time (Graves et al[2454], p. 170), and refined mixing apparatus hardware and control software to reduce the interval between mixing and analysis from 300 ms to 55 ms[2460-1]. They have also developed a dynamic temperature regulation unit that provides more accurate maintenance of sample temperature than was previously possible, and also permits analysis of cellular and chemical responses to rapid temperature changes[2427].

With a view toward improving sample throughput as well as refining kinetic measurements, Durack et al[1535] devel-

oped the technique of **time interval gating** to allow data from kinetic experiments involving multiple samples, each of which is measured briefly (i.e., for a few seconds) at intervals of several minutes, to be collected in a single large list mode data file, facilitating subsequent analysis of the data.

The technology of **flow injection analysis**, a rapid analytical chemical method pioneered by Ruzicka [147], has been applied by him and his colleagues to cytometry[1148-9,1536]. Their methodology uses computer-controlled systems of pumps and valves to handle samples, and software to facilitate non-equilibrium analysis.

Sklar et al[2462-4] have also developed systems for **high throughput flow cytometry**; the most advanced[2463] introduces 1-3 µL "slugs" of sample, interspersed with air bubbles to reduce sample carryover, into the cytometer at a rate of one sample every 1.3 seconds. The MoSkeeto™ sampler now offered by **DakoCytomation** appears to be a commercial version.

Time as a Quality Control Parameter

Watson[8,9] cleverly extended the earlier observations of cell arrival times[351] to permit the use of **time measurements for quality control** within individual sample runs. The interval between cell arrival or acquisition times is used to derive a real-time measurement of **sample flow rate**, which is displayed against time; in addition, a measured parameter of interest (DNA content, in the case illustrated in Figure 7-22) is shown (schematically) vs. time in a two-dimensional display.

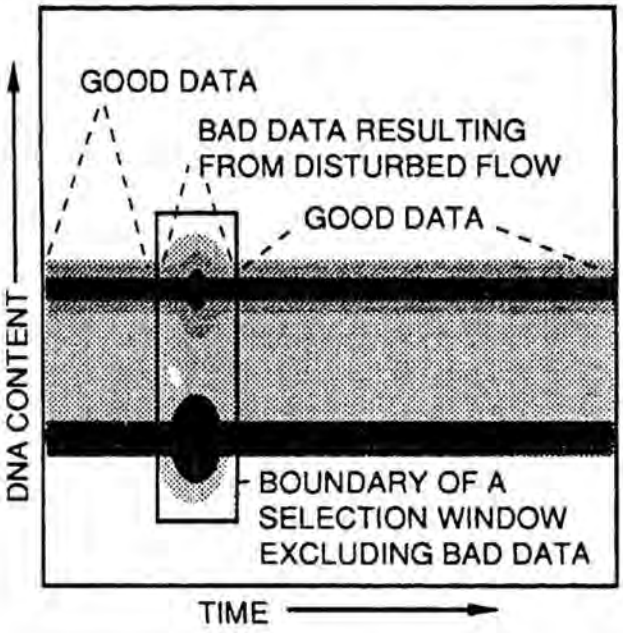


Figure 7-22. Time used as a quality control parameter in DNA content analysis (after Watson[819]).

The "lumps" in the middle of the display result from a temporary degradation in measurement precision caused by a transient disturbance of the flow pattern. Before and after the transient, the measurement precision is considerably better. However, if the DNA content distribution were calculated for the entire sample, the coefficient of variation would be increased by inclusion of the relatively small number of cells from which data were acquired during the flow disturbance. By defining a selection window as shown in the figure, it is possible to exclude the bad data points from the DNA content distribution. In many cases, e.g., when one analyzes cells from a very small sample of tumor tissue, being able to "rescue" data in this fashion may make the difference between getting and not getting enough information to characterize a specimen. Even when there is plenty of sample available, however, Watson's technique provides a convenient means of assessing the quality of measurements. Kusuda and Melamed[2725] used the chronological order of events in a list mode file as a time parameter and were able, in some cases, to correct for drift in data values.

Slooowww Flooowww

Conventional flow cytometers use flow velocities of 1-10 m/sec, making it almost impossible to examine the same cell twice at time intervals separated by more than a few hundred µsec. The relatively high flow velocities also result in shear stress on cells in the sample, making the use of flow cytometry for studies of cell adhesion suspect.

Amblard et al[3387] developed a chamber for flow cytometric analysis over an extended range of stream velocity which, with adaptations to the electronics, permitted standard analysis at velocities as low as 0.01 m/sec in a B-D FACS instrument. Conjugates formed by the adhesion between human B and resting T lymphocytes, disrupted in conventional flow cytometers, could be detected and precisely quantified provided analysis velocity was kept below 0.1 m/sec. A much longer chamber, operating at this velocity, with widely separated measurement stations, could allow multiple observations of an individual cell to be made over periods of tens to hundreds of seconds; nobody seems to have built one yet. However, slow flow systems are in use for such applications as DNA fragment sizing, which will be discussed in Chapter 10.

7.II LABELED LIGAND BINDING

The flow cytometric analysis of ligand binding to cell surface and intracellular receptors, or to receptors on beads or other particles, can be approached in several ways, the most obvious of which is probably the use of labeled ligands. To produce labeled ligands, you need to have enough relatively pure unlabeled ligand on hand to yield an adequate supply of labeled material for your experiments. Acquiring the starting material used to be the first hard part of the project; the second part was verifying that labeling the ligand didn't drastically alter its binding characteristics. In this age of cloning, a respectable variety of labeled ligands such as cytokines and hormones are available off-the-shelf, and you probably won't have to worry about getting and labeling

your ligand; if not, this isn't the place to look for help. This is a good place to mention that antibodies also count as ligands; their binding to cells can be analyzed using exactly the same techniques.

The production of labeled ligands, and the flow cytometric analysis of ligand binding, have become so common that I have given up trying to keep track of every ligand. Any listing or tabulation you see will be incomplete by the time it appears; I've run across several papers on flow cytometry of ligand binding that didn't turn up in my literature search on flow cytometry. As was the case with immunofluorescence, I'll have to stick to the basics with selective excursions into specifics.

Just as **cell surface receptors** for ligands ranging from small peptide hormones to viruses and bacteria can be demonstrated by flow cytometry using fluorescently labeled analogues, **cytosolic** and **nuclear receptors** for ligands such as steroid and thyroid hormones may also be demonstrable. However, since the ligands themselves are relatively small molecules, it is, in general, more difficult to find or synthesize analogues with binding characteristics similar to those of the natural ligands than to prepare usable fluorescent derivatives of larger molecules. In order to demonstrate receptors within intact cells, the fluorescent ligand used must obviously have the capability of crossing or being carried across the cell membrane, as well as the binding characteristics previously mentioned. The size and specificity constraints on analogues make it infeasible to attach more than one fluorochrome moiety to each molecule of ligand; since the number of intracellular receptor sites is generally small, one can therefore expect only weak fluorescence signals at best from cells stained with the labeled ligand analogue.

The flow cytometric literature in this area recounts numerous attempts at demonstration and quantitation of **estrogen receptors** in cells. The presence or absence of estrogen receptors, as determined by radioligand binding analysis of biopsy specimens, is of significance in assessing prognosis and planning therapy of breast cancer. Fisher et al[378], using fluoresceinated estrone (17-FE) and fluorescence microscopy, demonstrated heterogeneous binding patterns of the material in tissue samples; Tyrer et al[379], Kute et al[380], and Van et al[381] made flow cytometric measurements using 17-FE and other fluorescent analogues.

A study by Benz, Wiznitzer, and Lee[828], who utilized bovine serum albumin (BSA) as a carrier molecule to which an average of 25 estradiol and 4 fluorescein molecules were bound, generated a heated exchange of correspondence between Ashcroft[829] and Benz[830], which did not resolve the issue of how accurately one can study binding sites for small molecules using much larger ones. If you simply want to determine the number of binding sites, **anti-receptor antibodies** may be preferable to labeled ligand analogues as reagents, but, if the objective is to measure binding affinities, you may be literally and figuratively stuck with labeled ligands. On the bright side, Marchetti et al[1443] found comparable results in analyses of **glucocorticoid receptors** using flow cytometry with antireceptor antibodies and fluorescein-labeled steroid analogues and classical radioligand binding methods.

Labeling Strategies

The procedure described by Shechter et al[302], in which small peptide hormones such as insulin are fluorescently labeled by reaction with fluorescein- or tetramethylrhodamine-labeled **lactalbumin**, provides a way of getting a few more fluorescent molecules onto a ligand than can be done by direct labeling. This becomes important when the number of binding sites per cell is small; under these circumstances, flow cytometry may allow detection and quantitation of amounts of bound ligand too small to be seen under the fluorescence microscope. This degree of sensitivity is also associated with the technique of **video intensification microscopy (VIM)**[445-6], which offers the advantage of allowing the same cell to be observed over a period of time. A paper by MacInnes et al[447] illustrates the use of VIM and cell sorting in a complementary fashion to study luteinizing hormone releasing hormone binding sites on rat pituitary cells.

When the ligand for which receptors are to be demonstrated is a small molecule, covalent attachment of ligand molecules to a fluorescently tagged protein, or, alternatively, a phycobiliprotein, may yield a material that will still bind specifically to receptors, thereby enabling attachment of more than a single fluorochrome moiety to each receptor site. This technique was used by Osband et al[448] to show that a subpopulation of T cells bore H_2 (cimetidine-reactive) histamine receptors; the reagent employed was a conjugate of histamine with fluoresceinated albumin. Hallberg et al[449] used the same material to demonstrate H_1 and H_2 receptors on platelets; Muirhead et al[849], however, found only nonspecific binding of similar conjugates to lymphocytes.

Formal Analysis of Ligand Binding

Bohn[313,326-7] was probably the first to appreciate that flow cytometry could be used for **quantitative analyses of fluorescent ligand binding** to cells that yielded essentially the same data as were obtained from conventional radioligand binding assays; in some circumstances, bulk fluorimetry must be used in addition to flow cytometry for precise determination of the ratio of bound to unbound ligand, but, in other cases, information can be derived solely from flow cytometric data.

The flow cytometric method offers a particular advantage over radioisotope techniques in that the binding equilibrium is not disturbed because there is no need to wash away unbound ligand; the speed of the flow cytometric analysis and the elimination of the necessity to deal with and dispose of radioactive materials also weigh in its favor. Sklar and Finney[450] demonstrated the capabilities of flow cytometry in an analysis of steady-state binding of a fluoresceinated chemotactic peptide to neutrophils.

The study of Benz, Wiznitzer, and Lee[828] on estrogen receptors provides good examples of the calculation of binding

affinities and the construction of the **Scatchard** and **Lineweaver-Burk plots** commonly used with radioligand assays. Other older examples of quantitative analysis, dealing with binding of antibodies to cell surface receptors, were published by Uckun et al[1444] and Krause et al[1445]; Ziegler et al studied labeled **insulin**[2313]. In addition, Chatelier and Ashcroft[797] proposed an alternate method, **isoparametric analysis**, for determining ligand-binding characteristics.

More recently, Gordon[2726] described a Scatchard analysis of **concanavalin A** binding to lymphocytes, noting that cytometric plots seemed more precise than those obtained using radiolabeled ligands, particularly at low ligand concentrations. Macho[2727] et al quantified **fatty acid** binding to cells using a BODIPY-labeled dodecanoic acid. Cherukuri et al[2728] used flow cytometry, fluorescence spectroscopy, and two-photon microscopy in an elaborate mechanistic study of binding and internalization of fluoresceinated poly-D-lysine by murine macrophages. Waller et al[2729] established the fluorescence characteristics of six fluorescently labeled ***N*-formyl peptides**, which bind to neutrophils, and developed and validated competitive binding protocols to determine binding constants of unlabeled ligands. Lauer et al[2730] performed real-time quantitative flow cytometric analysis of the interaction of fluoresceinated cholera toxin subunits with ganglioside receptors inserted into phospholipid membranes supported by glass beads. All of the reports just described may be helpful to anyone interested in doing quantitative work with other ligands.

Labeled Ligands versus Anti-Receptor Antibodies

In some cases, there is a choice of labeled ligands available that will bind to a particular receptor; for example, transferrin receptors can be demonstrated using either fluoresceinated transferrin[451] or a monoclonal antibody such as Ortho's OKT9, which reacts with the transferrin receptor[452] (CD71). Flow cytometry can, of course, be utilized to establish the fact that two different ligands do react with the same or closely spaced sites on cell surfaces. Fingerroth et al[453] used flow cytometric analysis to study the binding of fluorescently tagged viruses, antibodies, and complement components to human B cells, and showed that the Epstein-Barr virus binds to the type 2 complement receptor.

Ligand Binding Detected by Functional Changes

When the ligand of interest is not available in sufficient quantity or purity to permit ligand labeling, the only feasible way to identify cells that have bound and responded to ligand is by demonstrating induced changes in functional parameters. This approach was taken by Price et al[417], who isolated erythroid and myeloid precursor cells from bone marrow based upon SCM responses to impure preparations of growth stimulatory factors, and by Osband et al[454], who isolated H_2 receptor bearing T cells based on membrane potential responses to a combination of histamine and an H_1 receptor antagonist. For this approach to work, it is necessary to have stable and reproducible flow cytometric methods for the detection of physiologic changes induced by ligand binding; while such methods have improved over the years, the methods of preparing labeled ligands and the sensitivity of instruments have also improved, making it easier to detect binding *per se*. However, demonstration of functional changes remains useful for distinguishing between cells that merely bind ligand and cells that respond. Tordai et al[1446], for example, used binding of labeled antireceptor antibodies and induction of calcium fluxes by unlabeled thrombin to identify functional thrombin receptors on T-lymphoblastoid cells.

Fluorescent Ligand Binding: Some Examples

High-density lipoprotein (HDL) binding was studied by Schmitz et al[1447], using a rhodamine label; they compared fluorescent and radiolabeled ligand binding and estimated the number of binding sites on human lymphocytes, monocytes, and granulocytes. Traill et al[1448] used **"DiI" (dioctadecyl-indocarbocyanine)** to label HDL and quantified its binding to lymphocytes; they also[1449,50] studied binding of DiI-labeled **low-density lipoprotein (LDL)**. Corsetti et al[1451,2] labeled LDL with *N,N*-dipentadecylaminostyryl-pyridinium iodide, which has very flow fluorescence in aqueous solution and which therefore should yield lower fluorescence background in HDL-binding measurements.

Laborda et al[1453] and Torres et al[1454] examined the binding and endocytosis of fluorescently labeled **α-fetoprotein** and **transferrin** by lymphoid cells. Midoux, Roche, and Monsigny[1455] studied binding, uptake, and degradation of fluoresceinated **neoglycoproteins**. Others have used fluorescein as a label for the clotting **factor VIII**[1460], for **anaphylotoxin C5a**[1461], and for **low molecular weight heparin**[2731]. Richerson et al[1462] and Sumaroka et al[1463] examined binding of mycobacterial **muramyl dipeptides** to macrophages, while other groups studied binding of bacteria to platelets[1464,5] and gastrointestinal cells[1466]. Szabo and Damjanovich[1467] reported the used of a fluorescein-labeled, protein A-rich strain of *Staphylococcus* as a second-step reagent for immunofluorescence.

While some work has been done with fluoresceinated preparations of the **lipopolysaccharide (LPS)**[1456-9] derived from various species of Gram-negative bacteria, Triantafilou et al[2732] used Alexa 488 hydrazide to label the oligosaccharide core of the material, avoiding interaction of the label with lipid A, which is the portion of the molecule involved in binding. Their labeled preparation can be used at concentrations in the range of those reported to be present in the blood of patients with Gram-negative sepsis.

Indirect staining was used by Gabrilovich et al[1468] to quantify binding of the **HIV gp120 protein** to cells; the protein was labeled with biotin and subsequently reacted with Texas Red-streptavidin. Indirect staining also provides amplification to facilitate detection of small numbers of binding sites for **growth factors** and **cytokines**. Wognum et al used biotinylated ligands followed by phycoerythrin-streptavidin to detect receptors for **erythropoietin**[1469] and for

interleukin-6 (IL-6)[1470]; further amplification with biotin-anti-phycoerythrin antibody increases the signal for erythropoietin receptor detection[1471]. Biotinylated ligands have also been used to quantify receptors for **IL-2**[1472-3] and **IL-4**[1474-5], enabling demonstration of fewer than 100 receptor sites/cell in some cases. Lawrence et al[2733] used a fluorescein-labeled high-affinity opioid in studies of the kappa opioid receptor, amplifying signals by staining the bound ligand with a phycoerythrin-labeled anti-fluorescein antibody.

Both direct fluorescent labeling and biotinylation of small molecules may, as mentioned before, result in loss of activity and even in the labeled ligand acquiring the characteristics of an antagonist, as was shown by Helmreich et al[1476], who fluoresceinated **glucagon** at different sites, and by Newman et al[1477-8], who made biotinylated derivatives of **parathyroid hormone**. Jans et al[1479] used spacers to preserve biological activity when preparing biotinylated **vasopressin**. In other cases, even small molecules may be labeled without apparent loss of activity and binding specificity; active fluorescein derivatives of **transforming growth factor beta**[1480], of **chemotactic peptides**[701], and of the fibronectin peptide **arg-gly-asp-ser**[1481] have been prepared, as has an active fluorescent **NBD-phorbol ester**[1482]. Chianelli et al[1483] have used fluorescence flow cytometry with a labeled antireceptor antibody in competitive assays to determine whether derivatization of IL-2 to various nonfluorescent products affects binding.

Moving from very small ligands to very large ones, I should mention the use of labeled **aggregated human immunoglobulin** in a flow cytometric assay for **circulating immune complexes** based on competitive binding to surface receptors on Raji cells[1484], and a couple of papers on binding of fluorescein-labeled viruses to cells[1485-6].

Since ligand binding is one of the phenomena investigated in the context of **high throughput screening** of lead (I don't mean Pb!) compounds in drug development, I should point out that a high throughput flow cytometer could be gainfully employed in this area.

7.12 FUNCTIONAL PARAMETERS I

The distinction I have made between structural and functional parameters is, to some extent, arbitrary; it is, after all, the function of cells that determines, maintains, and/or changes their structure. In general, what I have called **structural parameters** describe cells' stable morphologic characteristics and their content of specific chemical constituents, while the classification of **functional parameters** has been reserved for physical and chemical properties of cells which are defined operationally and/or which change rapidly. Both of these characteristics of functional parameters tend to make them more difficult to measure, by flow cytometry or otherwise, than are structural parameters. The motivation to make such measurements is provided by the perceptions that **functional heterogeneity** within cell populations is of biological significance and that observations of their functional characteristics can aid in understanding and predicting cells' behavior. I'll start with the outside of the cell here and work my way in.

Cell Surface Charge

Valet et al[372-377] have estimated **cell surface charge** from the binding of **fluoresceinated polycations** to the cell surface. They believe their flow cytometric method can be somewhat more informative than can the conventional measurement of surface charge based upon cells' electrophoretic mobilities, since flow cytometric analysis can be done using labeled polycations of different molecular weights and structures to define the accessibility, as well as the number, of various charged sites on cell surfaces.

Cell Membrane Characteristics

Membrane Integrity versus "Viability": Dye Exclusion Tests

I have discussed **membrane integrity** in connection with vital staining on pp. 299-301. Most people who work with cells are familiar with tests of "viability" based on cells' capacity to exclude acid dyes such as **trypan blue**, **eosin**, **erythrosin**, **nigrosin**, and **primulin** and some basic dyes such as **propidium**. The basis of all of these **dye exclusion tests** is the same; the dyes used are **impermeant**, and do not normally cross intact cell membranes.

A slight variation on the same theme is provided by using **esters** of **fluorescein** or related compounds as the reagents; **fluorescein diacetate (FDA)**, introduced on pp. 24-7, is lipophilic, uncharged, and nonfluorescent, and thus readily crosses cell membranes. Once inside cells, FDA is hydrolyzed by nonspecific esterases to produce the fluorescent fluorescein anion, which is retained (for a few minutes, at least) by cells with intact membranes and lost by cells with damaged membranes[335].

When doing dye exclusion tests, bear in mind that **propidium and ethidium are not interchangeable.** Although the two dyes behave nearly identically as intercalating stains for double-stranded nucleic acid, ethidium will enter intact cells slowly, especially if the pH of the medium is high, but is likely to be pumped out by an efflux pump. Propidium, which has one more positive charge, will not enter normally enter intact cells; you should thus use propidium, not ethidium, to test membrane integrity.

In these days of multicolor immunofluorescence measurements, it is preferable to have a dye exclusion indicator that emits at a longer wavelength than propidium; Schmid et al[1516] have shown that **7-amino-actinomycin D (7-AAD)** is useful for this purpose, and the red-excited **TO-PRO-3**, which, like propidium, is doubly charged and impermeant (pp. 300-1), is also useful. In work with bacteria using a dual-laser instrument and adding both dyes to samples, I have noted that cells that take up propidium take up TO-PRO-3 and *vice versa*; I never observed cells that took up only one of the dyes. I have not had the opportunity to play the same game with either propidium or TO-PRO-3 and

SYTOX Green, which is a current favorite for membrane integrity testing, especially in bacteria[2734], and which I suspect has at least three positive charges.

Bhuyan et al[336] and Roper and Drewinko[337], in papers published in 1976, showed independently that cells treated with lethal doses of cytotoxic drugs remained "viable" by dye exclusion tests for several days, and that the fraction of viable cells estimated by dye exclusion or ^{51}Cr release[336] was considerably higher than the fraction of cells that retained clonogenicity in culture. **As a general rule, cells that let in trypan blue or propidium are dead, but cells that don't are not necessarily viable.**

Despite the limitations of dye exclusion tests, there have been a lot of papers published that describe various flow cytometric adaptations and applications[4, 165, 197, 240, 313, 326-7, 338-45]. As might be expected, dye exclusion provides a somewhat more accurate measure of viability when applied to situations in which cells are killed by punching holes in their membranes, e.g., freezing or immune lysis[4,344].

Cells that do have holes in the membrane usually admit molecules considerably bigger than dyes, and thus tend to stain nonspecifically with fluorescent antibodies, lectins, etc. If fluorescein is being used to label such ligands, the addition of erythrosin or ethidium (or, preferably, propidium) to the sample produces strong fluorescence in the dead cells, allowing them to be gated out of analyses[165, 313, 326-7]. Khaw et al[342] worked the other side of this street, detecting loss of membrane integrity in cardiac muscle cells by their binding of fluorescent spheres coated with an antimyosin antibody. Stöhr and Vogt-Schaden[339] used a mixture of Hoechst 33342 and propidium to stain DNA in unfixed cells; this allows discrimination of cells with intact (blue fluorescence) and damaged (red fluorescence) membranes. Wallen et al showed that this test, like other dye exclusion tests, does not give a good indication of reproductive viability[345], reinforcing the conclusions others reached earlier[336-7].

If you think about it, you'll probably guess that it is easier to see a cell lightly stained with eosin or propidium, under a fluorescence microscope, than it is to see a cell lightly stained with trypan blue under a transmitted light microscope. This would suggest that dye exclusion tests done by absorption and fluorescence might give different results. During the development of the Block differential leukocyte counter, some comparative studies of trypan blue and ethidium (we didn't appreciate the difference between ethidium and propidium at the time) exclusion were done; the proportion of damaged cells estimated by ethidium fluorescence was always higher than that estimated by trypan blue absorption, and the ratio of the two remained nearly constant from sample to sample (K. F. Mead, unpublished). Others[807] have made similar observations.

Berglund et al[808] examined the UV-excited, blue fluorescent sulfonated dye **Calcofluor White M2R (CFW)**, an optical brightener closely related to the "LN" used in the Block differential staining system, as a dye for assessing membrane integrity; it works well with animal cells but may stain the walls of plant cells even when the membranes are intact. There may be some advantage to using CFW or dyes with similar spectral characteristics when one is trying to measure other parameters by green and orange or red fluorescence using 488 nm excitation. However, as a general rule, one can use propidium as a membrane integrity indicator even when utilizing orange fluorescence measurements for other purposes, by adjusting the propidium concentration; if not, 7-AAD (or TO-PRO-3 if you have a red laser and a 488 nm laser) will almost certainly work.

If, for example, you are trying to measure pyronin Y fluorescence in intact cells, adding propidium at a concentration of 1-2 µg/ml to the sample (this is less than 1/20 the concentration at which the dye is commonly used for stoichiometric DNA staining) will produce strong red fluorescence in cells with damaged membranes. If the detector used for the pyronin measurement has a response extending to at least 600 nm, the cells with damaged membranes will show up off-scale in this channel; alternatively, a separate red (600-640 nm) detector could be used. When using cells stained with Hoechst 33342, bear in mind that, if it gets in, propidium will strongly quench the Hoechst dye fluorescence; if you are triggering on the Hoechst dye fluorescence signal, you may have to adjust the threshold accordingly. 7-AAD, in my experience, does not quench Hoechst dye fluorescence to the same extent.

Even if a cell does let propidium or the other "excluded" dyes in, you can't be absolutely sure it's nonviable. Cell membranes can be transiently **permeabilized**, not only to dyes, but to macromolecules, by physical or chemical means, as discussed on pp. 300-301, and then resealed with retention of viability - even reproductive viability. If you permeabilize cells in a solution of propidium, reseal them, and add FDA (to a final concentration between 500 ng and 1 µg/ml, from a 0.5-1 mg/ml working solution in DMSO), any cells that weren't permeabilized will show only green cytoplasmic fluorescence, any cells that didn't reseal or had damaged membranes to start with will show only red nuclear fluorescence, and cells that were permeabilized and resealed will show green cytoplasm and red nuclei (F. Tsang & H. Shapiro, unpublished). Jones and Senft[809] noted that air-dried smears could be made from cell suspensions stained with FDA and propidium, allowing the fraction of cells with intact membranes to be determined as long as a week after slides are made.

Two compounds closely related to FDA and originally developed for intracellular pH estimation also offer advantages for dye exclusion tests because the fluorescent materials formed from them in cells are lost from the cells much more slowly than is fluorescein. The compounds are **carboxyfluorescein (COF)** and **2',7'-bis'(carboxyethyl)-5-(6')-carboxyfluorescein (BCECF)**; they will be discussed in more detail in the section on pH measurement. However, when it comes to dye-exclusion tests, the real champion among fluorescein-based dyes is **calcein**, introduced into cells as the **acetoxymethyl (AM)** ester; once the ester is

cleaved, the free dye stays inside cells much longer than the others (but see pp. 376-7). In general, however, people now seem to use nucleic acid dyes in preference to various fluorescein derivatives for dye exclusion testing, because the objective is often to eliminate dead cells from immunofluorescence analysis, and any of the fluorescein derivatives will produce enough of a green fluorescence signal to make it difficult or impossible to measure immunofluorescence using fluorescein, PE, or even PE tandems.

Detecting "Dead" Cells in Fixed Samples

Many of the samples now run through flow cytometers have been fixed before they are run. Identification of cells that had lost membrane integrity before fixation is useful, because when a sample is stained with fluorescent antibodies to a cell surface antigen or antigens prior to fixation, antibodies will enter cells with damaged membranes, and those cells will therefore exhibit high levels of nonspecific fluorescence. Riedy et al[1516] developed a method for identification of membrane-damaged cells using the irreversible binding of photoactivated **ethidium monoazide (EMA)**. EMA is a positively charged molecule containing the nucleic acid-binding fluorophore ethidium; it is excluded by "viable" cells with intact membranes and enters cells with damaged membranes. When added to a sample prior to fixation, EMA can be covalently bound to nucleic acids by photochemical crosslinking using visible light. When the samples are subsequently washed, stained with antibodies and fixed, ethidium is retained only in those cells that had damaged membranes prior to fixation; at appropriate levels, the ethidium fluorescence is distinguishable from fluorescein and phycoerythrin emission.

These days, there's an easier way to do things. Schmid et al[2735] found that, if cells were exposed to 4 μg/mL **7-AAD** before fixation, the dye could be kept in only those cells that had lost membrane integrity prior to fixation by addition of 4 μg/mL **actinomycin D (AD)** to subsequent fix, stain and wash solutions and buffers. AD is nonfluorescent, but competes with 7-AAD for binding sites on DNA. The 7-AAD/AD combination was used with fluorescein- and PE-labeled antibodies and TO-PRO-3 (with added RNAse) to permit two-color immunophenotyping and DNA content determination in cells that were not membrane-damaged prior to fixation.

Membrane Fusion and Turnover; Cell Tracking

It is often desirable to be able to attach permanent or relatively permanent fluorescent labels to cell membranes, for such purposes as detecting cell aggregation or hybridization, determining the localization and fate of cells isolated from and reinjected into an animal, and establishing the rate of turnover of various components of the membrane itself. A number of fluorescent compounds have been used for such membrane labeling.

Covalent labels such as FITC and XRITC bind to membrane proteins; Abernethy et al[1540] found that lymphocytes labeled with both dyes and reinjected into sheep could be followed over periods of days, and that FITC-labeled cells isolated from lymph could be double labeled with XRITC and recovered. Capo et al[1541] noted that there was some nonspecific transfer of fluorescein molecules (about 10,000 molecules) from FITC-labeled to unlabeled cells *in vitro*, suggesting that FITC is not the stablest of labels. Weston and Parish[1542] reported that Hoechst 33342-, BCECF-, and calcein-labeled cells could be followed for 2-3 days, while cells covalently labeled with **carboxyfluorescein succinimidyl ester (CFSE)** retained label for weeks.

Another class of labels are incorporated into the membrane lipid bilayer itself. Among the most widely studied are derivatives of cyanine dyes, including **"DiI" (dioctadecylindocarbocyanine)** and **"DiO" (dioctadecyloxacarbocyanine)**[1543], both of which can be excited at 488 nm, with DiO emitting green fluorescence at about 500 nm and DiI emitting yellow fluorescence at about 565 nm. Fluorescein and phycoerythrin emission filters are, respectively, well suited for measurement of DiO and DiI. The octadecyl (C_{18}) side chains of these probes reside in the lipid bilayer; the chromophores remain at the surface.

When used to label neuronal cells, which do not divide, DiI and DiO persist in the membrane for months; they were found to remain detectable for more than a week in cultured endothelial and smooth muscle cells[1544]. St. John[1545] found that DiI was toxic to embryonic rat neurons, and that the C_{12} derivative, which was not, could be used effectively for long-term labeling. Ledley et al[1546] used DiI as a marker to determine localization of transplanted hepatocytes and thyroid follicular cells by flow cytometry.

Paul Horan and his colleagues[1547-52] introduced the **PKH** series of tracking dyes, which includes both radioactively and fluorescently labeled compounds incorporating longer aliphatic chains (e.g., C_{24}) than are found in DiI and DiO. Presumably, these molecules incorporate into the outer layer of the lipid membrane bilayer and penetrate into the inner layer, providing stronger binding and, as a result, longer persistence. These dyes have been used to study peritoneal macrophages[1548-9], to discriminate target from effector cells in cytotoxicity assays[1550], to track lymphocytes used in adoptive immunotherapy[1552], and to label bacteria for studies of phagocytosis[1553]. I know of no comparative study of PKH dyes vs. DiI and DiO, but the PKH dyes demonstrably work.

Cell Proliferation Analyzed Using Tracking Dyes

As Horan et al pointed out[1547], if a cell labeled with a tracking dye subsequently proliferates, each daughter cell will get approximately half the label; in the next generation, each daughter cell will carry one-quarter of the label, and so on. Analysis of the labeling intensities of cells grown *in vivo* or *in vitro* after labeling will, therefore, allow determination of the number of division cycles through which each cell has progressed since the label was applied. While similar information can be obtained from bromodeoxyuridine labeling

studies, it is much simpler to obtain using tracking dyes. Ashley et al[1554] used the yellow-fluorescent dye PKH26 GL to follow leukemic cells through several cell divisions; more recently, Yamamura et al[1555] developed a method for analysis of proliferation of stimulated lymphocytes using the same dye. Results are shown in Figure 7-23.

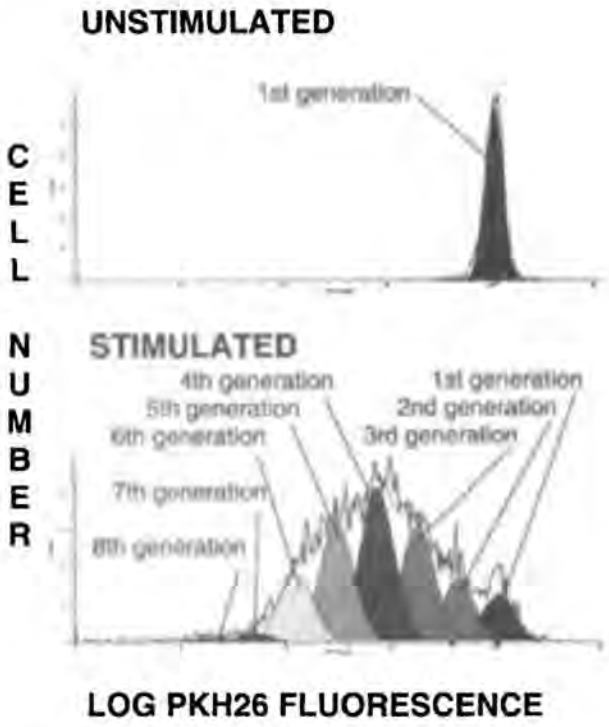


Figure 7-23. Cell proliferation indicated by dilution of fluorescence of the tracking dye PKH26 in mitogen-stimulated and unstimulated peripheral lymphocytes after 5 days in culture[1555]. The data and figures were provided by Yasuhiro Yamamura (Ponce (PR) School of Medicine), Abraham Schwartz (Flow Cytometry Standards Corp.), Bruce Bagwell, and Don Herbert (Verity Software House).

The raw histograms in the figure were analyzed using a variant of the ModFit DNA analysis program from **Verity Software House**, which does nonlinear least squares fits to decompose a histogram into a sum of distributions representing successive generations of daughter cells. The model can identify as many as ten successive generations of cells. This cannot be done using BrUdR or tritiated thymidine; the tracking dye technique also has the advantage of being usable with live cells, permitting sorting of cells in different generations. The PKH dyes and analysis software are available from **Sigma**.

Tracking dyes are particularly useful for analysis of responses of lymphocytes to antigens. When mitogens are used for lymphocyte stimulation, a majority of cells respond. In antigen stimulation, the fraction of responsive cells ranges from 1 or 2 percent, in mixed lymphocyte reactions, to one cell in several hundred thousand, which is typical when cells are exposed to viral antigens. In a lymphocyte population, there should be a spectrum of affinities for a particular antigen; cells that begin proliferating earlier may have different characteristics from those that begin proliferating later. This could be established by sorting of cells with different labeling intensities following stimulation by antigen, which might also provide clones useful for their cellular or humoral specificities. Sorting experiments along this line should also be able to determine to what extent late proliferative responses in antigen-stimulated cells are the result of recruitment, rather than of specific reaction to antigen.

A look at Figure 7-23, however, shows that the fluorescence distributions calculated by the model for successive generations of PKH26-labeled cells are rather broad and overlap considerably, meaning that sorting from any particular segment of the distribution would be likely to yield a mixture of cells from several generations. Since the previous edition of this book appeared, **carboxyfluorescein succinimidyl ester (CFSE)**[2349,50] has become widely used as a tracking dye for analysis of cell proliferation.

CFSE is, as Dick Haugland points out in the Molecular Probes *Handbook*[2352], an inappropriate term to describe the succinimidyl ester of 5(6)-carboxyfluorescein diacetate. He suggests CFDA SE; the name may not sell, but the sales receipts from the chemical itself should salve any wounds to his pride. CFSE is not properly a membrane label; it, like FDA and the esters of carboxyfluorescein and calcein, readily crosses cell membranes and enters cells, and is cleaved by intracellular esterases to a fluorescent form. Unlike fluorescein, carboxyfluorescein, and calcein, the fluorescent form of CFSE carries a reactive succinimidyl group, allowing it to bind covalently to amino groups in proteins; this accounts for the extremely long-term retention of the dye.

Both CFSE and the PKH dyes have been reported to be toxic to cells; this limits the concentrations at which they can be used to label a first generation, and, since tenth-generation cells will, on average, be only 1/1024 as bright as first generation cells, starting with lower concentrations of tracking dyes will usually decrease the number of generations discernible by flow cytometric measurements.

The PKH dyes and other long-chain fatty acid derivatives are more difficult to load into cells than is CFSE, but it is possible to make measurements of fluorescein- and PE-Cy5-labeled antibodies in cells stained with PKH26, while it is impossible to use fluorescein-labeled antibodies and difficult to use PE and some of its tandems in CFSE-stained cells. However, as can be seen from a comparison of Figures 7-23 and 7-24, CFSE clearly outdoes PKH26 in delineating generations. It is likely that if cells were sorted from a narrow middle region of one of the peaks shown in the bottom panel of Figure 7-24, the sorted population would come predominantly from a single generation.

Tracking dyes have had a substantial impact on studies of cellular immunology in recent years, and their applications to the study of lymphocyte development and activation will be discussed further in Chapter 10 (p. 501).

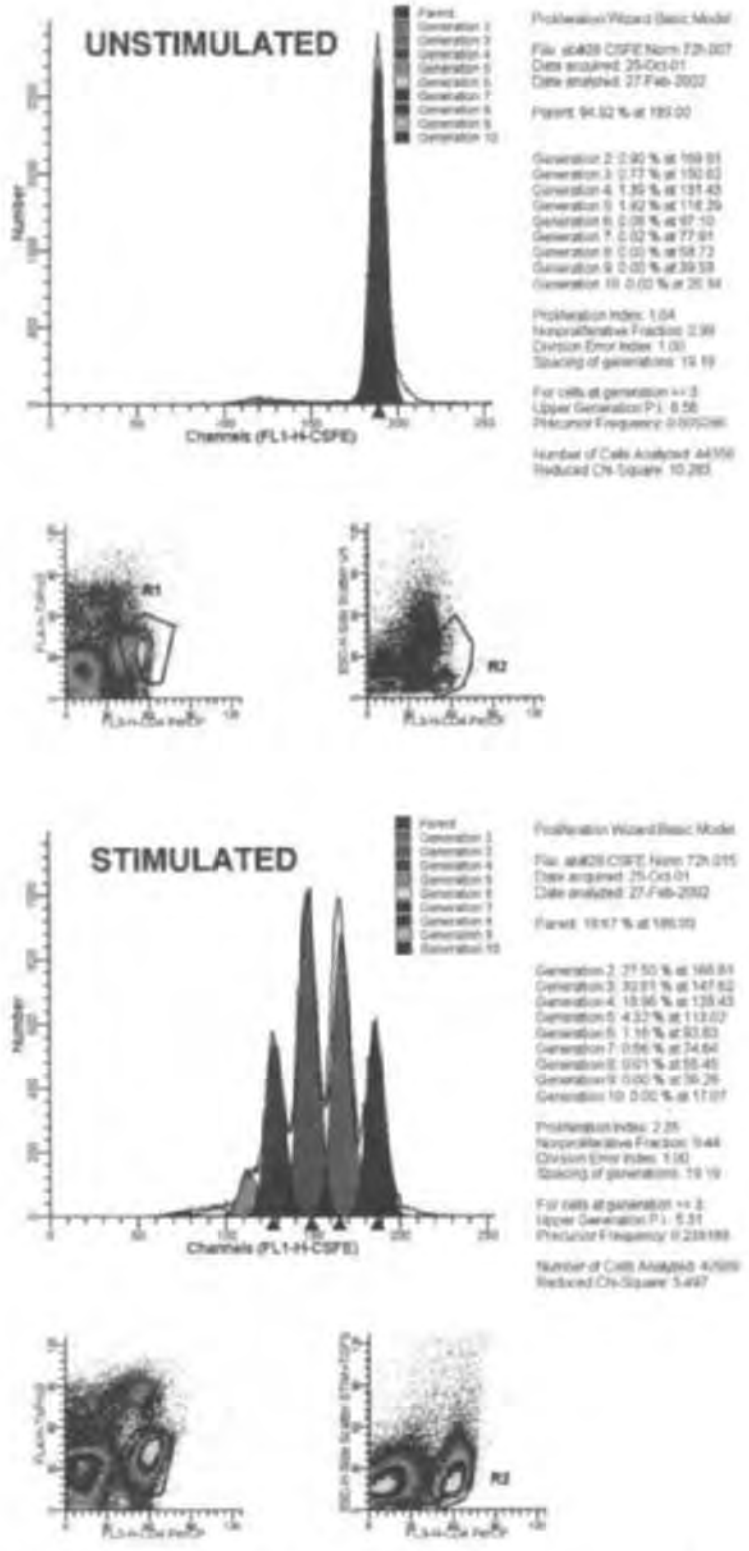


Figure 7-24. Cell proliferation of CD4+ lymphocytes indicated by dilution of fluorescence of carboxyfluorescein introduced into cells as the succinimidyl ester of carboxyfluorescein diacetate (CFSE). TO-PRO-3 was used to exclude dead cells. The data and figures were provided by Jonni Moore (U. of Pennsylvania) and Don Herbert (Verity Software House).

It would be desirable to have a tracking dye that did not emit fluorescence in the range of major popular antibody labels. There is nothing magic about the chromophores of either the PKH dyes or CFSE. The critical components of a PKH dye or of DiO, DiI, etc. are the long chain alkyl groups, which lock the compound into the lipid bilayer; the cyanine dye serves as a label. The chromophore in PKH26 is basically Cy3; Molecular Probes offers similar dyes with C_{18} alkyl groups and red-excited (Cy5-like) or near-infrared-excited (Cy7-like) chromophores.

The key characteristics of a CFSE-like dye are permeancy and a reactive group (amine-reactive, in the case of CFSE). Molecular probes sells a succinimidyl ester of SNARF-1 carboxylic acid, acetate, this compound, for which I'll accept any nickname with fewer than 10 letters, can be excited at 488 nm, but emits at longer wavelengths than does CFSE. Unfortunately, SNARF-1 itself was designed as a pH indicator; its emission characteristics change rather more dramatically with pH than do those of fluorescein, and any changes in intracellular pH with cell cycle stage or generation number might compromise the use of the SNARF-1 derivative for proliferation studies. A permeant reactive dye with spectral characteristics similar to Cy5.5 or rhodamine 800 (red-excited, emission >690 nm) would permit use of fluorescein, PE and almost all PE tandems for antibody labeling and gating, and could be very useful.

Membrane Organization and Fluidity/Viscosity

Lipid Packing Assessed with Merocyanine 540

Merocyanine 540 (MC540) is a green-excited, orange-fluorescent dye that, when it is applied to nerve axons, exhibits very small (0.3%) fluorescence changes in response to changes in membrane potential[457]. It can be excited at 488 nm, and its fluorescence is detectable through the 575 nm filters normally used for phycoerythrin measurement. MC540 was found by Valinsky, Easton, and Reich[4607] to stain immature and leukemic but not normal leukocytes; the staining was independent of membrane potential. The dye was subsequently used to photosensitize leukemic[1556] and malaria-infected[1557] cells.

McEvoy et al[1558] noted that MC540 binds preferentially to membranes with loosely packed lipids, and provides a flow cytometric method for assessing lipid organization in individual cells. Analyses of cells stained simultaneously with MC540 and 1-[4-trimethylammo-niumphenyl]-6-phenyl-1,3,5-hexatriene, which gave a signal to normalize for surface area, showed that all leukocytes in peripheral blood bound equivalent amounts of dye per unit surface area, indicating that lipids of the plasma membranes of all cell types are organized similarly. Lymphocytes, monocytes, and neutrophils activated by appropriate stimuli all bound increased amounts of dye per unit surface area, indicating a change in lipid organization to a less-ordered state.

Bright staining with MC540 is often achieved by UV illumination of the cells during staining. Szabo et al[1559] showed that staining of mouse spleen cells was greatly enhanced by UV illumination before addition of the dye, and that UV treatment caused an increased permeability toward propidium iodide and, later, intracellular fluorescein as well as increased MC540 staining, although the increase in MC540 fluorescence preceded the other effects. They suggested that penetration of MC540 to the more fluid inner membrane structures explains the fluorescence increase.

Double-staining experiments by Belloc et al[566], using MC540 and Hoechst 33342 on total bone marrow or peripheral blood cells, confirmed that the MC540-stained population included all the cycling cells, indicating that MC540 can be used as a marker for human hemopoietic cells. However, Pyatt[2736] et al found more total and committed progenitor cells in the CD34+MC540 dim population than in the CD34+MC540 bright population.

Around the time Annexin V[2353-4] was recognized as detecting membrane asymmetry in apoptotic cells, it was reported that MC540 was also effective for that purpose[2737-8,2970], staining apoptotic cells more brightly. The dye has been used only sporadically to detect apoptosis, but appears to be effective[2739-40]. Changes in MC540 fluorescence occur during platelet activation, and are apparently dependent on changes in accessibility of phosphatidylserine[2741-2].

I suspect that at least some oxonol dyes may also respond to membrane asymmetry; I will have more to say about this in the section on membrane potential dyes.

Membrane Fluidity and Microviscosity: Assessment Using Fluorescence Polarization

The concepts of **membrane fluidity** and **microviscosity** date from the early 1970's[397-8], by which time the Singer-Nicholson **fluid mosaic model of the cell membrane**[399] was generally appreciated, if not equally generally accepted. While it had become clear that many of the processes involved in the regulation of cell growth, differentiation, and differentiated function were mediated by receptor proteins extending through the cell membrane, it was also evident that the function of these proteins might be altered by local or global changes in the state of the **lipid bilayer** portion of the membrane.

Shinitzky and Inbar[397-8] investigated this possibility using measurements of the polarization of fluorescence of a hydrophobic probe bound to the membrane bilayer to derive an estimate of the physical state of membrane lipids. As was noted on pp. 114 and 283, when fluorescence is excited by polarized light, both the **polarization** and the **anisotropy** of fluorescence increase as the motion of chromophores becomes more restricted, i.e., as the "viscosity" of the medium increases, or as the "fluidity" decreases[400].

The probe Shinitzky and Inbar used for their measurements is **1,6-diphenyl-1,3,5-hexatriene (DPH)**. This is an uncharged hydrophobic material that is essentially nonfluorescent when coerced into aqueous media but which exhibits UV-excited blue fluorescence in nonpolar solvents. When a small amount of DPH in tetrahydrofuran (THF) or di-

methyl sulfoxide (DMSO) is added to a cell suspension, examination of cells under the fluorescence microscope shows DPH in most membranous and lipid-containing structures, inside the cells as well as in the outer membrane. If you look at cells such as peripheral blood lymphocytes, in which there aren't a lot of intracellular membranous structures, you'll get the idea that DPH is only in the outer membrane, but it ain't so.

The original measurements of DPH fluorescence polarization were done on cuvettes full of cells in a modified spectrofluorometer; an instrument specially designed for such measurements was later manufactured by Elscint (Haifa, Israel). Differences in anisotropy were found between normal and leukemic cells[401-2], and between cells before and after exposure to mitogens[403] and viruses[404-5]. These findings stimulated Arndt-Jovin et al to implement anisotropy measurements on their cell sorter[400,406]; they found changes in anisotropy during DMSO-induced differentiation of Friend leukemia cells.

Several factors that affect membrane microviscosity have now been identified. The cholesterol content and the fractions of unsaturated and saturated fatty acids in membrane lipids exert strong influences; cholesterol depletion decreases viscosity while increasing cholesterol content has the opposite effect. Increasing the content of unsaturated fatty acids decreases microviscosity[398]. Changes in lipid composition could explain some of the slower changes in microviscosity observed in connection with interactions of various materials with the membrane; however, it is more difficult to explain rapid changes in microviscosity on this basis.

In experiments with phospholipid membrane vesicles, Lelkes[411] showed that measured microviscosity increases with the electrical potential difference across the cell membrane, whether the interior of the vesicles is made electrically negative with respect to the exterior (as is the case with cells) or vice versa. He suggested that this results from an electrostrictive effect, i.e., that the high electric field, proportional to the transmembrane potential difference, which exists only across the thickness of the membrane bilayer maintains orientation of the lipid molecules and that mobility is decreased as field strength increases.

Karnovsky et al[412] have attacked the concept of membrane microviscosity as being too simplistic; they have amassed abundant evidence that the mobility of lipids differs in different domains of the membranes of individual cells. One certainly cannot expect to derive detailed information from measurements of the average anisotropy of a few million cells in a cuvette. This doesn't mean that there may not be a place for flow cytometric measurements of DPH emission anisotropy in single cells, but nobody seems to have found a good use for them yet. Later studies, which have looked at fluorescence polarization using DPH[845] and other membrane probes[413,846] have not provided a major impetus for a resurrection in this area.

Details of flow cytometric measurement techniques have been given by Fox and Delohery[845], and, more recently, by Bock et al[1561-2] and Collins and Grogan[1563,1032]. Collins and Grogan[1565] compared bulk fluorometric and flow cytometric measurements and found them equivalent; they also[1564] examined a series of *n*-(9-anthroyloxy) fatty acid probes, where *n* = 2, 3, 6, 7, 9, 12, and 16, showing that the anthroyloxy moieties of the probes located at a graded series of depths in the outer leaflet of the plasma membrane of living HeLa cells. For different n, the efficiency of quenching with an aqueous phase quencher, Cu^{++}, decreased with increasing n. The probes should therefore be usable for measurements of dynamic parameters related to membrane fluidity at different depths in the plasma membrane. Vincent-Genod et al[2762] noted a decrease of 3-, 6-, and 9-(9-anthroyloxy) fluorescence anisotropy values in late apoptotic lymphocytes, compared to viable lymphocytes.

Seidl et al[2518] devised a method for estimating membrane microviscosity that does not require fluorescence polarization measurements. Pyrene decanoic acid (PDA), a UV-excited lipid probe, normally emits at 397 nm; collision between two molecules, one in the excited state and one in the ground state, forms an excimer that emits at 485 nm. Since the rate of excimer formation depends on the rate of diffusion of molecules in the membrane, a change in microviscosity results in a change of the excimer/monomer fluorescence emission ratio.

Lipid Peroxidation

Hedley and Chow[1565] described a method for measuring lipid peroxidation using time resolved flow cytometry. The naturally fluorescent fatty acid ***cis*-parinaric acid**, which is readily consumed in lipid peroxidation reactions, losing its fluorescence, was loaded into cells by exposure to a 5 μM concentration for 60 minutes at 37 °C. Fluorescence microscopy showed diffuse staining of surface and internal membranes. A 325 nm HeCd laser was used to excite parinaric acid fluorescence at 405 nm. Addition of the oxidant *t*-butyl hydroperoxide resulted in a burst of intracellular oxidation, demonstrated by simultaneously loading the cells with dichlorofluorescein, and in loss of parinaric acid fluorescence over time; this was followed by cell death, indicated by decreased forward light scatter and propidium uptake. Pretreatment of the cells with the antioxidant alpha-tocopherol (200 μM) reduced the rate of loss of parinaric acid fluorescence and delayed the onset of cell death. Simultaneous biochemical analysis of the lipid peroxidation breakdown product malondialdehyde revealed a close temporal correlation with loss of parinaric acid fluorescence, with and without alpha-tocopherol treatment, suggesting that the flow cytometric assay for lipid peroxidation is comparable to standard methods.

The problem with parinaric acid is that, while it is very well excited by a 325 nm He-Cd laser, it is very poorly excited at 350 nm and above, meaning that you will get almost no signal out of the probe even if you use a big argon or krypton laser for UV excitation. That didn't stop somebody I knew from publishing some essentially meaningless figures

in which the distributions (mostly of noise) just happened to shift around in the right directions to support his hypothesis – and in a good single-name journal, too. As I have said before, bad flow happens to good journals.

If you only have a 488 nm laser, you can measure lipid peroxidation using a fluorescence emission ratio from hexadecanoyl-BODIPY-FL[2743], or by measuring decreasing fluorescence from 5-dodecanoylfluorescein[2744-5] or fluoresceinated phosphatidylethanolamine[2746-7].

Membrane Permeability to Dyes and Drugs: The Drug Efflux Pump(s) Revisited

Differences in the temporal pattern of uptake and retention of **Hoechst 33342** were used in the late 1970's for studies of lymphocyte activation[248-50,253,257]; this led to the demonstration in 1981 by Lalande, Ling, and Miller that dye retention was determined by the operation of what is now recognized as the **P-glycoprotein (Pgp) drug efflux pump**. This subject has already been discussed briefly on p. 309; we will return to it presently.

Flow cytometry of the kinetics of intracellular accumulation of **fluorescent drugs**, e.g., **anthracyclines** such as **doxorubicin** (daunomycin) and **adriamycin**[352,3,814], and **drug analogues**, such as **fluoresceinated derivatives of methotrexate**[354-6], can obviously be useful in pharmacology, e.g., for determination of mechanisms of drug resistance. The methotrexate analogues, in particular, have been valuable in studies of gene amplification[357]. Krishan, who has done extensive studies of anthracycline uptake and retention patterns in tumor cells and of their perturbation by phenothiazines and calcium channel blockers[352,815-7], has reviewed the general area of flow cytometric analysis of drugs and drug analogues on several occasions over the years[818,2748-50].

The cationic dye **rhodamine 123** had been shown by Lan Bo Chen and his colleagues in 1980 to stain mitochondria[489]; it was established in 1981 that the retention of the dye in mitochondria after washing was dependent on the existence of an interior-negative mitochondrial **membrane potential**[490]. By this time, it had already been noted that lymphocyte stimulation was accompanied by increased rhodamine 123 staining[494]. From 1981 to 1985, rhodamine 123 retention was thought to depend primarily on membrane potential. In 1985, Sonka et al[1566] found that in cells of an anthracycline-resistant tumor line, which was known to have an active efflux pump, rhodamine 123 retention was low. Substances such as verapamil, which were known to increase the sensitivity of anthracycline-resistant cells, inhibiting the pump, also shifted the cells from low to high rhodamine 123 retention. Tapiero et al[1567] reported similar findings in 1986.

At about the same time, Bertoncello, Hodgson, and Bradley[1568] and, slightly later, Mulder and Visser[1569] observed that bone marrow stem cells exhibited low rhodamine 123 retention. This characteristic remains useful for isolation of cells with marrow repopulating activity; it is now known to be due to the operation of an efflux pump, although it was originally attributed to decreased mitochondrial respiration.

Since the mid-1980's, various authors have described methods for demonstrating **multidrug resistance**, i.e., the presence and activity of the drug efflux pump, in tumor cells, based on uptake and or retention of Hoechst 33342[1570], anthracyclines[1571-3, 1576], rhodamine 123[1575, 1577], cyanine dyes[1577], and antibodies against P-glycoprotein[1573-4, 1576]. The pump has also been demonstrated in human peripheral lymphoid cells (B and NK, but not most T cells)[1578], trypanosomes[1579-80], and some bacteria[1581-2]. Kessel et al[1577], in an article well worth reading, demonstrated efflux from drug resistant cells of Hoechst 33342, rhodamine 123, and several cyanine dyes commonly used as membrane potential probes, and cautioned against drawing the conclusion that changes in rhodamine and cyanine dye fluorescence in cells are due to membrane potential changes. Oxonol dye probes of membrane potential, which are anionic and which apparently do not enter cells in large amounts, are largely unaffected by the operation of the pump. I will return to this issue in the discussion of membrane potential probes.

Assaraf and others[1583-5] further characterized methotrexate (MTX) resistance mechanisms, demonstrating that fluorescein-MTX is not transported by the carrier responsible for MTX uptake, but enters cells by passive diffusion. The labeled compound, however, remains useful for demonstration of increased intracellular dihydrofolate reductase, which causes one type of resistance to MTX. A second type appears to be due to decreased transport of MTX analogues into cells.

Fluorescent probes for specific transporters have been developed; Wiley et al[1586] described a fluorescein-labeled probe for the nucleoside transporter, while Knaus et al[1587-8] made BODIPY-labeled probes which bind to calcium channels. Using a somewhat less specific probe, the blue-violet-excited, green fluorescent anionic dye **lucifer yellow**, loaded into cells by electroporation, Dinchuk et al[1589] showed that an anion transport mechanism was responsible for efflux of the dye from lymphocytes. Aller et al[2751] and Natarajan and Srienc[2752-3], respectively, used **NBD-glucose** to study glucose uptake kinetics in rat brain cells and *E. coli*.

Dordal et al[2754] used a three-compartment mathematical model and kinetic measurements to assess the pharmacokinetics of doxorubicin.

We now know that there are multiple pumps that can mediate efflux of drugs and dyes from cells[2748-50]. In addition to P-glycoprotein, there are (at least) **multidrug resistance protein (MRP)**[2755-7], **breast cancer resistance protein (BCRP)**[2758], and **lung resistance protein (LRP)**[2748], and some tumors and cell lines contain more than one active pump. Whole-cell studies of one pump in such cells therefore require either the use of a substrate that is processed by only that pump or the use of a selective inhibitor(s) for the pump(s) not under study.

P-glycoprotein transports Hoechst 33342, rhodamine 123, and doxorubicin, among other compounds, and many acetoxymethyl (AM) esters, including indo-1 AM and fluo-3 AM, both used for cytoplasmic calcium measurements, and

calcein AM, used in tests of membrane integrity (pp. 370-1). If you are using these probes, it is advisable to determine whether or not the cells you are studying contain pumps that might interfere with your experiment. P-glycoprotein is inhibited by **cyclosporin A**, which competes with substrate molecules for binding sites, by the calcium channel blocker **verapamil**, which does not, by phenothiazines such as **trifluoperazine** and **prochlorperazine**, and, for those with a taste for political controversy, by **RU 486**.

MRP is a particularly effective transporter of glutathione S-conjugates of drugs; it also effluxes both **calcein AM** and free **calcein**, and carboxy-2',7'-dichlorofluorescein[2756], but not BCECF-AM[2757]. MRP is inhibited by **indomethacin**, **MK571**, and **probenecid**. **Mitoxantrone** was found by Minderman et al[2758] to be the only material that served as a substrate for BCRP in all of the cell lines they tested; fumitremorgin C inhibited mitoxantrone efflux by BCRP.

It is advisable to use antibodies[2755,2759], particularly antibodies that do not interfere with pump activity, as well as substrates to detect and quantitate protein pumps. Chen and Simon[2760] transfected cells with a Pgp-GFP fusion protein, allowing simultaneous quantitation of cells' content of protein and substrate, and showed that the degree of efflux depended only on the quantity of Pgp present in cells; Wang et al[2761] have standardized a flow cytometric assay for identification and evaluation of Pgp inhibitors.

There is a great deal more biochemical diversity among the prokaryotes than among the mammals, and we know that bacteria have a broader range of pumps than we do, and use some them to resist the actions of antibiotics. This is probably a good place to point out that all membranes are not created equal; the outer membrane of **Gram-negative bacteria** is, under normal conditions, impermeable to many lipophilic materials, such as cyanine dyes, which readily and rapidly enter almost all other types of cells, although it can be permeabilized, e.g., with EDTA. I have used this property as the basis of a "flow cytometric Gram stain" which will be discussed in Chapter 10; Molecular Probes advertises sets of nucleic acid dyes for the same purpose.

Endocytosis of Macromolecules and Particles

Fluorescently tagged macromolecules[368-70], plastic microparticles[371-3], and bacteria[374-5] have been used to study **endocytosis** and **phagocytosis**. Murphy et al[369] and Bassøe et al[375] have examined pH changes in the environment of endocytosed material, utilizing the pH-dependence of fluorescein fluorescence[202-4]. A refinement in this technique was introduced by Murphy et al[376]; cells are incubated with a mixture of fluorescein- and rhodamine B-labeled ligands, and excited at 488 nm, and the ratio of fluorescein and rhodamine fluorescence is calculated.

The emission intensity of fluorescein, when the dye is excited at 488 nm, decreases with decreasing pH over the range 8-4. The spectrum of rhodamine B does not change appreciably with pH over this range. The ratio of fluorescence signals from the two labels can thus be used to provide a measure of the ambient pH that does not vary with variations in the total amount of material in different cells. Additional material relating to this technique will appear in the later section on pH measurements.

As one would expect, work on flow cytometric measurement of endocytosis has concentrated heavily on analysis of blood mononuclear and polymorphonuclear phagocytic cells. Terstappen et al[825] developed a flow cytometric assay for circulating immune complexes in serum based upon phagocytosis of the complexes by granulocytes from healthy donors and subsequent immunofluorescent detection of intracellular complexes. Davis et al[826] have used fluorescein-labeled dextran to study fluid pinocytosis induced in polymorphonuclear cells by chemotactic peptides.

When microparticles, rather than macromolecules, are used to demonstrate endocytosis, it is possible to precisely specify both the size and spectral characteristics of the indicator used, and, if one cares, to obtain histograms of the numbers of particles taken up by cells, provided the particles are relatively bright and uniform. The particles themselves may also be coated with specific ligands. To permit simultaneous immunofluorescence analysis using fluorescein antibodies and measurement of phagocytosis, Rolland et al[827] employed 0.3 μm plastic particles labeled with ethidium as targets; the red fluorescence of the particles could readily be discriminated from fluorescein fluorescence.

Newer phagocytosis assays have added extra tricks. Using two-color analysis, orange- or red-labeled bacteria, and **2,7-dichlorofluorescin diacetate (DCFH-DA)**, an indicator of oxidative metabolism which will be further discussed shortly, it is possible to determine both phagocytosis and the subsequent occurrence of the respiratory burst[1590-1]; approximately the same trick can be done using green-labeled bacteria and **hydroethidine**, a derivative of ethidium that is oxidized to the red fluorescent form during the respiratory burst[1592]. Phagocytosis can also be assayed, with somewhat less certainty, with DCFH-DA and unlabeled bacteria, using the occurrence of the burst as an indicator of phagocytosis[1593]. Phagocytosis of immunoglobulin-coated sheep erythrocytes and respiratory burst can be assayed using the fluorescent product of DCFH-DA for the latter and changes in scatter signals for the former[1594].

Differentiating between green-labeled phagocytic targets attached to the cell surface or in solution and those that have actually been ingested can be accomplished by adding trypan blue[1595] or crystal violet[1596] to the sample. A somewhat more informative technique allows neutrophils to phagocytose fluorescein-labeled, heat-killed yeast cells, after which ethidium bromide is added to the solution. Ingested yeast cells are green; those attached to the cell surface are red[1597]. Similar distinctions can be made by allowing cells to ingest opsonized green fluorescent beads and then adding red fluorescent antibodies to stain the externally bound, but not the ingested beads[1598]; this requires lots of fluorescence compensation, and would probably work better with red beads and green antibodies. If all you want to measure is the ingested

labeled bacteria, a commercial lysing reagent can be used to get rid of the rest[1599].

Ma et al[1600] used crystal violet to quench fluorescence from antibody in solution, which allowed them to demonstrate internalization of fluorescein-conjugated IgG from normal serum or serum containing anti-ribonucleoprotein by normal lymphocytes. The results showed that 54% of normal lymphocytes were penetrated by anti-RNP antibody and 23% by normal IgG, respectively. Suzuki et al[1601] employed DiI as a label to study endocytosis of lipid microspheres. Haynes et al[1602] studied intracellular digestion of endocytosed albumin by labeling the protein so heavily with rhodamine that the fluorescence of the dye was largely quenched; as protein was hydrolyzed within phagocytic vacuoles, small rhodamine-labeled fragments were released into solution, and became more fluorescent.

Wang Yang et al[1603] used pulse width and area measurements to analyze capping and endocytosis of fluorescein-immunoglobulin by mouse B cells; fluorescence decreased when the material entered acidic subcellular compartments. Chanh and Alderete[1604], however, observed decreases in fluorescence intensity associated with capping itself.

Bassøe et al[2763] have recently described the use of multiple probes, flow cytometry, and confocal microscopy to dissect the phagocytic process in neutrophils; they were able to demonstrate formation of reactive oxygen species (ROS) (pp. 379-80) in phagosomes.

Moving away from the mammals, flow cytometry and mathematical modeling have been applied to analysis of feeding by the ciliated protozoan *Tetrahymena pyriformis*[1605-7]. Flow cytometric analysis of the distribution of various types of microplankton before and after exposure to larger organisms has also been used to determine the dietary habits of the latter[1608]. Nature is red in cilium and pseudopod...and if it isn't, we can stain it so it is.

Enzyme Activity

Enzyme activity in single cells can be demonstrated and quantified by flow cytometry following incubation of the cells with **chromogenic** or **fluorogenic substrates**[358], which, respectively, yield colored and fluorescent products. Some products normally detected by their absorption are fluorescent, so the distinction between chromogenic and fluorogenic substrates can be blurry. The colored reaction products of chromogenic substrates are detected by scatter and absorption or extinction measurements; forward and side scatter as measured in conventional fluorescence flow cytometers are generally usable for this type of analysis. We have already encountered fluorogenic substrates in the contexts of membrane integrity determination (fluorescein diacetate, etc.) and signal amplification (p. 344).

Bayer's instruments for differential leukocyte counting (developed by Technicon, which was acquired by Bayer) measure **peroxidase** activity by scatter and absorption to discriminate among lymphocytes, monocytes, neutrophils, and eosinophils[84-5]. These systems have also been adapted to study staining kinetics[348] and, using immunoenzyme staining methods, to analyze T cell subsets in peripheral blood[279,1585]. Ross et al[1609] described a pararosaniline method for esterase staining and a naphthyl phosphate method for alkaline phosphatase staining, allowing the Technicon H-1 to be used for those relatively common cytochemical analyses. Kaplow, who collaborated in this work, had earlier used chromogenic substrates with the Bio/Physics Cytograf, which measured scattering and extinction of 633 nm laser light, to demonstrate esterase, peroxidase, and phosphatase activities in blood cells[359-60].

Dolbeare and Smith[358] discussed a number of **fluorogenic substrates** that can be used to demonstrate the activity of enzymes in, and in some cases on, cells; many such materials are available commercially. The majority of fluorogenic substrates are derivatives of **fluorescein**, of **coumarins** such as **4-methylumbelliferone (4-MU)**, or of **α-naphthol**. Substrates derived from fluorescein, as would be expected, yield blue-excited, green fluorescent products, while coumarin and α-naphthol-based substrates, the latter more often employed as chromogens rather than fluorogens, form products that require UV or violet excitation and emit in the blue and green regions of the spectrum. Derivatives of **resorufin**, which form green- to yellow-excited, orange-red fluorescent products, can be used to demonstrate esterases and oxidative enzymes. The excitation maximum of resorufin, however, is at about 570 nm, necessitating the use of a krypton laser at 568 nm, a dye laser, or, possibly, the 577 nm line of a mercury arc lamp for excitation.

Dolbeare and Vanderlaan[363] and Smith and Dean[364] have described fluorogenic reagents based upon other UV-excited, blue fluorescent and blue- excited, green fluorescent materials for flow cytometric demonstration of peptidases and acid phosphatases. Fluorogenic substrates suitable for use with red excitation are uncommon; Lee, Berry, and Chen[1610] described one candidate, **vita blue**, which could be derivatized for use as both an esterase substrate and a pH indicator, but the dye hasn't made it to the majors yet, as far as I know.

If cells are incubated with two (or more) fluorogenic substrates that yield products with different spectral characteristics, it is possible to demonstrate and monitor several enzymes in cells using multiparameter flow cytometric analysis[364-7]. Since the amount of detectable reaction product in a cell at any given time depends on many factors, including the rate of accumulation of substrate in the cell, the rate of entry of substrate to the cellular compartment containing enzyme, the rate of enzymatic reaction, and the rate of efflux of reaction product from the cell, it is not surprising that cellular fluorescence values, especially for different cell types, do not always correlate with enzyme activities[824].

Dick Haugland reviewed fluorogenic substrates and their uses in an 8-page article in 1995[2764]; since his current (2002) edition of the Molecular Probes *Handbook*[2332] devotes 56 pages to the subject and probably has at least 8 pages of references, you probably don't need to look for the article. Both article and handbook emphasize the desirability of

trapping the fluorescent product in or on cells for flow cytometry and other assays done on cell suspensions.

Indicators of Oxidative Metabolism I: Tetrazolium Dye Reduction

Blairet al[820] used forward and side scatter measurements to demonstrate the development of oxidative enzyme activity in HL-60 human promyelocytic leukemia cells stimulated to granulocytic differentiation by dimethyl sulfoxide. The reagent employed was **nitroblue tetrazolium (NBT)**[361], widely used in histo- and cytochemistry for demonstration of **oxidative enzymes**. NBT, which is colorless, is reduced to a diformazan that forms an amorphous blue-black precipitate. **NBT reduction** is used as an indicator of the function of mature granulocytes and to determine whether morphologically immature cells belong to the myeloid series.

Blair et al found that the forward scatter signal was decreased, and the orthogonal light scatter signal was increased, in cells that reduced NBT. This work was noteworthy because it called attention to a general method for demonstrating color reactions in cells (or at least those which yield products with very high extinction) that requires neither absorption nor extinction measurements, and thus could be used with most flow cytometers.

Huet et al[1611] used side scatter signals for flow cytometric analysis of **3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT)** reduction; the color reaction produced when MTT is reduced is commonly used as a viability indicator in cell cultures. Fattorossi et al[1612] devised an alternate method for measurement of tetrazolium dye reduction by neutrophils; the cells were labeled with fluorescein-con A, the fluorescence of which was quenched by the formazan product of NBT reduction. Van Noorden, Dolbeare, and Aten[1613] proposed a more general method for detection of enzymatic reactions yielding colored formazan products; the formazan quenches glutaraldehyde-induced "autofluorescence" in cells.

Fluorescent tetrazolium dyes were investigated by Severin and Stellmach[821-2], who measured oxidative activity of living cells using **cyanoditolyl tetrazolium chloride (CTC)**, which is reduced to a water-insoluble fluorescent formazan that has an excitation maximum at 450 nm and emits in the range from 580 to 660 nm. CTC generates more product than NBT[1614]; Huang and Severin[1615] used it with lactate, fumarate, and other metabolic intermediates to characterize the activities of six different dehydrogenases in Ehrlich ascites cells, and it has become fairly popular for studies of bacteria[2252].

Indicators of Oxidative Metabolism II: 2,7-Dichlorofluorescin Diacetate (DCFH-DA), etc.

Bass et al[362] used another fluorogenic material to demonstrate oxidative metabolism of blood granulocytes. **2,7-dichlorodihydrofluorescein diacetate**, also known as **dichlorofluores*cin* diacetate (DCFH-DA** or **H_2DCFDA)**, like FDA, is uncharged, nonfluorescent, and lipid soluble, and readily penetrates cells. Nonspecific intracellular esterases transform the ester into a nonfluorescent intermediate, DCFH. In the presence of **peroxidase**, and of H_2O_2 formed during the **respiratory burst** in activated granulocytes, DCFH is converted to the fluorescent **2,7-dichlorofluorescein (DCF)**. Duque and Ward[821] described the use of DCFH-DA and other dyes for quantitative assessment of neutrophil function. Cells are loaded with 5 μM DCFH-DA for 15 minutes at 37 °C, after which gated fluorescence analysis of the neutrophil population, identified by forward and side scattering characteristics, is done over time following administration of a stimulus such as a phorbol ester. Like fluorescein, DCF does leak out of cells; Molecular Probes offers **carboxy-H_2DCFDA**[2765], which responds to the respiratory burst by forming carboxydichlorofluorescein (carboxy-DCF), which is better retained by cells than is DCF[2442].

DCFH-DA has been used in combination with various particles for combined analyses of phagocytosis and respiratory burst in neutrophils[1590-1,1593-4]. The presence of peroxidase in neutrophils is evidently important for the development of the fluorescent product; monocytes, which have much less peroxidase, don't become as brightly fluorescent as neutrophils, even when H_2O_2 is added[1616].

Maresh and Monnat[1618], studying cells derived from myeloid leukemias, note the existence of a DCFH-DA oxidative pathway stimulated by fluoride, which appears distinct from that involved in the respiratory burst, and wonder whether a similar pathway in normal cells might confound some results obtained with this reagent.

Indicators of Oxidative Metabolism III: Hydroethidine (Dihydroethidium)

Dihydroethidium is probably more widely known under the name **hydroethidine (HE)**, which is a trademark of Prescott Laboratories. It is the product of reduction of ethidium by sodium borohydride; described in 1984 by Gallop et al[973]. The UV-excited, blue fluorescent HE, unlike ethidium, readily enters live cells, in which it may be oxidized to ethidium, which then exhibits its characteristic blue- or green-excited red fluorescence, enhanced by binding to double-stranded nucleic acids. HE has been used as an indicator of viability[1619]; when it is added at low concentrations, little ethidium accumulates in the nuclei of dead cells, while those of live cells become red fluorescent. HE has also been used as an indicator of the neutrophil respiratory burst[1592]; while DCFH-DA responds primarily to **hydrogen peroxide** generation, HE is more affected by **superoxide**, and the two dyes, used separately, allow discrimination of these two types of **reactive oxygen species (ROS)**[1620-1].

Indicators of Oxidative Metabolism IV: Dihydrorhodamine 123

In 1988, Rothe, Oser, and Valet[1622] introduced **dihydrorhodamine 123**, which is oxidized to the green fluorescent cationic dye **rhodamine 123**, to detect the respiratory burst in neutrophils. According to Henderson and Chap-

pell[1623], rhodamine 123 fluorescence is produced only in cells containing peroxidase, meaning that the dye is relatively insensitive to superoxide production. Dihydrorhodamine is said to provide brighter signals than DCFH-DA.

Indicators of Oxidative Metabolism V: Detection of Hypoxic Cells

Some of us, e.g., radiobiologists and radiotherapists, are interested in finding cells that aren't getting and/or using their share of oxygen. Hypoxic cells in transplanted tumors and tumor cell spheroids had been distinguished by their pattern of uptake of Hoechst 33342[1624-6], but attempts were later made to develop more specific markers. Hodgkiss et al[1627-8] prepared 2-nitroimidazole derivatives with coumarin and indolizine chromophores that appeared useful for hypoxic cell detection. A more recent report[2766] utilized 2-nitroimidazole bound to theophylline (NITP). Under hypoxic conditions, this molecule is bioreductively bound to cellular macromolecules; it is then detected using anti-theophylline antibodies.

Detection of Caspase Activity

Caspases (cysteine-aspartic acid specific **proteases**) are activated by cell death inducing stimuli, and several reagents have been described for detection of caspase activity in apoptotic cells. **PhiPhiLux®** fluorogenic caspase substrates[2556-7] with either green or yellow emission, excitable at 488 nm, are available from **OncoImmunin**. Molecular Probes offers UV-excited, blue fluorescent (aminomethylcoumarin-based) and blue-green excited, green fluorescent (rhodamine 110-based) substrates for caspase-8, which is activated early in apoptosis, and caspase-3, which is activated later[2332]. Caspases can also be detected using fluorescent inhibitors. **Benzyloxycarbonyl-valinealanine-aspartic acid-fluoromethyl ketone (zVAD-FMK)** is a potent broad-spectrum inhibitor of caspases that binds irreversibly (covalently) to the enzyme active site and can block apoptosis. **Serologicals Corporation** offers a line of caspase inhibitors (originally Intergen's) usable for cytometry[2769]. **FAM-VAD-FMK** is a carboxyfluorescein (FAM) derivative of zVAD-FMK, and blocks apoptosis[2770]; there are also FAM-labeled inhibitors selective to various degrees for individual caspases, including caspase-3 and caspase-8, and yellow fluorescent sulforhodamine B-labeled (SR) derivatives of zVAD-FMK and of a caspase-3 inhibitor. **Immunochemistry Technologies** is an alternate source. Since pockets of caspase activity may be localized within cells, it may be advantageous to use scanning rather than flow cytometry for studies of caspases in apoptosis[2770-2].

Other Enzymes

Cathepsin L activity[1633-4] can be measured flow cytometrically in single viable cells by the intracellular cleavage of non-fluorescent (Z-Phe-Arg)2-rhodamine 110 to the green fluorescent monoamide Z-Phe-Arg-rhodamine 110 and rhodamine 110. (Z-Phe-Arg)2-R110 and (Z-Arg-Arg)2-R110 are several hundredfold more selective for cathepsin L than for cathepsin B, providing sensitivity for the former. Rhodamine-110-based substrates for nonspecific **aminopeptidases** have also been developed[2773].

For assessment of activities of **lysosomal enzymes**, van Noorden[1635] reports that the best results have been obtained with methods based on naphthol AS-TR derivatives and with methods for the demonstration of protease activity using methoxynaphthylamine derivatives as substrates and 5'-nitrosalicylaldehyde as coupling reagent.

Dive, Workman, and Watson[1636] used flow cytometry to measure the activity of **gamma-glutamyl transpeptidase**, by monitoring the conversion of gamma-glutamyl aminomethylcoumarin to aminomethylcoumarin. This was technically difficult because of the location of the enzyme on the cell exterior, resulting in rapid escape of the product.

Huang et al[1639-40] have developed fluorogenic substrates for **alkaline phosphatase**. Molecular Probes' **ELF-97 phosphate**[2774-5] produces a violet-excited, green-yellow fluorescent insoluble precipitate when cleaved by either alkaline or acid phosphatases; it can be used to demonstrate phosphatases in cells or for enzyme amplification using alkaline phosphatase-tagged antibodies or nucleotide probes (p. 344). This should be a good reagent to use with violet diode lasers.

Meshulam et al[1369] measured **phospholipase A** activation using bis-BODIPY-phosphatidylcholine, which localizes in the inner leaflet of the cell membrane. Sidhu et al[1641] used ethoxyresorufin and scanning laser cytometry to measure the activities of **cytochrome P-4501A1** and **NADPH DT-diaphorase**.

Detection of Enzymes and Products by Antibodies

Like other proteins, enzymes can be detected in or on cells using antibodies, although antibody reactivity doesn't absolutely indicate enzyme activity.

Tran-Paterson et al[1629] compared levels of the neutral endopeptidase-24.11, also known as the CD10 or CALLA antigen (Common Acute Lymphocytic Leukemia Antigen) on normal granulocytes, leukemic cells, and transfected COS-1 cells, as detected using fluorescent anti-CD10 antibody and by analysis of enzyme activity in cell suspensions; they found good correlation between results obtained by the disparate methods. Milhiet et al[1630] used a fluorescent inhibitor, *N*-[fluoresceinyl]-*N'*-[1-(6-(3-mercapto-2-benzyl-1-oxopropyl) amino-1-hexyl]thiocarbamide (FTI), for flow cytometric detection of CD10 as an enzyme.

Antibody detection of the products of enzymatic reactions is a more direct indicator of enzyme activity than is antibody detection of the enzyme protein itself. Antibodies to **phosphotyrosine**[1631-2] have been used for flow cytometric quantification of **tyrosine kinase** activity.

Enzyme Kinetics in Single Cells

By making appropriate timed multiparameter flow cytometric measurements, it is possible, as Dive, Workman, and Watson[813,1637-8] have shown, to characterize intracellular enzyme reactions in considerable detail, determining relevant

parameters of reaction and inhibition kinetics. As is always the case when kinetic measurements are made using flow cytometry, it is necessary to validate the assumption that the time courses of reactions are similar in at least a substantial fraction of the cells in the sample. Scanning laser cytometry can be used to study enzyme kinetics in individual cells over time when this is necessary or desirable[2776-8].

Sulfhydryl (Thiol) Groups; Glutathione

Sulfhydryl or **thiol** groups in protein, and non-protein thiol compounds such as the tripeptide glutamyl-cysteinyl-glycine, more commonly known as **glutathione**, play an important role in cells' oxidative metabolism. When cells become hypoxic, the sulfhydryl/disulfide ratio increases, and the free radical scavenging properties of thiols are believed to play a pivotal role in increasing the resistance of hypoxic tumor cells to ionizing radiation[332] and to some chemotherapeutic agents. Flow cytometry of glutathione levels, in particular, has acquired new importance because of their relation to drug resistance in cancer cells[801,1116] and the progression of HIV infection[1117-8].

Olive and Durand[333,4] examined several maleimide and **bromobimane** derivatives as reagents for determination of cellular thiols. A maleimide derivative of coumarin emits blue fluorescence and can be used with UV excitation; **monobromobimane (MBB)** and didansylcysteine have similar spectral characteristics. **Fluorescein-5-maleimide** penetrates viable cells less well, but excites at 488 nm.

Treumer and Valet[800] and Rice et al[801], respectively, characterized ***o*-phthaldialdehyde (OPT)** and **monochlorobimane (MCB)**, both of which form UV-excited, blue fluorescent products with reduced glutathione (GSH), as rapid and specific vital stains for this substance. These reagents were used to demonstrate heterogeneity in GSH content in tumor cell populations. O'Connor et al[1649] advocated the use of **mercury orange**, and Poot et al[1650] the use of **chloromethylfluorescein**, both of which can be excited at 488 nm with, respectively, orange and green emission, for glutathione measurement.

While MCB is probably the most specific reagent for glutathione, because its reaction with the peptide is catalyzed by glutathione S-transferase, the form of this enzyme predominant in human cells (GST-pi) is much less effective in catalysis than is the form found in rodents[1651-2]. In 1994, Hedley and Chow[1653] evaluated a wide variety of methods for measurement of glutathione by flow cytometry, concluding that MBB was the reagent of choice for work with human cells. They found that MBB could be adequately excited by the 325 nm line of a He-Cd laser; its absorption maximum is 394 nm, making a violet diode laser a near ideal source for exciting the probe. MBB emission can be measured adequately at 450 nm; the emission maximum is at 490 nm, but one would normally want to cut off detection well below this wavelength to avoid interference from 488 nm laser light. Mercury orange was found usable, although results were less consistent and background fluorescence was higher. Chow and Hedley[2779] have measured GSH in clinical samples, attempting to correlate GSH concentration and drug resistance. MBB staining of intracellular glutathione is illustrated in Figure 7-25.

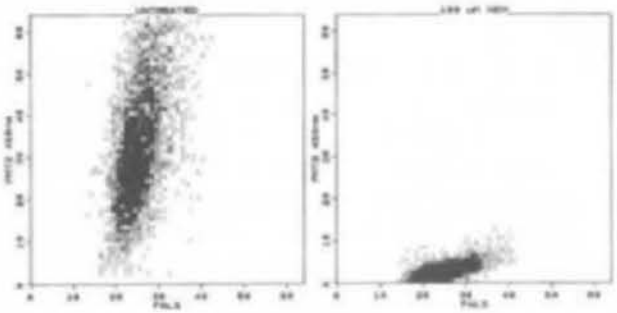


Figure 7-25. Specific staining of glutathione in cells by monobromobimane (MBB). The x-axis shows forward scatter, the y-axis MBB fluorescence, which is greatly reduced in the sample shown in the right panel, which was treated with N-ethylmaleimide (NEM) to deplete intracellular glutathione. The figure was provided by David Hedley (Princess Margaret Hospital, Toronto).

7.13 FUNCTIONAL PROBES II: INDICATORS OF CELL ACTIVATION

Introduction

The functional parameters I will emphasize in this section, i.e., **"structuredness of cytoplasmic matrix" (SCM)**, **cytoplasmic and mitochondrial membrane potential**, **"membrane-bound"** and **cytoplasmic calcium ion concentration**, and **intracellular pH**, and the effects of cell surface ligand-receptor interactions on these parameters, have been the subject of a good deal of research and a large number of publications.

Much of the earlier work, and of the earlier literature, did not deal with flow cytometry or with alternative methods of analysis of functional parameters in single cells. Most investigators now working in this area are well aware of the capabilities of flow cytometry, and are in possession of at least some of the information that has already been gained from flow cytometric studies of functional parameters and of the probes used for their measurement.

Cytoplasmic calcium ion concentration ($[Ca^{++}]$) and distribution, intracellular pH, and membrane potential ($\Delta\Psi$) have all been observed to change during the early stages of **surface receptor-mediated activation processes** related to the development, differentiated function, and pathology of a large number of cell types. Accordingly, $[Ca^{++}]$, pH, and $\Delta\Psi$ have, individually and collectively, been envisioned as "**second messengers**" mediating responses to cell surface ligand-receptor interactions.

The investigation of changes in the cellular ionic environment during activation has been actively pursued in

hopes of clarifying the mechanisms of receptor function and of developing more precise tests of cell function for clinical and research use. Studies in this area have been facilitated by the development of fluorescent probes that allow estimation of [Ca^{++}], pH, and $\Delta\Psi$ in cell populations and, in conjunction with instrumental methods such as flow cytometry, in individual cells.

While the current state of the art appears to allow fairly precise quantitation of [Ca^{++}], pH, and $\Delta\Psi$ in terms of population averages, the use of flow cytometry to measure the same probes in single cells can reveal and has revealed marked heterogeneity even within supposedly homogeneous cell populations. Flow cytometric analysis of the effects of ligands, agonists, and antagonists on individual cells may yield otherwise unobtainable information about mechanisms of receptor function. It can also provide considerable insight into the sources of inaccuracy in measurements based on existing fluorescent probe technology.

Changes in the Cellular Ionic Environment Following Activation by Ligand Interaction With Cell Surface Receptors

In 1977, when I got interested in this subject, I read a then current review by Sonenberg and Schneider[421] dealing with physical and chemical changes in receptors, in the membrane, and in other cellular structures following ligand interactions with cell surface receptors. Few of the details had been worked out back then (for example, we didn't know JAK about kinases, and NO way did we suspect that a gas could act as a messenger), but the review and a few other articles written in that era did have the big picture:

The earliest detectable biochemical changes occur in milliseconds to seconds after the number of occupied receptor sites increases following exposure of cells to ligand. They include alterations in transmembrane ion fluxes, in intracellular [Ca^{++}], pH, and $\Delta\Psi$, and in activities of membrane-associated enzymes such as adenylate cyclase, protein kinases, and phospholipid methyltransferases[422].

Within seconds to minutes, there may be changes in cyclic nucleotide concentrations, increased protein phosphorylation, and alterations of membrane uptake of sugars, amino acids, and fatty acids. Ca^{++} redistributions within the cell and changes in the mobility of membrane lipids and proteins also may occur in this time frame. The biologic response to surface stimulation may occur in milliseconds, as happens in nerve impulse transmission, or after days, as in mitogenic activation of lymphocytes.

The observation that changes in [Ca^{++}], pH, and/or $\Delta\Psi$ occurred early in the course of interaction of many different ligands with cell surface receptors in diverse cell types led to investigation of the role of ionic species in signal transduction between the cell surface and the nucleus and cytoplasmic organelles such as mitochondria. Ion flux changes were observed, and assigned a role in signal transduction, in the activation of lymphocytes[423-7], platelets[204,428-31], mast cells[432-5], and neutrophils[436-8], and in growth factor action[439-44], as well as in many other cell activation processes.

A single pattern of ionic events has been observed in most of the cell activation processes that have been examined to date. Following ligand binding to cell surface receptors, there is a rapid influx of Ca^{++}, usually accompanied by release of intracellular "membrane-bound" Ca^{++}, and resulting in a transient **rise in free cytoplasmic [Ca^{++}]**, which lasts for a few minutes at most. There is also typically an **increase in intracellular pH**, resulting from **Na^+/H^+ exchange** or **antiport**, which can be inhibited by amiloride. Changes in **membrane potential ($\Delta\Psi$)**, when they are observed, can go either in the direction of **depolarization (decrease in transmembrane potential)** or **hyperpolarization (increase in transmembrane potential)**, and appear to be due primarily to Na^+ and/or K^+ shifts across the membrane.

In some cases, **crosslinking of two or more receptors by a multivalent ligand** is required to induce ion flux changes; in others, binding of monovalent ligands to receptors is all that is necessary. When ion flux changes are initiated by crosslinking, bivalent antibodies to the receptor can produce the same effect as the natural ligand, while monovalent fragments cannot[432]. In most cases, materials that induce ion flux changes of the same nature and magnitude as those induced by natural ligands also induce the biologic responses induced by those ligands. For example, the **calcium ionophore A23187** is mitogenic for T lymphocytes, induces platelet aggregation, stimulates histamine release from mast cells, etc.[444].

If one monitors the average [Ca^{++}], $\Delta\Psi$, or pH of millions of cells in a suspension, it is possible to demonstrate **dose-response relationships** between the amounts of active ligand added and the magnitude of changes observed in the measured parameters. When the same parameters are followed in single cells, a correlation between ligand dose and degree of response may also be observed[362]. However, when the biologic responses of individual cells are examined, the usual pattern of response appears to be **all-or-none**[194]. Neurons exposed to neurotransmitters either generate a propagated action potential or they do not; lymphocytes either reproduce or do not, and so on.

It is generally accepted that an individual neuron is subject to the excitatory and inhibitory influences of various neurotransmitters, which are secreted by neighboring cells and which act on its dendrites and cell body, raising or lowering its resting membrane potential and, accordingly, changing its firing threshold. The membrane is a locus for summation of excitatory and inhibitory effects.

It is also known that the biologic responses of so-called "non-excitable" cells, such as lymphocytes, can be modulated by a great variety of cytokines, peptide hormones, neurotransmitters, and other ligands that react with cell surface receptors. It was hypothesized that, if the signals from these ligand-receptor interactions were transduced by ion fluxes across the cell membrane, the membranes of non-excitable

cells, like those of neurons, might serve as "summing nodes" for excitatory and inhibitory stimuli.

The fact that ionic responses to ligand binding to surface receptors seem, even at the single cell level, to be dose-dependent, while biologic responses tend to be all-or-none, suggested that there might be [Ca^{++}] and/or $\Delta\Psi$ or pH threshold levels that must be reached for biologic responses to occur. Investigation of this possibility would require means to detect and quantify heterogeneity of cell populations with respect to ligand binding, [Ca^{++}], $\Delta\Psi$, pH, and changes in these parameters. Doing this nondestructively should allow one to make serial observations of cells, which could establish relationships between the nature and magnitude of cells' early responses to ligands and the same cells' subsequent biologic behavior.

It seemed to me at the time that cytometry, flow and otherwise, might usefully and profitably be applied to such studies. I knew there existed some fluorescent probe techniques for estimation of $\Delta\Psi$, [Ca^{++}], and pH; and I thought it would be fairly simple to adapt them for use with flow cytometry, and not much harder to answer all of the biological questions raised above. This view, needless to say, was excessively optimistic, for many reasons, some of which I will explain after the upcoming grand tour of functional parameters.

"Structuredness of Cytoplasmic Matrix" (SCM) and the Cercek Test for Cancer

"Structuredness of cytoplasmic matrix," or SCM[414], was probably the first functional parameter measured by flow cytometry. The measurement of SCM, like that of membrane fluidity, requires fluorescence polarization measurements. In this case, the fluorescence is emitted by **fluorescein**, produced by the action of nonspecific cellular esterases on **fluorescein diacetate (FDA)**, about which a fair amount has already been said (pp. 24-7, 369-71).

The original work in this area was done, and the term SCM was coined, in the early 1970's by Lea and Boris Cercek[415], then working in Manchester, England. They measured fluorescein fluorescence polarization in cell suspensions in cuvettes using a modified spectrofluorometer, and, in analyses of synchronized cell cultures, found changes during different phases of the cell cycle. The **fluorescence polarization**, calculated as described on p. 114, was taken as a measure of SCM.

In studies done over a period of several years (summarized in reference 414), the Cerceks found that SCM changed within a few hours after human lymphocytes were exposed to mitogenic lectins or antigens. They also reported that lymphocytes from cancer patients exhibited diminished SCM responses (i.e., smaller changes in polarization after exposure) to the lectin PHA when compared to cells from controls, and that lymphocytes from patients with cancer showed more marked SCM responses after exposure to preparations containing tumor antigens than did cells from controls. These results seemed to promise a rapid method for cancer diagnosis, and therefore excited considerable interest among cancer researchers and oncologists and in the diagnostics industry.

From about 1975 on, several groups of investigators attempted to duplicate the Cerceks' results with cancer patients, and at least as many failed as succeeded; this led, among other things, to everybody criticizing everybody else's methodology. It therefore seemed logical to try to put things on a more objective basis by making measurements of single cells, which has since been done by microfluorometry[416] as well as by flow cytometry[338,407-9,417-19]. Stewart et al[338] reported differences in the fluorescein polarization responses of lymphocytes from breast cancer patients and controls following exposure to PHA and pooled tumor antigen; their study was designed to exclude observer bias. Hartmann et al[418] noted diminished polarization responses of lymphocytes from cancer patients compared to cells from controls.

On another front, Price et al[417] demonstrated changes in fluorescence polarization of bone marrow cells within a few hours following addition of preparations of (granulocyte) colony stimulating factor and erythropoietin. By sorting cells with the most marked responses, they were able to obtain suspensions enriched in granulocytic or erythrocytic precursors dependent upon which cell growth factor was used as the stimulus. This suggested that changes in SCM, whatever their physicochemical explanation might be, could serve as general indicators of the effect of activators such as mitogens and growth factors on responsive cells.

Udkoff et al[419] analyzed the effects of the concentration of lectin, FDA, Ca^{++}, and K^{+} on the polarization responses of lymphocytes from normal donors, and found that all of the above might influence the shape of both fluorescence polarization and intensity distributions. To put it in lay terms, sometimes it works and sometimes it doesn't, and, when it doesn't, you can't always tell why (G. Price, R. Miller, L. Kamentsky, R. Udkoff, S. Chan, W. Eisert, W. Beisker, H. Steen, L. Scherr, and others, personal communications). I might add that SCM is not the only indicator of cell activation with which this problem has been encountered. This inhibited commercial development of an SCM-based cancer diagnostic test, but left the way open for further investigation and application of the effect.

Meisingset and Steen[420] investigated binding of fluorescein to proteins and lymphocytes, and noted both polarization and spectral changes on binding. Clearly, the rotational mobility of free cytoplasmic fluorescein will be greater than that of fluorescein bound to intracellular macromolecules, and changes in the ratio of free and bound dye will therefore change the polarization measured in a cell or cell suspension. Based upon studies using **time-resolved spectroscopy**, Kinoshita et al[847] concluded that the fraction of bound molecules and the anisotropy produced by binding are the primary determinants of intracellular fluorescein fluorescence anisotropy; they did not suggest a mechanism by which changes might occur in activation. Prosperi et al[848] found that various membrane-active agents changed rates of

fluorescein influx and efflux; similar changes during activation might affect SCM by changing the ratio of free to bound dye.

The difficulty of measuring fluorescence polarization by flow cytometry has impeded investigation of the physicochemical basis of SCM response, although work with carefully modified commercial apparatus[1668-9] has established that polarization changes are routinely detectable in activated cells, and that such changes do not occur in the absence of calcium or in the presence of cytochalasin B.

The SCM test has been intensively studied by two Israeli physicists, Mordechai Deutsch and the late Aryeh Weinreb, and their colleagues at several hospitals and research institutions in Israel and elsewhere. Initial success with the SCM test using bulk fluorometry in the 1980's[1670-2] led Deutsch and Weinreb to develop a static cytometer, the Cellscan[1145], which uses a single photon counting technique (p. 164) to maximize the precision of measurements of fluorescence polarization of fluorescein and other dyes in single cells. A commercial version of this apparatus is available from **Medis Technology**; Deutsch et al have since developed a next generation apparatus, the Individual Cell Scanner[2780], which also makes high-precision polarization measurements.

Measurement precision is critical for SCM measurements because the largest changes in polarization tend to occur in those cells with the lowest intensity of intracellular fluorescence. The precision of a fluorescence intensity measurement made in a flow cytometer, in which observation time is constant, decreases as intensity decreases because fewer photoelectrons contribute to lower intensity measurements. The polarization values calculated from low intensity fluorescence measurements will also be less precise. The Cellscan and Individual Cell Scanner count a preset number of photons (usually 10,000 or 20,000) in each fluorescence measurement channel, and derive fluorescence intensity values from the time taken to reach the preset photon count; the contributions to variance from photoelectron statistics are therefore the same for both low and high intensity fluorescence measurements.

The polarization of intracellular fluorescein fluorescence in T cells decreases when the cells are exposed to mitogens; the average polarization of mitogen-treated cells is 9.7% lower than that of control cells at 15 min following addition of mitogen, and 13.5% less at 180 min[2781]. The coefficient of variation of the distribution of polarization values, presumably reflecting biologic variation, is large, approximately 75 percent, and, if the measurement technique itself is not highly precise, it may not be possible to distinguish differences between treated and control cells.

We now know a great deal about SCM and changes in SCM. The effect can be demonstrated using carboxyfluorescein[2782] and BCECF[2783] as well as fluorescein. Decreases in polarization, beginning at 40 min after exposure and lasting 24 hr, are induced by the T cell mitogens PHA, Con A, and anti-CD3 antibody, by phorbol esters, and by the calcium ionophore ionomycin, but not by pokeweed mitogen (PWM), a B cell mitogen[2781,2784]. The effects of ConA can be counteracted by addition of a competing sugar, methyl α-D-mannopyrannoside within 1 hr, but not after 5 hr[2781]. The mitogen-induced decrease in fluorescence polarization is inhibited by inhibitors of energy metabolism (NaN_3, NaF, KCN, and a proton ionophore), by proton kinase C inhibitors (H7, staurosporin) and by agents that disrupt cytoskeletal microtubules (colchicine, the *Vinca* alkaloid vinblastine) and microfilaments (cytochalasin B)[2781,2785].

Intracellular fluorescein fluorescence polarization values measured in cells from the Jurkat T-lymphoblastoid line were lower in S and G_2/M phase cells than in G_0/G_1 cells[2786]. Fluorescein fluorescence polarization in human lung-derived fibroblasts was decreased by IL 1-α and IL 1-β, and by TNF-α; the effects of these cytokines were inhibited by vinblastine[2787]. Increases in fluorescence polarization in mouse thymocytes and Jurkat cells occur in the early stages of spontaneous apoptosis and of apoptosis occurring in response to treatment with glucocorticoids, Fas ligand, or cancer chemotherapeutic agents; the increase in polarization is unaffected by treatment with caspase-3 inhibitors. The polarization changes in apoptosis appear to be associated with cell dehydration and shrinkage[2788-9].

In an earlier edition of this book, I suggested that intracellular pH changes related to cell activation might play a part in the SCM response. The excitation spectra of fluorescein, and of carboxyfluorescein and BCECF which can also serve as indicators of SCM response, are pH-sensitive, and changes in cytoplasmic pH occur in at least some cell activation processes, as will be discussed later, and could conceivably affect both the spectrum and the binding of the dye. However, after extensive discussions with Motti Deutsch and Reuven Tirosh, and consideration of recent findings[2780-9], I am persuaded that the effect is primarily due to changes in the organizational state and mobility of cytoplasmic proteins, and differences in free and bound amounts of different ionic forms of the dye. SCM changes are real; they're just hard to measure, which has given the whole procedure an undeservedly bad reputation.

Reports of clinical trials of the SCM test in cancer diagnosis continue to emerge[2790-8]; it seems to work overall, but is probably not quite sensitive or specific enough to be widely adopted. However, if you look at the data that have been obtained to date with single cell measurements, something very interesting emerges. Two phenomena are observed in the Cercek cancer test that supposedly differentiate between people with cancer and people without cancer. First, T cells from people with cancer don't show as much of a response to mitogens such as con A and phytohemagglutinin (PHA) as do T cells from people without cancer. That's old news; reports of diminished immune response in cancer, e.g., loss of skin reactions to tuberculin and other antigens, go back for generations. What isn't old news, however, is the observation that a substantial fraction, meaning 10 percent and sometimes several times that, of T cells from people with cancer show an SCM response, or evidence of activation, on

exposure to tumor-derived antigens, which have little or no effect on T cells from people without cancer. This suggests that people with cancer either have a greatly expanded clone or clones of antigen-responsive T cells, or that some recruitment phenomenon is occurring in people with cancer but not in normals.

As I hope will be made clear by the end of this book, there are a lot of ways of measuring lymphocyte activation cytometrically, ranging from looking at calcium fluxes in the first few minutes to measuring activation antigens such as CD69 and CD98 (4F2) and cytokine production after 4-8 hours to measuring increased RNA and DNA synthesis after 24 and 36 hours. We also now have tetramers that allow us to identify cells that respond specifically to antigens.

The time course of SCM changes is roughly that of calcium changes; none of the proponents of the SCM test seem to have followed up and found out whether the activated cells go on to express activation antigens and reproduce. The studies done on SCM in blood cell precursors by Price et al[417] offer some encouragement, but we don't have the data for lymphocytes. Assuming that the cells from cancer patients that are activated by tumor antigens are doing what comes naturally for specifically activated lymphocytes, we ought to be able to detect activation by any of the several means just mentioned, all of which are much easier to implement than fluorescence polarization measurements, and most of which can be implemented on commercial flow cytometers. If the Cercek cancer test really works, this could be a big shot in the arm for clinical flow cytometry, because a lot of people will be getting tested every year or two. If the cancer test doesn't work, at least we might learn something about tumor immunology by looking further into patients' T cell responses to antigen.

The Cerceks themselves moved from England to Southern California around 1980, and went to work for Beckman. Shortly after they got there, I helped some folks at Beckman to build a flow cytometer that was supposed to be used for SCM work. The flow cytometer project stopped, and nothing further was heard until 1993, when the Cerceks published three papers[1673-5] describing the isolation of a cancer-associated, SCM-recognition, immunedefense-suppressing, and serine protease-protecting (CRISPP) peptide from the blood plasma of cancer patients. A consensus, synthetic 29 amino acid CRISPP peptide (CRISPPs) has the same cancer SCM-recognition (CR) activity and SCM-response modifying effects as the natural peptides; in other words, if you add it to normal T cells, they behave like T cells from people with cancer. The Cerceks subsequently attempted to determine the genetic origin of the CRISPP peptide[2799], and reported that CRISPP peptide increased DNA synthesis in cultured hepatocytes, while increasing DNA synthesis at low doses and decreasing DNA synthesis in higher doses when added to PHA-stimulated cultures of human lymphocytes[2800]. Before Beckman and Coulter merged, Coulter looked into the SCM test and decided not to pursue it; it doesn't look as if Beckman was any more interested in the CRISPP peptides than in the SCM test.

Thus far, neither the Cellscan nor the Individual Cell Scanner seem to have been used to make a multiparameter measurement of fluorescein fluorescence polarization and of another activation-related parameter (e.g., cytoplasmic Ca^{++}, pH, cytoplasmic or mitochondrial membrane potential, early activation antigen expression, or cytokine production), or even to detect phenotypic differences between responding and nonresponding cells. It would be nice to look into all of that. Every couple of years or so, somebody runs across the SCM literature and asks me about measuring fluorescence polarization of fluorescein in lymphocytes using a flow cytometer, and I have to say that my limited experience bears out Motti Deutsch's contention that flow cytometry isn't precise enough to be useful. Lou Kamentsky was trying to implement the SCM test using Ortho's flow cytometers in the late 1970's, without much luck, and I convinced him to look for another indicator of activation that might be easier to measure. I proposed membrane potential, which brings me (and you) to the next topic.

Optical Probes of Cell Membrane Potential ($\Delta\Psi$)

Membrane Potential and Its Physicochemical Bases

Electrical potential differences exist across the membranes of most prokaryotic and eukaryotic cells. These potential differences are due in part to the existence of **concentration gradients** of Na^+, K^+, and Cl^- ions across the cell membrane, and in part to the operation of various **electrogenic pumps**.

The potential differences across the cytoplasmic membranes of resting mammalian cells range in magnitude from about 10 to 90 mV, the cell interior being negative with respect to the exterior. There is also a potential difference of 100 mV or more across the membranes of **energized mitochondria**, with the mitochondrial interior negative with respect to the cytosol; this potential is dissipated when **energy metabolism** is inhibited.

In **prokaryotes**, the enzymes responsible for energy metabolism are located on the inner surface of the cytoplasmic membrane, and the potential difference across this membrane, which is typically 100-200 mV, depends largely on energy metabolism.

The resting potential across the cytoplasmic membrane of mammalian cells is frequently estimated from the **Goldman equation**[455]:

$$\Delta\Psi = \frac{RT}{F} \ln \frac{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o}{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}$$

where $\Delta\Psi$ is the membrane potential, R is the gas constant, T is the temperature in degrees Kelvin, F is the Faraday, $[X]_i$ is the concentration of X ions inside the cell, $[X]_o$ is the con-

centration of X ions outside the cell, and P_X is the permeability of the membrane to X ions.

$\Delta\Psi$ Measurement Using Microelectrodes

Membrane potentials can be measured directly using **implanted microelectrodes**, but this becomes increasingly difficult as the technique is applied to smaller cells. Changes in $\Delta\Psi$ in response to ligand-receptor interactions have, however, been detected by such direct measurements. For example, Taki[456] reported that lymphocytes were electrically **depolarized** (i.e., the potential difference across the membrane decreased in magnitude) within 10 minutes following exposure to PHA; depolarization reached a maximum within a few hours, following which **hyperpolarization** (increase in magnitude of the potential difference) occurred over a period of several days. See p. 408 for a follow-up.

Indirect $\Delta\Psi$ Measurement in Cell Suspensions Using Distributional Probes

Indirect estimates of $\Delta\Psi$ can be obtained by monitoring the **distribution** of **radiolabeled lipophilic cationic indicators** (e.g., ^{3}H-triphenylmethylphos-phonium, or TPMP$^+$, which was described by Bakeeva et al in 1970[459]) or of **lipophilic cationic dyes**, such as cyanines and safranins, between cells and the suspending medium. Lipophilic indicators are used because this characteristic enables indicator molecules to pass freely through the lipid portion of the membrane; thus, the concentration gradient of an indicator species C^+ across the membrane is determined by the potential difference across the membrane according to the **Nernst equation:**

$$[C^+]_i/[C^+]_o = e^{-FE/RT},$$

where the notation is the same as was used previously. A ratio $[C+]_i/[C+]_o$ of 10 corresponds to a potential difference of 61 mV at 37 °C. Because their response to changes in $\Delta\Psi$ is dependent upon their distribution across the cell membrane, lipophilic indicator cations are often described as **distributional probes**.

Once cells have been equilibrated with an indicator cation, **depolarization** of the cells will cause **release of indicator from cells into the medium**, while **hyperpolarization** will make **cells take up additional indicator from the medium**. The indicator distribution will not adequately represent the new value of $\Delta\Psi$ until equilibrium has again been reached; this process requires periods ranging from a few seconds to several minutes. Thus, while distributional probes may be suitable for detection of slow changes in $\Delta\Psi$, they cannot be used to monitor the faster changes that occur during the propagation of action potentials in tissues such as nerve and muscle.

Most of the dyes now used as probes of $\Delta\Psi$ were developed during a systematic search by Lawrence Cohen, of Yale University, and his coworkers[457] for materials that would exhibit rapid enough changes in optical characteristics to respond to action potentials in nerve cells. Among the dyes evaluated were many that proved unsuitable because of their tendencies to redistribute across cell membranes in response to slow potential changes. As might be expected, most lipophilic cationic dyes fell into this category. One of these, **3,3'-dihexyloxacarbocyanine [$DiOC_6(3)$]**, was studied by Hoffman and Laris[458], who were able to make estimates of $\Delta\Psi$ in red blood cell suspensions based on the partitioning of the dye into the cells.

The use of a lipophilic cationic dye as a distributional probe of $\Delta\Psi$ involves only a slight departure from the distributional probe technique for $\Delta\Psi$ estimation using radiolabeled lipophilic cations The radioisotope method requires that a known volume of cells (or organelles, e.g., mitochondria) in suspension be equilibrated with the indicator; the intracellular and extracellular indicator concentrations are calculated from a determination of the amount of cell-bound indicator done by scintillation counting of the cells after washing to remove unbound indicator.

Estimation of $\Delta\Psi$ of cells in suspension using symmetric cyanine dyes[458] is done in a spectrofluorometer. Addition of cells to micromolar solutions of dyes such as $DiOC_6(3)$ produces a suspension with lower fluorescence than that of the original solution, indicating that, **at micromolar external concentrations, the fluorescence of dye taken into cells is quenched**. When intracellular and extracellular ion concentrations are manipulated to hyperpolarize the cells, increasing cellular uptake of dye, the fluorescence of the suspension decreases further; when the cells are depolarized, releasing dye into the medium, the fluorescence of the suspension increases. The results obtained by Hoffman and Laris using this method to estimate $\Delta\Psi$ in giant red cells from *Amphiuma* compared favorably with the results these authors obtained by microelectrode measurements.

This success led to a comparative study by Sims et al[460] in which 29 cyanine dyes were examined as indicators of $\Delta\Psi$ in red cells and lipid membrane vesicles. This paper introduced the **abbreviated nomenclature for the cyanine dyes** that is now in general use, explained in Figure 7-12 (p. 313). The lipophilicity of cyanine dyes increases with the length of the alkyl side chains, i.e., as n in the formula "**$DiYC_{n+1}(2m+1)$**" increases. The wavelengths of maximum absorption and emission are essentially independent of the alkyl side chain length, denoted in the formula by (n + 1), but increase with the length of the polymethine bridge between the rings, i.e., as m in the formula increases[461]. The quantum efficiency of the dyes is increased, and the absorption and emission maxima are shifted to longer wavelengths, in nonpolar solvents. Spectra of some symmetric cyanine dyes and of tandem conjugates of phycobiliproteins and cyanine dyes appear in Fig. 7-9 (p. 296); the wide range of spectral characteristics available results in large part from the early development of the dyes by Eastman Kodak and their exploitation as photographic sensitizers[461].

The report of Sims et al[460] gave particular prominence to two dyes, **dipentyloxacarbocyanine** [$DiOC_5(3)$] and

dipropylthiadicarbocyanine [DiSC$_3$(5)], because they exhibited larger fluorescence changes than most of the other dyes in response to maximal hyperpolarization of the cells induced by the **potassium-selective ionophore valinomycin** (see reference 444 for an extensive discussion of ionophores), and because they were relatively stable and did not cause excessive lysis of cells.

Some people mistakenly assumed that DiOC$_2$(3) and DiSC$_3$(5) were the only cyanine dyes suitable for $\Delta\Psi$ estimation; this caused some problems because these dyes, and many of the others described by the Yale group, were not available commercially. The many investigators worldwide who became interested in using dyes for $\Delta\Psi$ measurements were supplied with them by Alan Waggoner[462], now at Carnegie Mellon University, who was responsible for most of the chemistry in the Yale project. Most people did not realize that dyes such as DiOC$_6$(3) and DiSC$_3$(5), which, respectively, can be substituted for DiOC$_2$(3) and DiSC$_3$(5), were readily available from Eastman and other companies (Eastman's product line is now sold by Acros Organics, but the dyes remain widely available). Results typical of those obtained in cuvette measurements with cyanine dyes are shown in Figure 7-26.

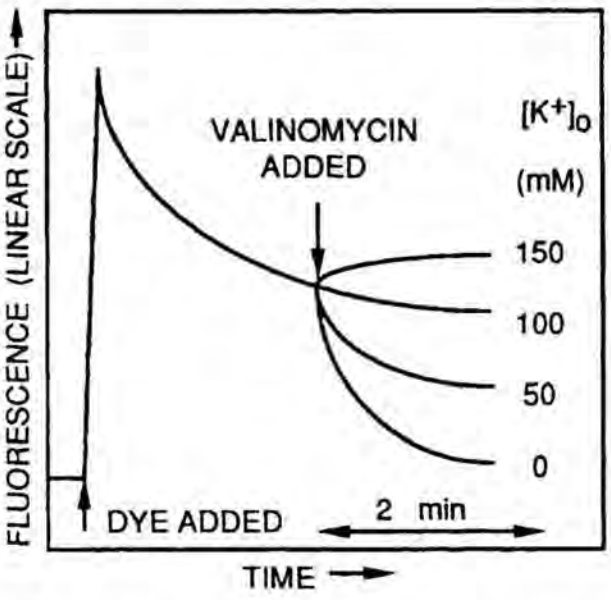


Figure 7-26. Valinomycin-induced changes in fluorescence intensity of red cell suspensions equilibrating with a cyanine dye at various external K^+ concentrations.

Under normal circumstances, the intracellular concentration of K^+, $[K^+]_i$, is considerably higher than the extracellular concentration $[K^+]_o$, while the intracellular concentration of Na^+, $[Na^+]_i$, is considerably lower than the extracellular concentration $[Na^+]_o$. The ionophore **valinomycin (VMC)**, which is lipophilic, forms a complex with K^+ ions and can thus readily transport them across cell membranes. Addition of VMC thus effectively increases the cells' potassium permeability (P_K in the Goldman equation), to the point at which membrane potential is determined almost entirely by the transmembrane $[K^+]$ gradient. If $[K^+]_o$ is low, VMC addition hyperpolarizes cells; if $[K^+]_o$ is high, VMC addition depolarizes cells, and, if $[K^+]_o = [K^+]_i$, VMC addition does not change $\Delta\Psi$.

Data such as those shown in Fig. 7-26 are obtained by normalizing curves so that the levels of fluorescence in each sample prior to VMC addition are identical. This normalization is necessary because even when the concentrations of cells and dye added to clean cuvettes are carefully controlled, equilibrium fluorescence readings obtained from cell suspensions vary due to such factors as dye adhesion to the cuvette walls. Despite this, people who worked with this technique for $\Delta\Psi$ estimation developed the notion that cyanine dye fluorescence could somehow be calibrated to read out $\Delta\Psi$ to the nearest millivolt. This was credible only as long as nobody looked at cyanine dye uptake at the single cell level. As far as I can determine (L. Cohen, personal communication; A. Waggoner, personal communication), cyanine dye-stained cells, other than nerve or muscle cells, weren't even looked at under a fluorescence microscope until I started playing with $\Delta\Psi$ probes in the mid-1970's.

At that time, the literature stressed the point that $\Delta\Psi$ estimation with cyanine dyes depended on **quenching of the fluorescence of intracellular dye**; in essence, the assumption was being made that all of the fluorescence measured in the cell suspension was coming from free dye in solution, or at least that the contribution of cell-associated dye to the total fluorescence signal remained constant. When I first looked at cells stained with micromolar concentrations of cyanine dyes, such as were used for $\Delta\Psi$ estimation in suspensions, I expected to see dark cells against a fluorescent background, which would certainly not make it easy to adapt cyanine dye $\Delta\Psi$ probes for flow cytometry. I was pleasantly surprised when I found brilliant fluorescence in the cells, and somewhat disappointed when I examined the broad fluorescence distributions (Figure 7-27, next page) obtained when they were run through a flow cytometer[424].

Single Cell Measurements with Distributional Probes

According to the Nernst equation (p. 386), $[C^+]_i/[C^+]_o$, i.e., the ratio of the intracellular and extracellular **concentrations** of a membrane-permeant cationic dye C^+, varies with $\Delta\Psi$. Since the extracellular concentration $[C^+]_o$ is the same for all cells in a suspension, the intracellular concentration $[C^+]_i$ should provide an indication of $\Delta\Psi$. If $\Delta\Psi$ is the same in all the cells, $[C^+]_i$ should be the same. However, the fluorescence distribution is not a distribution of dye concentration, i.e., of $[C^+]_i$, but of the **total fluorescence**, which we hope is proportional to the total amount of dye, per cell. To obtain a distribution of $[C^+]_i$, we need to divide the fluorescence value for each cell by the cell's volume, obtained by an electronic volume sensor or estimated from forward scatter or extinction; this is rarely done in practice.

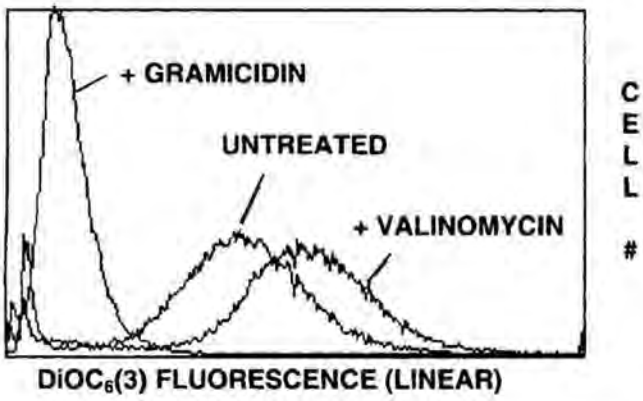


Figure 7-27. Distributions of the fluorescence of $DiOC_6(3)$ in CCRF-CEM T-lymphoblasts equilibrated with 50 nM dye for 15 min. A: untreated cells, B: cells depolarized with gramicidin, C: cells hyperpolarized with valinomycin. The residual peaks at the left of the distributions represent signals from debris.

While volume measurements were not made of the cells that provided the data of Fig. 7-27, experience tells us that the coefficient of variation (CV) of the cell volume distribution of human peripheral blood lymphocytes is no more than 15%, while the CV of the cyanine dye fluorescence distribution observed in these cells is about 30%. Thus, variance of cell volume cannot explain all of the variance of the fluorescence distribution. Does this mean that there is a broad distribution of membrane potentials in lymphocytes? Not necessarily. The cells treated with the ionophore **gramicidin (GRM)**, which forms channels in the membrane that permit most mono- and divalent ions to pass through, should be completely depolarized, yet their fluorescence distribution also shows a greater variance than could be explained by cell-to-cell differences in volume.

Recalling that there are large potential gradients across mitochondrial membranes, you might be tempted to think that **mitochondrial uptake** of dye was responsible for the variance in fluorescence in the distributions of Fig. 7-27. This possibility was investigated; lymphocytes in which the mitochondrial potential was abolished using a combination of antimycin, dinitrophenol, and oligomycin did show less cyanine dye fluorescence (by 15-20%) than cells that were not exposed to these inhibitors, but the variance of the fluorescence distribution was not significantly reduced.

In cells treated with GRM, $\Delta\Psi$ should be zero; and the concentrations of dye inside and outside the cells should therefore be the same. How is it, then, that we can manage to see the little buggers? Well, there are two reasons. The first is that the fluorescence of cyanine dyes is enhanced (approximately sixfold in the case of $DiOC_6(3)$, less for other dyes) when the dye is in a hydrophobic environment[460]. The second, which is quantitatively more important, is that the lipophilic character of the dye causes it to be concentrated in cells in the absence of a potential gradient because it binds to intracellular material, predominantly lipids and membranous structures (a 1997 paper[280] actually describes $DiOC_6(3)$ as a "specific" stain for endoplasmic reticulum).

In the study of Sims et al[460], symmetric cyanine dyes with different alkyl side chain lengths were equilibrated with VMC-treated suspensions of red blood cells, which had been estimated to have a membrane potential of –40 mV. The Nernst equation would predict that the ratio of intracellular and extracellular dye concentrations should be less than 10; the ratio observed for diethylthiadicarbocyanine [$DiSC_2(5)$], the least lipophilic dye in the series, was over 100, and the ratios observed using other thiadicarbocyanine dyes increased with the length of the alkyl side chains, reaching a value above 10,000 for dihexylthiadicarbocyanine [$DiSC_6(5)$]. Thus, while uptake of cyanine dyes does provide an indication of membrane potential, the association of these dyes with cells is distinctly "Non-Nernstian."

Don't try to measure membrane potential with cyanine dyes with heptyl or longer side chains, e.g., $DiOC_7(3)$. In the series running from C_1 through C_6, the longer chain dyes, which are more lipophilic, get in faster, which is an advantage. At C_7 and above, the cyanines start to take on characteristics associated with the **cyanine tracking dyes** such as "DiI," "DiO," and the PKH series, discussed on pp. 45-6 and 371-4; they feel so warm and cozy in the lipid bilayer that they're not in any hurry to leave, even if the potential changes. The diheptyl (C_7) cyanines, which are not widely used, can be thought of as the "missing link" between membrane potential probes and tracking dyes, which typically have akyl side chains containing at least 14 carbons.

Non-Nernstian binding of probe is also encountered when $\Delta\Psi$ is estimated with radiolabeled lipophilic cations such as ^{3}H-TPMP$^+$. In order to get accurate values of cytoplasmic $\Delta\Psi$ using lipophilic cationic indicators, it is necessary to inhibit the mitochondria and to correct the results to account for uptake in the absence of a potential gradient. Felber and Brand[465,6] produced a few papers that resolved several controversies in the previous literature regarding lymphocyte membrane potentials. Their work, unfortunately, did not extend to single cell measurements.

This still leaves us looking for an explanation of the variance of cyanine dye fluorescence distributions. Further comparison of flow cytometric data[112] with data obtained from cuvette measurements can be helpful in this regard. When flow cytometry is done on cells exposed to different concentrations of cyanine dyes, a point is reached at which increasing the dye concentration does not increase fluorescence in the cells. If the dye used is $DiOC_6(3)$, this happens when cells at a concentration of 10^6/ml are incubated with 2 μM dye. The variance of the fluorescence distribution remains large, suggesting that cyanine dye fluorescence in cells results from fluorescence enhancement of dye bound to hydrophobic regions, and that the variance of fluorescence is due primarily to cell-to-cell variations in the number of binding sites.

Further evidence in support of this hypothesis comes from the observation that when the hydrophobic binding sites are saturated, at a dye concentration of 2 μM, the fluorescence distributions of cells are identical whether they are suspended in isotonic NaCl, in which they maintain normal $\Delta\Psi$, or in isotonic KCl, in which they are depolarized. In both cases, addition of VMC or GRM does not change cellular fluorescence. In other words, the cellular fluorescence no longer responds to changes in $\Delta\Psi$. However, if the fluorescence of cell suspensions equilibrated with 2 μM $DiOC_6(3)$ in NaCl and KCl is measured in a spectrofluorometer, the potential difference will be detectable, and hyperpolarization caused by addition of VMC to the cells in NaCl will produce a demonstrable fluorescence change. This means that the cells contain dye that is essentially nonfluorescent due to quenching, as well as dye bound to hydrophobic sites in which fluorescence is enhanced. VMC addition causes uptake of additional dye, which is also quenched once it enters the cells. Quenching of the fluorescence of symmetric cyanine dyes typically involves the formation of **aggregates**. So-called **H-aggregates** are nonfluorescent, while fluorescent **J-aggregates** typically emit at wavelengths substantially longer than do single dye molecules.

The dye concentration at which saturation of binding sites occurs is determined primarily by the lipophilicity of the dye; the fluorescence of cells (at 10^6/ml) equilibrated with 2 μM $DiOC_2(3)$, which is less lipophilic than $DiOC_6(3)$, is less than the fluorescence of cells equilibrated with the C_6 dye, and does change when $\Delta\Psi$ is changed by ionophore addition or by manipulation of ion concentrations in the medium. In fact, if cells in 2 μM $DiOC_2(3)$ in NaCl (normal $\Delta\Psi$, higher fluorescence) are mixed with an equal volume of cells in 2 μM $DiOC_2(3)$ in KCl (depolarized, lower fluorescence), the cells and dye reequilibrate within a few minutes, as shown in Figure 7-28, yielding a fluorescence distribution that reflects the intermediate value of $\Delta\Psi$ resulting from the ionic composition of the mixed suspending medium.

The distributions shown in Figure 7-28 are distributions of $\Delta\Psi$ values estimated from the ratio of fluorescence and extinction signal amplitudes. This was done in an attempt to compensate for the effects of cell size variation in the cultured cell line used for the experiments, which results in broadening of the fluorescence distributions. The variance of the distributions of the fluorescence/extinction ratio remains fairly large, indicating that factors other than cell size contribute substantially to the variance in the number of sites to which dye binds with fluorescence enhancement.

From what has gone before, we can conclude that the cyanine dyes, under the best conditions, are not going to give us absolute values of $\Delta\Psi$ to the nearest millivolt, whether we measure cell suspensions in cuvettes or individual cells in flow cytometers or microphotometers. However, while the fluorescence of cell-associated cyanine dye cannot provide an absolute measure of $\Delta\Psi$, it can provide a reasonably rapid indication of substantial changes in $\Delta\Psi$ that occur over periods of seconds to hours, and allow us to examine correlations between $\Delta\Psi$ and other parameters.

The breadth of the distributions obtained by flow cytometry means that cyanine dye fluorescence has only limited capability for detecting heterogeneity of $\Delta\Psi$ in populations; if 50% of the cells have $\Delta\Psi$ values that are approximately half those of the other 50%, you'll know it, while if 5% of the cells exhibit a 15% increase in $\Delta\Psi$, you won't. Are there, then, any better dye probes of $\Delta\Psi$ than the cyanines?

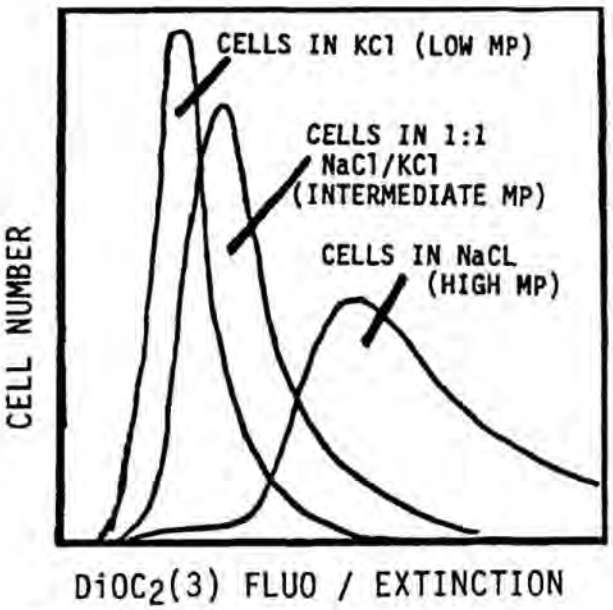


Figure 7-28. Distributions of MP ($\Delta\Psi$) (estimated from the ratio of $DiOC_2(3)$ fluorescence to extinction) in cultured CEM lymphoblasts suspended in isotonic NaCl, isotonic KCl, and a mixture of the two.

It has already been mentioned that any cationic dye that crosses cell membranes readily can serve as a distributional probe of $\Delta\Psi$. Among the classes of such dyes that have been investigated by Cohen, Waggoner et al[457,461,462] and by others are **cyanines** (e.g., the classical mitochondrial stain **pinacyanol** as well as the compounds discussed above), **acridines** (yes, acridine orange works as an $\Delta\Psi$ probe if you use it at about 10 nM, but who needs it?), **oxazines** (e.g., Nile blue), **pyronins**, **rhodamines** (e.g., **rhodamine 123**), **safranins** (e.g., **Janus green**, well known as a mitochondrial stain, and **safranin O**) **styryl compounds** and **triarylmethane dyes** (e.g., **crystal violet**). I have done flow cytometry of cells stained with a reasonable number of cationic dyes from this list, and none of the dyes offers any obvious advantages from a metrologic point of view in flow cytometry, although some may be less toxic and/or more stable than the cyanines. Plášek and Sigler and their colleagues have refined calibration of cuvette measurements of $\Delta\Psi$ using cyanine dyes, but have only obtained semiquantitative results using flow cytometry[2802-4].

Leslie Loew and his colleagues[1676-8] have described the use of the relatively hydrophilic methyl and ethyl esters of

tetramethylrhodamine for "Nernstian" membrane potential measurement by imaging microspectrofluorometry; by measuring dye concentrations in various spatial regions, these probes allow simultaneous determination of cytoplasmic and mitochondrial membrane potential, once corrections are made for contributions for dye from out-of-focus regions and for non-Nernstian binding.

Oxonol Dyes as Membrane Potential Probes

Oxonols[426,850-1] (Figure 7-29), which are negatively charged, bind to the cytoplasmic membrane but do not accumulate in intact cells; probably because they do not enter cells at appreciable concentrations, they are much less toxic than cyanines and other cationic $\Delta\Psi$ probes. For the same reason, oxonol fluorescence, unlike fluorescence from cationic probes, is not greatly influenced by potential-dependent uptake of dye into mitochondria. These desirable characteristics of oxonols are offset somewhat by their weaker fluorescence, as compared to cyanines, and, since they don't produce distributions with any less variance, than do cyanines, I don't use oxonols much. I also find that the bright staining of damaged cells by oxonols, which, being negatively charged and highly lipophilic, stick like crazy to everything inside cells once they get in, is something of a nuisance. This may be dealt with by adding a dye such as crystal violet or trypan blue to quench the offending fluorescence, but it gets to be too much of a production for my taste.

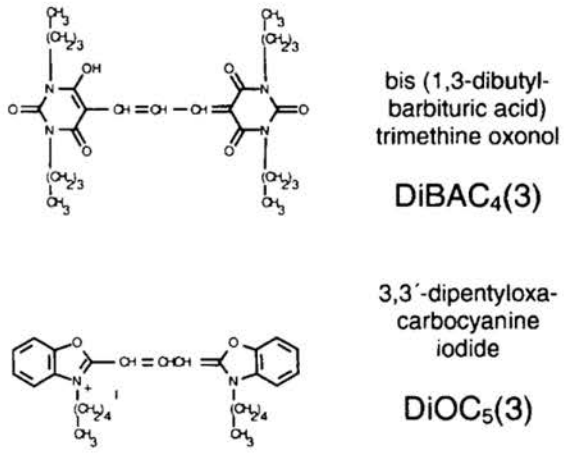


Figure 7-29. Structures of two of the more popular membrane potential probes, an oxonol and a cyanine. Structures were provided by Molecular Probes.

When I have compared oxonols and cyanines in rat and human leukocytes, T cells, and human lymphoblastoid cell lines, I have gotten pretty much the same results in terms of being able to detect depolarization and hyperpolarization. (**oxonol-stained cells** are **brighter** when **depolarized** and **dimmer** when **hyperpolarized**, a mirror image, if you will, of cyanine dye response). This is probably so because I make measurements over short time periods, which, combined with the relative resistance of the cells I study to cyanine toxicity and the absence of significant mitochondrial interference with fluorescence, lets me get away with it. Ken Rosenthal and I grew cells in 2 μM $DiOC_6(3)$ for a few days, in the dark, of course. People whose cells won't stand up to that have good reason to use oxonol dyes.

I have confidence in the **estimates** (calling them **measurements** would imply more accuracy and precision than dyes and flow cytometry can give us) of $\Delta\Psi$ I get from cyanines, which do, after all correlate with microelectrode measurements[458,852], but oxonols work. Krasznai et al[280c] described a method for determination of "absolute membrane potential" of cells using the popular oxonol **$DiBAC_4(3)$**, a dye that is frequently referred to as "bis-oxonol" but should not be, because it is one member of a large family of bis-oxonols. A calibration curve is constructed from a plot of fluorescence intensity measured from stained cells vs. extracellular dye concentration; it is assumed that dye distribution is Nernstian, which is probably a risky assumption given the lipophilicity of oxonol dyes. However, Krasznai et al reported good agreement between their flow cytometric measurements and patch clamp measurements.

When cells are added to solutions of oxonol dyes, the fluorescence of the resulting suspensions is increased. Oxonols, being negatively charged, should tend to remain outside cells with interior-negative membrane potentials; a purely Nernstian distribution for cells with $\Delta\Psi = -61$ mV would make the internal oxonol concentration 1/10 of the external concentration. However, like most of the symmetric cyanines, the oxonols increase fluorescence in nonpolar solvents, with the result that any dye molecule bound to membranes or lipids becomes more fluorescent than it would be in aqueous solution. The lipophilicity of oxonols results in their being taken into cells against the electrochemical gradient across the cytoplasmic membrane, but the dyes are largely excluded from mitochondria by the electrochemical gradient across the mitochondrial membrane.

A careful choice of controls is required when oxonols are used. These dyes form complexes with valinomycin, complicating its use as a hyperpolarizing agent; monensin has been suggested as a replacement. The real danger, however, arises from using heat-killed or fixed cells as exemplars of cells with $\Delta\Psi = 0$, because both heat and fixation permeabilize cells and change the chemical structure of the membrane. The observed increased binding of oxonols is therefore likely to reflect influences of factors other than membrane potential, e.g., binding to intracellular proteins and changes in membrane asymmetry.

The binding of merocyanine 540, which can be envisioned as a chimerical combination of half a cyanine and half an oxonol, to cells is known to be influenced by changes in membrane asymmetry, hence the utility of that dye in detecting apoptotic cells. I have already (p. 374) noted my suspicion that at least some oxonol dyes may share the characteristics of their half-sibling, although I haven't done the experiments needed to prove the point. You're welcome to

try and beat me to it using merocyanine 540 and $DiBAC_4(3)$ alternately with APC-annexin V in an apoptosis model system. In the meantime, stick with adjustments of the external potassium concentration or oxonol-friendly ionophores to get control cells with zero membrane potential.

Possible Alternatives to Distributional Probes for Flow Cytometry of Membrane Potentials

Dyes that work as Nernstian probes of membrane potential are obviously just dandy for monitoring membrane potential changes in single cells in **static** or image cytometers; even with non-Nernstian dyes, making repeated observations of the same cell over time eliminates the otherwise vexing problem of cell-to-cell variability in fluorescence intensity. However, under the best of circumstances, distributional dyes can only be used to monitor relatively slow $\Delta\Psi$ changes, occurring over periods of seconds to minutes. Better results would be expected using faster responding dyes, which sense membrane potential by different mechanisms.

The hardware technology for making static or imaging measurements is a lot simpler than it used to be, and it certainly seems easier than building a multistation flow system 100 meters long, which some of us have also thought of as a way to make repeated observations of the same cell several seconds apart. Because of the photodynamic toxicity of most membrane potential probes, however, you have to be careful, when making static measurements, about how you treat the cells between observations. For example, if you stain cells with a green-excited dye, you do all of the hunting for the cells under low-level red illumination using phase contrast and/or image intensification, thereby avoiding light and heat damage to the cells. When you want to measure the cells, you give them no more than a few milliseconds worth of green illumination at a time, using an arc or quartz lamp (with heat filters!!!) and a shutter, or else a xenon flash lamp. This minimizes photodynamic damage to the dye as well as to the cells. As long as you have several measurement values for the same cell, all you need to calculate is the percentage change in fluorescence in response to the stimulus applied, and you are largely unconcerned by the large cell-to-cell variations in fluorescence intensity that plague you when you do $\Delta\Psi$ estimation by flow cytometry.

As I mentioned previously, the distributional dye probes of $\Delta\Psi$ emerged as a spinoff of a project designed to find dyes that could respond within milliseconds to changes in nerve membrane potentials. The first dyes from which Cohen et al got fast responses typically showed fluorescence or absorption changes of about 0.1% in response to a 100 mV change in $\Delta\Psi$. One of these, merocyanine 540[467] (see above), was found to stain immature and leukemic but not normal leukocytes; the staining appeared to be independent of $\Delta\Psi$, but if the fluorescence had changed by 0.1% on VMC addition, nobody would have noticed. No need to worry about membrane potential affecting merocyanine fluorescence in the experiment suggested above.

Better **fast-response $\Delta\Psi$ probes** have since emerged from continued development efforts; one of the best dyes available a few editions back was a styryl compound that exhibited a 21% fluorescence change in response to a 100 mV potential change[468]. Believe it or not, this still wasn't good enough for flow cytometry. If we are looking for a 10 mV depolarization in mitogen-stimulated lymphocytes[465], we can expect to get a 2% fluorescence change in the best dye probe under the best circumstances, and we'd need an instrument CV of less than 1% to be able to detect it. That's one flow cytometer I can't tell you how to build. If you could build it, it wouldn't help, because cells stained with the fast **oxonol**, **merocyanine**, and **styryl** dye probes produce fluorescence distributions with the same large variances seen in cyanine dye fluorescence distributions.

Ratiometric Probes for Membrane Potential

Is there a way to make this a real measurement, i.e., to improve the accuracy or, more to the point, decrease the variance, of distributions observed in flow cytometric $\Delta\Psi$ estimation? Several possibilities suggest themselves. I have mentioned that the ratio of $[C^-]_i/[C^-]_o$ in cells varies with the lipophilicity of the dye C^- as well as with $\Delta\Psi$. If two cyanine dyes of differing lipophilicities, e.g., a C_2 dye and a C_6 dye, are added to cells, the ratio of intracellular concentrations of the two dyes should vary with membrane potential. Since the dyes presumably bind to the same hydrophobic sites in cells, cells with more sites should take up more of both dyes. Thus, the cell-to-cell variation in numbers of binding sites should not affect the ratio of concentrations of the two dyes in cells. This ratio could therefore be used as an index of $\Delta\Psi$. In order to get this methodology to behave, however, you have to use a multistation system and two dyes with widely separated spectra; otherwise, energy transfer comes in and confuses things.

The **fast potential probes** respond to membrane potential changes by **changing their position and/or orientation in the membrane**; it is claimed that most do not penetrate to the cell interior. Since all of the transmembrane potential difference is developed across the thickness of the membrane, the electric field strength in the membrane itself can be quite high. Loew[469] has designed electrochromic $\Delta\Psi$ probes that undergo spectral changes in responses to changes in the applied electric field. The current champ is **di-4-ANEPPS** [1-(3-sulfonato-propyl)-4-[beta-[2-(di-*n*-butyl-amino)-6-naphthyl] vinyl] pyridinium betaine], which responds via a rapid (less than millisecond) spectral shift to membrane potential changes[1679]. The problem with this dye is that the electric field affects the excitation, rather than the emission, spectrum of the dye; you need a blue and a green excitation beam (441 nm from a He-Cd laser and 515 nm from an argon laser would do), and the membrane potential is calculated from the ratio of fluorescence intensities at 610 nm excited by the two sources. It may not be worth putting together a fancy flow cytometer to try the measurements; Chaloupka et al[2806] attempted cuvette measurements of

$\Delta\Psi$ in *Saccharomyces cerevisiae* using di-4-ANEPPS, and found that the probe was not localized to the cell membrane and that the magnitudes of responses increased with time, and I suspect that taking a ratio won't cure all of those ills.

Loew's group[1680] also reported that the nonlinear optical phenomenon of **second harmonic generation**, which is responsible for the frequency doubling properties of crystals used in the laser industry, is sensitive to membrane potential; they have found a dye that gives a big signal, but it would definitely require more in the way of excitation power than most of us have in our flow cytometers.

Looking toward simpler solutions, we might reason that if the variance in distributions of cationic dye fluorescence in cells arises because of cell-to-cell variations in the number of dye binding sites, we ought to be able to do dandy $\Delta\Psi$ measurements, at least on things like peripheral blood lymphocytes, if we can find a lipophilic cationic DNA fluorochrome. If we then add dye at lower concentrations than are needed to get stoichiometric staining of DNA, so as to be sure that some dye binding sites remain available, the fluorescence histogram we get should be a histogram of $\Delta\Psi$, shouldn't it? Yes, if the dye stains DNA and doesn't stain all kinds of other stuff in the cytoplasm. Most of the symmetric monomethine cyanine dyes, e.g., $DiOC_2(1)$, $DiSC_2(1)$, $DiQC_2(1)$, and $DiLC_5(1)$, stain cell nuclei, and show fluorescence enhancement when bound to DNA, but they stain cytoplasmic constituents as well. Some blue- and green-excited cationic DNA fluorochromes described by Latt et al[470] seem to present the same problems with nonspecific staining. The asymmetric cyanines, such as thiazole orange and thiazole blue, should be good candidates, except that they're not DNA-specific. I would bet that a suitable dye exists, but not on when someone will find it.

Farinas[2807] et al have managed to come up with a flow cytometric ratiometric technique for membrane potential measurement that uses an asymmetric cyanine dye; they measure fluorescence of $DiBAC_4(3)$ and SYTO-62 in a slow-flow microfluidic system. The ratio of fluorescence of the two dyes changes by approximately 2% for each 1 mV change in membrane potential. The measurement is not made at equilibrium; instead, mixing fluidics insure that the time interval between dye addition and observation is constant (see p. 365). I don't see why the measurement wouldn't work in a more conventional instrument with appropriate sample handling hardware.

Gonzalez and Tsien[2356,2808-9] have described (and, via **Aurora Biosciences**, now part of **PanVera**, patented) a high-sensitivity, fast-response ratiometric method for measuring cytoplasmic $\Delta\Psi$ that also uses oxonol dye fluorescence, but in a far more specific way. Oxonols bind to both the inner and the outer face of the cytoplasmic membrane; as cells depolarize, dye shifts from the outer face to the inner face, while, as cells hyperpolarize, dye shifts from the inner face to the outer face. Gonzales and Tsien made energy transfer (FRET) measurements using fluorescently labeled lectins[2808] and, later, fluorescently labeled fatty acids[2809] as donors, and oxonols as acceptors, using the ratio of acceptor fluorescence to donor fluorescence as an indicator of $\Delta\Psi$. They achieved the best sensitivity with coumarin-labeled phosphatidylethanolamine as the donor and bis(1,3-dihexyl-2-thiobarbiturate)trimethine oxonol as the acceptor, with the fluorescence ratio changing 50% for a 100 mV change in $\Delta\Psi$. This is the highest sensitivity ever reported for a fast response (< 2 ms) method. An even faster response (< 0.4 ms, shorter than the duration of nerve action potentials) was achieved using a pentamethine oxonol acceptor.

I'll discuss another, lower-tech, ratiometric membrane potential measurement technique, useful for bacteria, later on.

Using Cyanine Dyes for Flow Cytometric $\Delta\Psi$ Estimation, In Case You're Still Interested

Now, if things are as bad as I have said, why does anybody bother using cyanines – or other dyes – for flow cytometric estimation of cytoplasmic $\Delta\Psi$? I can tell you why I use them; I have a good idea of the limitations of the reagents and the technique and I only use them when I don't need a more precise estimation of $\Delta\Psi$ than they can give me. You now know most of what I know about the limitations; if you're still interested in using the technique, here's how.

The dye I personally use most often is **$DiIC_1(5)$**, otherwise called **hexamethylindodicarbocyanine** and sometimes known as HIDC, which is available from Molecular Probes and (as a laser dye) from several other companies. I use $DiIC_1(5)$ because its fluorescence can be excited with a red He-Ne or diode laser and measured through 665 nm long pass glass filters (or 660-670 nm bandpass interference filters) using an R928 or other red-sensitive PMT. When I work with 488 nm excitation, I usually use **dihexyloxacarbocyanine [$DiOC_6(3)$]** or **hexamethylindocarbocyanine [$DiIC_1(3)$]**, the latter also known as acronol phloxine. $DiOC_6(3)$ is green fluorescent and can be used with the same detector/filter combination used for fluorescein; $DiIC_1(3)$ is orange fluorescent. I tend to use the C_6 dye at a final concentration of 50 nM and the others at 100 nM to 1 µm.

I make up 1 mM stock solutions of dye in DMSO and keep them in the dark at room temperature. I make working solutions by diluting the stock solution with ethanol so that I can reach the desired final dye concentration by adding 5 µl of working solution to each 1 ml of cell suspension. Since the dye really hates to hang around in aqueous solution, the cells tend to suck up most of it. As a result, you need to keep the cell concentration with which you work fairly constant; I generally shoot for 10^6/ml because that's a good concentration to use for flow cytometry.

If you are working with cells in protein-free media, dye equilibration is usually complete after 15 minutes at room temperature. If there's protein in the medium, I give the cells about 30 minutes at 37 °C. It's important to keep the incubation temperature and the interval between dye addition and introduction of the sample into the flow cytometer

constant, and it's not a bad idea to run all samples in an experiment at the same flow rate, because this is an equilibrium staining procedure.

If the experiment you are doing extends over a period of a few hours, it's important to run controls during the course of the experiment as well as at the beginning and end. Controls should include an untreated cell sample, a sample of cells hyperpolarized by addition of 5 μM valinomycin (VMC), and another sample depolarized by addition of 10 μM gramicidin (GRM) (this assumes you are working with cells in a high-Na^+, low-K^+ medium). If you have any doubt about the health of your cells, make up a triple-dip sample of control cells, measure them after they have equilibrated with dye, and then add VMC to what's left of the sample. If the fluorescence of the cells doesn't increase, either the cells are dead or you've used too much dye. If it does increase, add GRM to the remaining cells; they should depolarize. If they don't, something's wrong. VMC, by the way, will not hyperpolarize cells after GRM addition has depolarized them, so make sure you do things in the right order.

I should also remind you that cyanines and other positively charged dye probes of membrane potential are subject to eviction from cells by the **glycoprotein drug efflux pump** (pp. 376-7). This is not as much of a problem when you're doing measurements of cytoplasmic membrane potential, and leaving cells in equilibrium with the dye, as it is when you're looking at mitochondria, and have to wash the cells. Washed cells with an active pump lose dye, period; equilibrated cells with an active pump come to equilibrium with lower concentrations of dye than they would if the pump weren't there, but the intracellular dye concentration still responds to membrane potential changes. If you're paranoid about the pump, use 100 nM $DiBAC_4(3)$; this oxonol dye is unaffected by it.

By following the procedures I have just described, you should be able to establish that the cells with which you are working have a nonzero potential difference across their cell membranes, and that the indicator dye you are using will respond to potential changes in either direction from the control value. You can then examine the effects of the biologic, chemical, or physical agents you are studying on the cells' $\Delta\Psi$.

It is advisable to keep the cells happy if you want them to continue responding over the course of a few hours. If the buffer capacity of the medium won't stand up to the metabolic output of the cells, you may get funny results because pH does have some ill-defined effects on $\Delta\Psi$ probes. If you are working with blood cells, avoid using NH_4Cl or a hypotonic medium to lyse red cells; the former depolarizes cells[471], and the latter may change cation contents (and thus presumably $\Delta\Psi$) and cell volumes of different cell types in different ways[472].

If you add practically any protein to a cell suspension that has been equilibrated with a cyanine or other lipophilic cationic indicator in a protein-free medium, the indicator concentration in cells will decrease because the indicator will bind to the protein in solution. When you do the measurement, it looks as if the cells have depolarized, but they probably haven't. If you're trying to find the effects of adding various proteins to cells, work in a medium with added protein, e.g., 1-10% albumin.

Similar artifactual "depolarization" may appear when you add appreciable concentrations of nonpolar solvents to a dyed cell suspension, which is why it's a good idea to keep the total amount of added DMSO, EtOH, etc. relatively constant from sample to sample and not add amounts of these solvents in excess of 1% of sample volume. If you are adding material dissolved in DMSO, make sure the sample is well mixed, because DMSO, left to its own devices, will sit at the bottom of an aqueous solution, and the hydrophobic molecules in it may be content to stay there unless prodded.

If you want to eliminate mitochondrial influences on the uptake of cyanines or other lipophilic cationic dyes, you can add an **uncoupler**. Felber and Brand[463] used 5-10 μM **carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP)** in their TPMP$^+$ procedure; I have tried this a few times and it seems to work. Waggoner[462] cautions that the related uncoupler **carbonyl cyanide chlorophenylhydrazone (CCCP)** may interfere with cuvette measurements using $DiSC_3(5)$; I have used CCCP at 5-10 μM for flow cytometry without problems.

For the record, I have tried to measure $\Delta\Psi$ in cell suspensions in cuvettes using cyanine dyes on several occasions, and have only succeeded once; the flow cytometric procedure done on cells from the same samples at the same time has worked. Oxonol dyes, which are nontoxic and which respond to $\Delta\Psi$ changes more rapidly than do cyanines, have been advocated as superior $\Delta\Psi$ probes for cuvette measurements by Tsien, Pozzan and Rink[426]; I'll try them if I ever have occasion to attempt more cuvette measurements

Also for the record, there have been occasions when we shut off a flow cytometer after running cyanine dye-stained lymphocytes from one donor on one day, turned the system on again the next day, and found that the peak of the distribution of fluorescence from another donor's cells was within a few channels of the peak of the distribution obtained the day before. From my point of view, the flow cytometric technique is more reproducible than is the bulk measurement method.

There have been a lot of bad things said about cyanine dyes in the literature, many of which are true. At micromolar concentrations, cyanines have been observed to be toxic to bacteria[473] and mammalian cells[474-7,1683]. The dyes themselves may perturb $\Delta\Psi$ directly by altering membrane conductivity; their inhibition of energy metabolism may also result in $\Delta\Psi$ changes. When used to monitor neutrophil $\Delta\Psi$ responses to chemotactic peptides, $DiOC_6(3)$ and TPMP$^+$ have been reported to give contradictory results[478], while $DiSC_3(5)$ was found to be destroyed by oxidation following neutrophil activation[479].

The affinity of cyanine dyes for cell constituents is so high, and their solubility in water so low, that you can end

up with inconclusive results when you use very low concentrations of dye and relatively high concentrations of cells, simply because the dye molecules grab on to the first cells they see[855]. Olive and Durand have taken advantage of this property and used $DiOC_5(3)$, which I told you not to use as an $\Delta\Psi$ probe, as an indicator of perfusion or penetration of materials into tumor cell spheroids[854].

The toxicity of the cyanine dyes is a liability shared in common with other families of cationic dyes and with lipophilic cations such as TPMP'; when the cyanines are used in flow cytometry, at concentrations of 5-100 nM, toxicity is less than when radiolabeled cations or cationic dyes are used at micromolar concentrations for bulk measurements. Different cell types appear to have different degrees of susceptibility to cyanine dye toxicity; Crissman et al[855] found that simultaneous staining with $DiOC_5(3)$ improved both Hoechst 33342 staining of live CHO cells and cell survival following sorting, possibly because of effects on the efflux pump, while the supposedly less toxic rhodamine 123 affected neither staining nor survival. While this may not lead to the routine appearance of cyanine dyes in multivitamin pills, it does remind us that one cell's poison may be another cell's medium.

Cytoplasmic Membrane Potential: Summing Up

It is hardly feasible to make microelectrode measurements of $\Delta\Psi$ in large numbers of cells, and impalement with microelectrodes is probably more traumatic to lymphocytes than to squid axons. Despite this, cationic dyes such as the cyanines, radiolabeled cations, the anionic oxonol dyes, and microelectrodes have produced comparable results in most systems to which two or more measurement methods have been applied.

Tasaki and Byrne[1684] studied the time course of the intrinsic birefringence change of axons that coincides with action potentials, and attribute it to swelling of the nerve brought about by movement of water into the superficial layer of the axon. A close relationship was also demonstrated between nerve swelling and changes in light scattering and in dye absorbance. The intrinsic optical changes, by the way, are on the order of a few parts per ten thousand; you won't be noticing them on your scatter channel, and we'll probably have to stick with using dyes for flow cytometric measurements.

In looking at some studies[1685-93] on flow cytometry that emerged prior to the previous edition, I noticed that at least as many people seemed to use cyanines[1687-9] as oxonols[1690-1,1693]. Seamer and Mandler[1691] applied my trick of dividing by a size measure (Figure 7-28, p. 389) to lower the CVs from oxonol measurements. Damjanovich and Pieri[1692] correlated fluorescence flow cytometry with patch clamp measurements in lymphocytes. Radosevic et al[1693] used DiBAC4(3) to study membrane potential in conjugates of cytotoxic NK cells, which were labeled with a tracking dye, and K562 target cells. Conjugates could be identified on the basis of tracking dye fluorescence and light scattering signals; using a slit-scan technique, the membrane potential of each cell in a conjugate was measured separately, showing that depolarization of the K562 cell occurs as a consequence of the cytotoxic activity of the NK cell. In at least some of the studies in this group[1687-8], in which cyanine dyes were used, I suspect that the efflux pump had some effect on results. Watch out.

Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Mitochondrial Staining with Rhodamine 123 Is Membrane Potential-Dependent

Since 1980, numerous investigators have used the lipophilic cationic dye **rhodamine 123** for investigations of mitochondrial structure and function; it is appropriate at this point to discuss the general issue of **estimation of mitochondrial membrane potential (which I will abbreviate as $\Delta\Psi_m$), as opposed to cytoplasmic membrane potential ($\Delta\Psi$)**, and to clear up some misconceptions that seem to have arisen concerning this particular dye.

As was mentioned on p. 385, there is normally a relatively large (100 mV or more, interior negative) gradient of electrical potential across mitochondrial membranes in cells. The electrical potential and pH gradients between the cytosol and the mitochondrial matrix are inextricably linked to the processes of **energy metabolism**[487-8]. When cells with normal mitochondrial function are allowed to equilibrate with lipophilic cations, potential differences alone generate gradients of cation concentration across mitochondrial and cytoplasmic membranes, with the concentration being highest in the mitochondria.

According to the **Nernst equation** (p. 386), the ratio of mitochondrial to cytosolic (free) cation concentrations should be 100:1 if the potential difference across the mitochondrial membrane is 120 mV, and 1000:1 if it is 180 mV. The presence of any high-affinity binding sites for the cation within mitochondria will result in the ratio of concentrations of (free + bound) cation in mitochondria and cytosol being higher than that predicted by the Nernst equation, unless there are much greater numbers of high-affinity binding sites within the cytosol.

If cell samples are allowed to come to equilibrium with equal concentrations of a series of cationic dyes of similar structure with increasing lipophilicity (e.g., cyanines), higher mitochondrial/cytosolic dye concentration ratios should be found for the dyes with higher lipophilicity (in the case of the cyanines, those dyes with longer alkyl side chains), due to increased binding to hydrophobic membrane structures, which are at least as abundant in mitochondria as in the cytoplasm. The ratio of intracellular and extracellular dye concentrations, $[C^+]_i/[C^+]_o$, should also increase with lipophilicity.

Under the equilibrium conditions just described, both cytoplasmic ($\Delta\Psi$) and mitochondrial ($\Delta\Psi_m$) membrane potential affect the total amount of dye taken up and bound by cells. The relative contributions of cytoplasm and mitochondria to the cytoplasmic "$\Delta\Psi$," as estimated by fluores-

cence of cells following equilibration with dyes such as cyanines, are, however, difficult to estimate *a priori*, because the total amount of dye in each compartment is the product of concentration and volume. In addition, one cannot expect to measure the same amounts of fluorescence emission from equal amounts of intra- and extramitochondrial dye, because quenching increases with dye concentration.

If cells are washed following a first equilibration with dye, the rate at which a new equilibrium between intramitochondrial, cytosolic, and extracellular dye concentrations is reached should be dependent upon mitochondrial affinity for the dye, because, after washing, the mitochondria serve as the primary source for redistribution of dye into the cytosol and thence into the extracellular medium. More and/or stronger mitochondrial binding of dye should be reflected in longer reequilibration times and, until equilibrium is reached, in higher ratios of intra- to extramitochondrial dye concentrations. Observation of such cells under a fluorescence microscope should show dye fluorescence to be confined to the mitochondria.

All that can be concluded, however, from the observation of fluorescence in the mitochondria of washed cells, or from microphotometric or flow cytometric measurement of cellular fluorescence under such conditions, is that the mitochondria were energized prior to dye addition and probably remained energized at the time of measurement; variations in $\Delta\Psi_m$ cannot be inferred from variations in cell fluorescence. This is so because the number and/or volume of mitochondria may vary from cell to cell and because mitochondrial fluorescence itself is likely not to be proportional to $\Delta\Psi_m$ due to concentration quenching. Recall (p. 388) that the ratio of concentrations of cyanine dyes inside and outside cells is typically at least several hundred, much higher than would be predicted by the Nernst equation; even if the ratio between cytosolic and mitochondrial concentrations is purely Nernstian, one can expect the concentration of dye in the mitochondria to be on the order of 10^4 times the external dye concentration. If the external dye concentration were only 10 nM, the mitochondrial concentration would be 100 μM, and one would expect dye fluorescence to be substantially quenched.

If the mitochondria are **deenergized**, i.e., if $\Delta\Psi_m$ is reduced to or near zero, before dye is initially added to the cells, e.g., by addition of a **metabolic inhibitor**, e.g., an **uncoupler** of oxidative phosphorylation, such as **CCCP**, no mitochondrial concentration of dye occurs in the first place, other than that which might result from dye binding to membranous structures in the absence of a potential gradient. Under these circumstances, the dye concentration in cells left in equilibrium with dye responds to differences in (cytoplasmic) $\Delta\Psi$, and flow cytometric fluorescence and cell size measurements can provide valid, semiquantitative estimates of this parameter provided dye concentrations are kept low, as discussed on p. 392, to minimize saturation and concentration quenching effects. Pretreatment with an inhibitor of mitochondrial energy metabolism similarly allows valid estimates of cytoplasmic $\Delta\Psi$ to be derived from uptake of $TPMP^+$ and similar substances.

If no inhibitor is added to cells, and they are examined while in equilibrium with dye, differences in fluorescence will be observed between cells with intact membranes and normal mitochondrial function, on the one hand, and damaged cells, on the other. Cells with deenergized mitochondria will, as just discussed, take up less dye than cells with intact energy metabolism; cells with cytoplasmic membrane damage sufficient to diminish or abolish the cytoplasmic $\Delta\Psi$ gradient will also take up less dye. Thus, equilibrium dye fluorescence measurements in cells can provide an indication of **cell viability** that is based upon **metabolic integrity** as well as membrane integrity.

Washing cells before measurement can be expected to produce some improvement in live-dead cell discrimination. If mitochondria are deenergized before equilibration with dye, very little fluorescence will be seen in washed cells even if the cytoplasmic membrane is intact. If mitochondria become deenergized after equilibration with dye, but before washing, some concentration of dye in mitochondria may be observed if high-affinity binding results in dye being lost relatively slowly from the mitochondria. If the mitochondria become deenergized after washing, dye should be lost from the mitochondria into the cytosol at a rate dependent upon the affinity of mitochondria for the dye.

Viewed against this background, the observed behavior of **rhodamine 123 (R123)** in mitochondria and cells is no different from what would be expected, and not markedly different from what would be expected and has been observed using other dyes. During the fall of 1979, when my initial publication on flow cytometric $\Delta\Psi$ estimation[424] and their first paper on R123 staining of mitochondria[489] were in press, Lan Bo Chen and Lincoln Johnson asked me if they could look at some R123-stained cells in one of my flow cytometers. We got to talking about membrane potentials; when I found out that R123 was a cationic dye I suggested that the mitochondrial staining they were seeing was potential-dependent, and we exchanged samples of dyes.

The R123 staining technique Johnson et al described[489] involved a 30 min equilibration of cells with 10 μg/ml (about 25 μM) R123, following which cells were washed and examined. Using that concentration of R123, I couldn't measure unwashed cells in the flow cytometer because of the intense background fluorescence. Backing off to 1 μM, I found (unpublished) that I could get R123 fluorescence to respond to the usual $\Delta\Psi$ manipulations involving VMC, GRM, and changes of (K^+) concentration in the same fashion as did the cyanines. Johnson and Chen[490] established that R123 staining was dependent upon the presence of the mitochondrial $\Delta\Psi$ gradient. They also observed that cells equilibrated with safranin and the cyanine dyes showed potential-dependent mitochondrial staining. In fact, cyanine dye-stained cells could be examined without washing, probably because cyanines are considerably more lipophilic than is R123, which should make the ratio of intra- and

extramitochondrial dye concentrations at least somewhat higher for the cyanines.

A few weeks after these experiments were done, Paul Horan and coworkers exhibited pictures of cyanine dye-stained fibroblasts at the 1979 Asilomar conference on Analytical Cytology; these showed dye concentrated in mitochondria. Horan et al found, as did Johnson et al using R123, that stimulation of confluent fibroblasts by wounding caused an increase within minutes in mitochondrial fluorescence in cells adjacent to the damaged areas[491]. Until both groups published in 1981, however, most people who were aware of the capacity of R123 to stain mitochondria were unaware of the central role of mitochondrial $\Delta\Psi$ in R123 staining.

James and Bohman[492], for example, used R123 and flow cytometry to study proliferation of mitochondria during the cell cycle of HL-60 cells, and Darzynkiewicz et al[493] reported increased R123 staining of stimulated lymphocytes beginning approximately 24 hr following exposure to lectins, attributing most of the increase in fluorescence to an increase in the number of mitochondria per cell, in work submitted for publication before reports of the potential dependence of R123 staining appeared in print.

The R123 fluorescence response in lymphocyte activation was not surprising to me because I and others[425,456,484] (also M. Brand, personal communication), using TPMP', microelectrodes, $DiOC_6(3)$, and TPMP', respectively had found apparent hyperpolarization of lectin-stimulated T lymphocytes beginning at 5-12 hours, and usually preceding increases in RNA content as measured with pyronin Y. I also noted (unpublished) that R123 staining increased in stimulated T cells; an example of typical results obtained from lymphocytes stained with Hoechst 33342 in combination with various other probes appears as Figure 7-30.

In retrospect, some of the differences in R123 fluorescence between resting and stimulated lymphocytes may reflect behavior of the glycoprotein pump, which appears to be more active in resting than in stimulated cells, leading to observable differences in the fluorescence of Hoechst 33342 in when cells are equilibrated at lower concentrations of the dye than are typically used for stoichiometric DNA staining[248-50,253,257] (see pp. 309 and 376).

I had suggested[494] that flow cytometric $\Delta\Psi$ measurements might provide a more sensitive indicator of **cell viability** than was given by dye exclusion tests. This was of interest to several of my colleagues because it might provide a fast means for determining the effects of chemotherapeutic agents on cancer cells. Sam Bernal, Lan Bo Chen and I[495] looked at the effects of various drugs on R123 fluorescence in L1210 mouse leukemia cells, and concluded that loss of R123 fluorescence was a better and earlier predictor of loss of clonogenicity than were dye exclusion tests. However, later work (E. Adams, B. K. Bhuyan et al, presentations at the 1984 Cell Kinetics Society meeting and personal communications) showed that, while loss of R123 fluorescence generally indicates loss of clonogenicity, cells exposed to lethal doses of some drugs may not lose R123 fluorescence for some time. Bhuyan et al demonstrated by biochemical analyses that **R123 fluorescence parallels mitochondrial ATP content**, and that the changes in this parameter following lethal doses of different agents occur with different time courses.

When R123 first came into use, great stress was placed upon its relatively low toxicity as compared to dyes such as the cyanines. Darzynkiewicz et al[340], however, found that R123 inhibited the growth and cell cycle progression of cultured cells, while uncharged rhodamine dyes, which were not taken into mitochondria, were not inhibitory. Chen's group, which had reported different patterns of retention of the dye in normal and transformed cells[496], thereafter discovered a selective toxic effect of R123 on carcinoma cells *in vitro*[497] and *in vivo*[498]. They have since established that the dye interferes with mitochondrial energy metabolism[499].

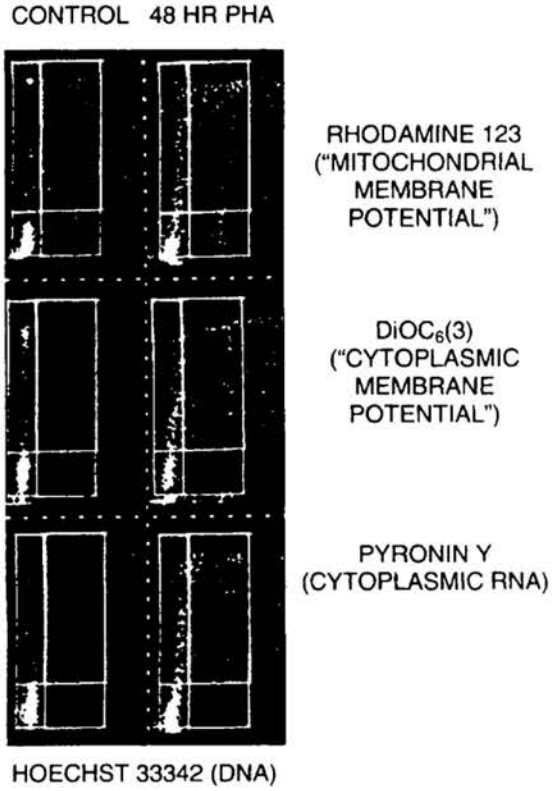


Figure 7-30. Two parameter flow cytometric analyses of lectin-stimulated human peripheral blood lymphocytes 48 hr after PHA addition.

The toxicity of the more lipophilic cyanine dyes, and of lipophilic cations such as TPMP', relative to R123 must be examined in the light of the fact that differences in lipophilicity exert profound effects on partitioning of the dye between mitochondria, cytosol, and medium. In cells equilibrated with equimolar concentrations of R123 and a lipophilic cyanine such as $DiOC_6(3)$, the intramitochondrial concentration of the cyanine dye may be hundreds of times higher than that of R123. Conversely, there exist other fluo-

rescent probes of mitochondrial energization which, like R123, are relatively water-soluble, and which are also said to be nontoxic; e.g., the styryl dyes **DASPMI (dimethylaminostyrylpyridinium iodide)** and **DASPEI** described in 1976 by Bereiter-Hahn[500]. The studies of R123 as an anticancer drug recalled the therapeutic use of $DiSC_2(5)$ as an antihelminthic agent under the name Dithiazanine[501]. I have often wondered whether some of the unexplained sudden deaths associated with this dye/drug were related to the observation by uninformed patients of brilliant blue stools following its oral administration. In a more serious vein, it should be pointed out that Zigman and Gilman[475] have related the toxicity of cyanine dyes to redox potential; their work should be read by anyone interested in the pharmacologic, as opposed to the flow cytometric, aspects of dye probes of cell membrane potential. It is also evident from the work of Chen's group that thiacyanine dyes are more toxic than indo- and oxacyanines[1683].

Since the late 1980's, a lot of interesting things have happened in the mitochondrial area, with flow cytometry playing a pivotal role.

Borth, Kral, and Katinger[1699] established that R123 fluorescence of hybridomas was responsive primarily to glucose concentration, rather than to aging or growth; this was significant since R123 fluorescence was proposed as a useful parameter that has been proposed for use in monitoring cells in bioreactors. Myc et al[1700] used Hoechst 33342 and R123 to determine that there was no variation with phases of the cell cycle in R123 retention characteristics in either normal lymphocytes or leukemic cells.

Irion et al[1706] and Rottele and Zimmermann[1707] studied the kinetics of uptake of a variety of dyes with different lipophilicities into HeLa cell mitochondria *in situ*. The kinetics of uptake were found to be limited by diffusion; more lipophilic molecules were taken up more rapidly. Dye molecules accumulated in strongly lipophilic areas of the mitochondria, and both the potential gradient and hydrophobic interactions contribute to strong dye binding. Styrylpyridinium dyes are of interest because, unlike cyanines, they exhibit very low fluorescence in aqueous solution.

The Search for Better $\Delta\Psi_m$ Probes: Round One

Maftah et al[1696] and others[1697-8] described the use of **nonyl acridine orange (NAO)**, which they reported stained mitochondria in a membrane potential-independent fashion. One of the problems with attempting to measure $\Delta\Psi_m$ using the fluorescence of R123 or other dyes is that the number and size, or mass, of mitochondria vary from cell to cell; it is therefore not possible to determine to what extent differences in dye fluorescence between cells reflect differences in $\Delta\Psi_m$, as opposed to differences in these other factors. In theory, NAO fluorescence would provide a measurement of mitochondrial mass. Since the fluorescence emission maxima of NAO and R123 are quite close to one another, it would not be feasible to attempt ratiometric measurements. However, I suggested in the previous edition that the ratio of fluorescence of a suitably spectrally separated cationic dye, e.g., $DiIC_1(5)$ and NAO might provide an indicator of $\Delta\Psi_m$ normalized for differences in mitochondrial mass; I was wrong. I also speculated that the nonyl group locked NAO into the mitochondrial membrane, and it looks as if I was right about that; I can't finish the story without bringing in some other developments.

O'Connor et al used flow cytometry with rhodamine 123[1703] and other dyes[1704] to analyze isolated individual mitochondria; staining with R123, safranin O, and $DiOC_6(3)$ were potential dependent, while staining with NAO was not, as would be expected. Wolf and Kapatos[1705] were able to discriminate isolated neuronal synaptosomes from free mitochondria because, under the experimental conditions used, the former maintained a membrane potential and the latter did not.

Juan et al[2310] described a kinetic measurement procedure using rhodamine 123 to assess mitochondrial membrane potential; they found that, if dye and cell concentrations were kept constant, the rate of uptake of R123 by isolated rat hepatocytes was relatively constant, with increasing concentrations of added glucose or other substrates increasing the dye uptake rate and addition of uncouplers decreasing the dye uptake rate.

Hahn et al[1702] described a photocrosslinking fluorescent indicator of mitochondrial membrane potential; a carbocyanine dye was derivatized with a photoreactive nitrophenylazide group so that illumination would covalently attach it to nearby molecules. The dye, **PhoCy** (photofixable cyanine), specifically stained mitochondria in living fibroblasts; when stained cells were illuminated and fixed with formaldehyde, staining was retained owing to cross-linking, while fixation without illumination eliminated mitochondrial staining. If the mitochondria in cells were energized in the first place, one could, in theory, label them with PhoCy, wash out excess dye, lock the PhoCy into the mitochondria by illuminating the cells, apply some treatment, and restain with a spectrally distinct cationic dye to determine whether or not $\Delta\Psi_m$ had decreased. However, PhoCy itself apparently didn't make it to market, although some other reactive cationic mitochondrial dye probes eventually did.

$\Delta\Psi_m$, JC-1, and Apoptosis

Lan Bo Chen published two reviews, in 1988[1694] and 1989[1695], reflecting what he and his coworkers had done with R123 and other dyes up to that point. In 1991, his group described the use of the symmetric cyanine dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (**JC-1**), which forms fluorescent aggregates, as a probe of mitochondrial membrane potential[1681-2]. When excited at 490 nm, JC-1 monomers show an emission maximum at 527 nm, while aggregates emit maximally at 590 nm. In theory, the orange to green fluorescence ratio should give you a measure of membrane potential.

I've tried JC-1 in cells and bacteria; the biggest problems I've found with it is that it aggregates in solution and that it

takes much longer to equilibrate to stable fluorescence values than do the more popular dyes such as $DiOC_5(3)$ and $DiOC_6(3)$. The fluorescence distributions in two dimensions are broad enough so that taking ratios doesn't help all that much. There are probably better dyes out there with similar properties if you want to do ratiometric measurements, but looking at two-color fluorescence from JC-1 has turned out to be very useful, even if it won't let you measure $\Delta\Psi_m$ to the nearest millivolt.

Chen's group and collaborators[1708], using NMR, found that R123 had different effects on intermediary metabolism of normal and cancer cells. On another front, the report by Chen's group[1692] that membrane potentials were different based on differences in JC-1 fluorescence at different points in mitochondria resonated with the conclusion drawn by Skulachev and his colleagues, based on analyses of fibroblast and cardiac myocyte mitochondria[1709] and on filamentous cyanobacteria[1710], that networks of mitochondria (or bacteria) act as power-transmitting protonic cables, with membrane potential changes propagating along the network[1711]. A cellular information superhighway? For what it's worth, Bedlack, Wei, and Loew[1712] observed localized membrane depolarizations that they believed to be involved in electric field-directed neurite growth. This gets us from subcellular power transmission lines to the kind a lot of people worry about these days; it's not at all illogical that electric field effects would be mediated via membrane potential changes, but I haven't seen anything along this line hit the newspapers yet. I haven't looked that hard at the journals. Chen's group published a methods paper and review on JC-1 in 1995[2810], but things didn't get really interesting until after that.

In 1993, Cossarizza et al[1701] reported using JC-1 for two-color flow cytometric measurement of mitochondrial membrane potential; while, as I said, it didn't give them values to the nearest millivolt, it did (and does) a much better job of discriminating cells with energized and deenergized mitochondria than did other popular dyes[2811].

In 1993, Richter[2812] suggested that maintenance of $\Delta\Psi_m$ was important in preventing **apoptosis**; in 1994, Cossarizza et al[2813] and Vayssiere et al[2814] reported that $\Delta\Psi_m$ was reduced in apoptosis, and, in 1995, Cossarizza et al[2815] reported that N-acetylcysteine stabilized $\Delta\Psi_m$ and prevented apoptosis in cells treated with agents that would normally induce the process. Richter and Cossarizza et al joined forces on a 1996 paper emphasizing the role of maintenance of $\Delta\Psi_m$ and ATP production in the prevention of apoptosis. The concept, and JC-1, started to get a lot of attention from that point on; I'll have more to say about the subject when I discuss apoptosis in Chapter 10. Meanwhile, I'll continue with the story of mitochondrial probes.

The Search for Better $\Delta\Psi_m$ Probes: Round Two

While, by 1997, JC-1 was recognized as more reliable in providing information on the state of energization of mitochondria than either $DiOC_6(3)$ or R123[2811], it did not provide a quantitative measure of $\Delta\Psi_m$, at least when applied to whole cells. Rottenberg and Wu[2817] noted in 1997 that $DiOC_6(3)$ itself induced apoptotic changes in lymphocytes from old mice, which led them to consider some attributes of this and other dyes that might affect their use as probes of $\Delta\Psi_m$, and, in 1998, to report that $DiOC_6(3)$ could be used for quantitative flow cytometric measurements of $\Delta\Psi_m$[2818].

It had been known for some time that DiOC6(3) and other oxacarbocyanine dyes inhibited NADH reductase in isolated mitochondria *in vitro*; Rottenberg and Wu reasoned that mitochondria *in situ* would be affected by substantially lower concentrations of dye in solution, because of the Nernstian and non-Nernstian concentration that occurs in cells. They found that the respiration of lymphocytes exposed to 40-100 nM $DiOC_6(3)$, a range of concentrations commonly used for assessment of was inhibited approximately 90 percent, suggesting that the probe itself was likely to alter the parameter they were trying to measure.

Apart from their effect on respiration and $\Delta\Psi_m$, extracellular dye concentrations in the 40-100 nM range create problems because, as mentioned on pp. 388 and 395, the concentration of dye in energized mitochondria can easily reach 10,000 times the extracellular dye concentration, and, at intramitochondrial concentrations in the 400-1000 μM range, the fluorescence of dye in mitochondria should be almost completely quenched.

I noted, in the discussion of measurements of cytoplasmic membrane potential, that $DiOC_6(3)$ fluorescence in cells, measured by flow cytometry, becomes independent of membrane potential at an extracellular dye concentration of 2 μM. I also noted that membrane potential changes in cells exposed to that concentration of dye can be measured in cuvettes, because the cuvette measurement measures the fluorescence of free dye in solution. In order to keep cellular fluorescence responsive to changes in cytoplasmic membrane potential, it is necessary to use lower concentrations of dye than might be used for cuvette measurements, and the 10-100 nM concentrations typically used for flow cytometry may be advantageous in terms of eliminating much of the effect of $\Delta\Psi_m$ on cytoplasmic membrane potential by virtue of extensive quenching of intramitochondrial dye.

When we are trying to measure $\Delta\Psi_m$ by flow cytometry, we cannot practically distinguish between fluorescence from intramitochondrial dye and fluorescence from dye in the extramitochondrial portions of the cell. We would prefer to have a monotonic relationship between measured fluorescence and $\Delta\Psi_m$; the fluorescence intensity measured from cells with energized mitochondria should be greater than that measured from cells with deenergized mitochondria, and cells with intermediate values of should yield intermediate values of fluorescence intensity. This is unlikely to happen when there is extensive quenching of dye in mitochondria; it is far more likely that a decrease in $\Delta\Psi_m$, resulting in release of dye into the cytosol, will lead to an increase in cellular fluorescence. Rottenberg and Wu[2818] found that 0.2 nM $DiOC_6(3)$ provided a monotonic fluorescence response across the range of $\Delta\Psi_m$.

Since the concentrations of JC-1 typically used for studies of $\Delta\Psi_m$ are in the range between 1 and 10 μM, it is not unlikely that perturbation by the dye will complicate any attempt to derive a quantitative measurement. Because the response of JC-1 to $\Delta\Psi_m$ is largely dependent on the concentration of dye being high enough to promote formation of J-aggregates, it is likely that reducing the concentration to the nanomolar range would eliminate the metachromatic fluorescence response that has provided most of the motivation for using the dye. Cossarizza et al[2819] did find a linear relationship between JC-1 orange (590 nm) fluorescence and $\Delta\Psi_m$ in isolated mitochondria, but it is likely that the intramitochondrial dye concentration in that experimental situation was lower than would be expected in mitochondria *in situ* due to the absence of the Nernstian and non-Nernstian concentration of dye into a cytoplasmic extramitochondrial compartment.

In 1996, Poot et al[2820], at Molecular Probes, described two reactive probes that responded to $\Delta\Psi_m$, **chloromethyl-X-rosamine (CMXRos)** and **CM-H_2XRos**, both given the **MitoTracker Red** trade name. Both probes are lipophilic and cationic, and are concentrated in mitochondria, where the chloromethyl groups on the probes bind covalently to thiols. CMXRos is fluorescent; its excitation maximum is at 578 nm, but some fluorescence is excitable at 488 nm. The emission maximum is at 599 nm. CM-H_2XRos, the reduced form of CMXRos, is nonfluorescent; once inside cells with an active energy metabolism, it is oxidized to CMXRos.

The ability of these probes to bind covalently to constituents of mitochondria allows them to withstand fixation, as did the PhoCy dye described on p. 397, but there has been a running controversy in the literature as to how faithfully CMXRos measurements of fixed cells represent the state of mitochondrial energization before fixation. In dual staining experiments, Macho et al[2821] found CMXRos fluorescence to be highly correlated with $DiOC_6(3)$ fluorescence, and pursued immunofluorescence measurements of intracellular proteins such as Bcl-2 in cells that had been fixed and permeabilized following CMXRos staining. Ferlini et al[2822] questioned whether some of the correlation between CMXRos and cyanine dye fluorescence represented spectral crosstalk, but Macho et al[2823], in response, cited additional evidence that CMXRos responded to $\Delta\Psi_m$.

Molecular Probes offers several fixable mitochondrial probes in addition to CMXRos and CM-H_2XRos; these include **MitoTracker Green FM**, which has spectral characteristics similar to fluorescein but is almost nonfluorescent in aqueous solution, and **MitoTracker Orange CM-H_2TMRos** and **CMTMRos**, which emit at 576 nm[2532]. Hollinshead et al[2824], in a paper demonstrating the advantages of antibiotin antibodies, which react with endogenous biotinylated proteins in mitochondria, for specific labeling of those organelles in fixed material, reported that MitoTracker Green FM staining, representative of the state of mitochondrial energization before fixation, was preserved in fixed tissue. Poot and Pierce[2825] reported that, in cells stained with 200 nm each of MitoTracker Green FM and CMXRos, the ratio of CMXRos fluorescence to MitoTracker Green fluorescence provided better discrimination of apoptotic cells than did CMXRos fluorescence alone. A challenge by Gilmore and Wilson[2826] was followed by a response to Ferlini et al by Poot and Pierce[2827], maintaining that changes in CMXRos fluorescence are sensitive indicators of early apoptosis. Salvioli et al[2828], in a study of HL-60 cells, conceded that, while JC-1 and CMXRos did not always give identical results, CMXRos was a reliable probe for assessing $\Delta\Psi_m$, but Mathur et al[2829], studying cultured cardiomyocytes, found that JC-1 staining did reflect $\Delta\Psi_m$, but that CMXRos staining did not.

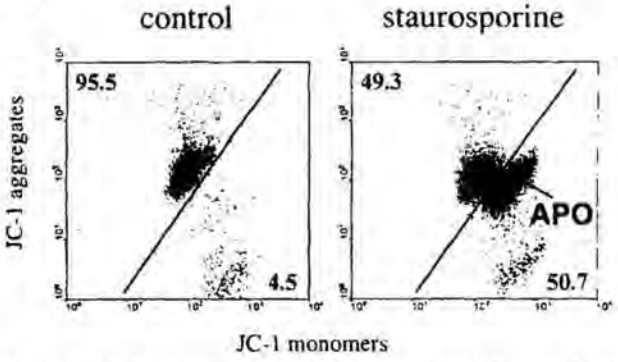


Figure 7-31. Demonstration of apoptotic HL-60 cells (APO) with deenergized mitochondria by JC-1 staining. Apoptosis was induced by staurosporine. The "JC-1 aggregates" axis displays 590 nm fluorescence; the "JC-1 monomers" axis displays 530 nm fluorescence. From: Salvioli et al, Cytometry 40:189-197, 2000 (Reference 2828), © John Wiley & Sons, Inc., used by permission.

If you decide to be conservative and rely on JC-1 as your probe for $\Delta\Psi_m$, Figure 7-31, above, gives you an idea of what you can expect. The apoptotic cells contain somewhat fewer JC-1 aggregates, as indicated by slightly lower 590 nm fluorescence, and substantially more JC-1 monomers, as indicated by 530 nm fluorescence, than do the unaffected or control cells. It would not be unusual to see lower values of 590 nm fluorescence in apoptotic cells than are shown in this figure.

In 2000, Keij et al[2830] reported that mitochondrial staining by NAO (p. 397), MitoTracker Green, and another Molecular Probes offering, **MitoFluor Green**, was affected by $\Delta\Psi_m$ changes induced by drugs prior to staining. A critique by Isola et al[2831] noted that most people stained first and applied treatments later. However, Keij et al have made the point that the mitochondrial uptake of all of the dyes they used is potential-dependent, which should bring us closer to the conclusion that neither NAO nor any of the MitoTracker dyes is a probe of "mitochondrial mass."

It had been alleged for many years that NAO binding to mitochondria was at least partly potential-independent, and reflected the affinity of the dye for **cardiolipin**, a polyunsaturated acidic phospholipid found exclusively in bacterial and mitochondrial membranes. NAO has, therefore, been

used as a probe for cellular cardiolipin content as well as a probe for mitochondrial mass; Jacobson et al[2832], who have confirmed that NAO binding responds to $\Delta\Psi_m$, as Keij et al[2830] reported, may have slain that dragon. Or maybe not. Rhodamine 123 had magical properties attributed to it for years before JC-1 came along, but the mitochondrial staining properties of both dyes are largely deducible from their lipophilicities, structures, and association constants. We know that the MitoTracker dyes stay in mitochondria because they bind covalently once they are driven in by cytoplasmic and mitochondrial membrane potential gradients and by lipophilicity, and we can be pretty sure that NAO, once driven in by the same factors, is locked into mitochondrial membranes by its tracking dye-like nonyl side chain. If you're looking for a mitochondrial mass probe, anti-biotin antibodies[2829] may not be a bad bet.

That's about it for mitochondria; I should mention that Pham et al[2765] have developed a neat technique for looking at the activity of individual components of the respiratory chain in digitonin-permeabilized cells, using either 40 nM $DiIC_1(5)$ or 90 nM CMXRos as a probe of $\Delta\Psi_m$, and simultaneously detecting reactive oxygen species with carboxy-DCF (p. 379).

Bacterial Membrane Potentials

Bacteria, unlike eukaryotes, do not compartmentalize their respiratory enzymes inside mitochondria; the enzymes are, instead, located on the inner surface of the cytoplasmic membrane. As a result, the 100-200 mV, inside-negative, potential gradient generated by bacterial energy metabolism exists across the cytoplasmic membrane, and some bacteria stain in more or less the same potential-dependent fashion with rhodamine 123, cyanine dyes, and other fluorescent lipophilic cationic dyes[856-7]. Other bacteria, however, have to be coaxed into staining. In general, there's not much of a problem getting **Gram-positive** bacteria to take up lipophilic dyes, because the dyes readily get through the bacterial cell wall. The **Gram-negative** bacteria are more of a problem; the lipopolysaccharide-containing **outer membrane**, really a cell wall and not a membrane, efficiently excludes lipophilic compounds. It is possible to render the outer membrane permeable to lipophilic materials using any one of a number of chemical agents; the most widely used is probably **ethylene diamine tetraacetic acid (EDTA)**, which removes calcium from the structure.

Since bacterial membrane potential changes rapidly in response to the availability or lack thereof of suitable energy sources, and is rapidly dissipated when the organism is killed by drugs or other agents, it is possible to exploit this parameter and potential-sensitive dyes in rapid cytometric procedures for bacterial detection, identification, and antibiotic susceptibility testing. This subject will be further discussed in Chapter 10; here, I will consider some particular problems in bacterial membrane potential measurement, and describe at least one possible solution.

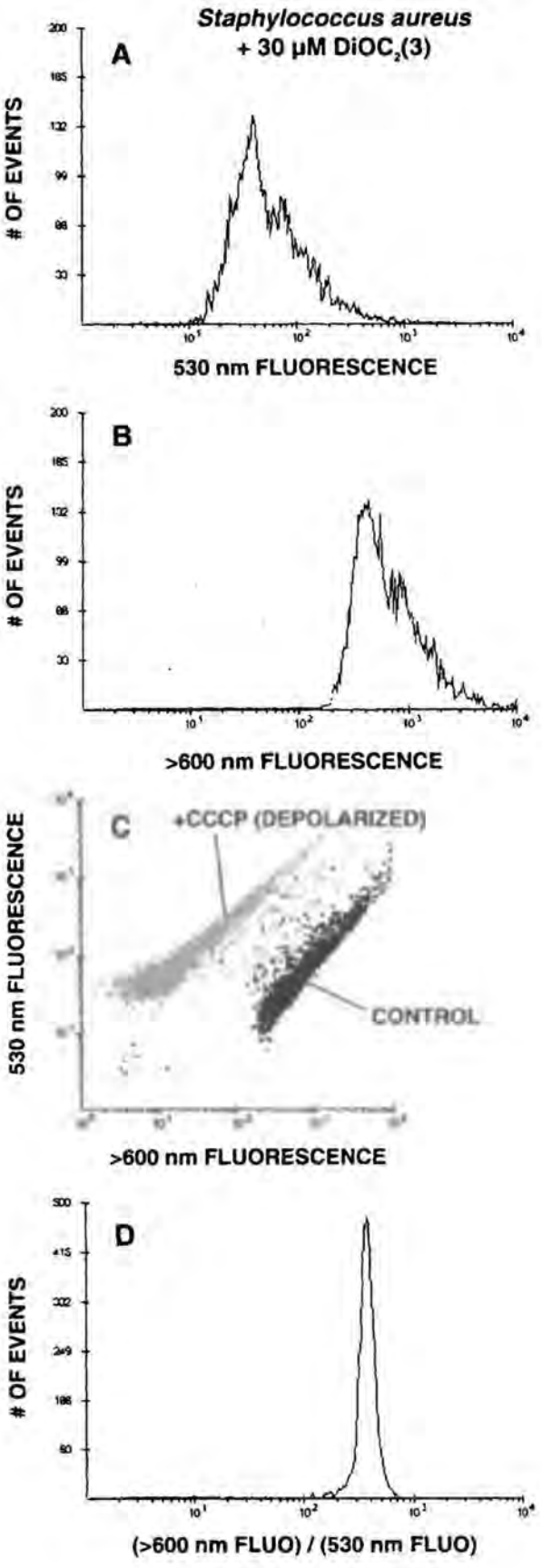


Figure 7-32 (legend on next page)

Figure 7-32 (opposite page). Measurement of the membrane potential of *Staphylococcus aureus* using $DiOC_2(3)$ and a ratiometric technique developed by Novo et al[2357]. Cells are loaded with 30 µM dye, and fluorescence, excited at 488 nm, is measured at 530 nm and at >600 nm. The curves and dots plotted in gray represent cells depolarized with CCCP; the curves and dots plotted in black represent control cells. Plots were created with FCS Express software (DeNovo Software).

Ratiometric ΔΨ Measurement in Bacteria

There are now a substantial number of papers in the literature (see Chapter 10 for references) describing flow cytometric measurement of ΔΨ in bacteria, both as a means of determining the metabolic state of bacteria in their natural environments and as a means of assessing response to antimicrobial agents or lack thereof. Rhodamine 123, $DiOC_6(3)$, and the oxonol dye $DiBAC_4(3)$ have all been used as probes, and they all share a disadvantage which can be appreciated from panels A and B of Figure 7-32 and from Figure 7-27 (p. 388). The distributions of dye fluorescence from cells that are maintaining normal membrane potential and from cells that have been completely depolarized by an ionophore (gramicidin A or CCCP) overlap enough so that one cannot clearly discriminate depolarized from control cells. This becomes a serious problem when one is attempting to determine whether bacteria will respond to a drug; killing 95 percent, or even 99 percent, of the population isn't good enough.

A few years back, David Novo, now known as the entrepreneurial genius behind **DeNovo Software** and its flagship product **FCS Express**, but then fresh out of college, came to my lab and did a creditable imitation of a very good postdoc. Dave wanted to develop a flow cytometric antimicrobial susceptibility test, and we started looking at cyanine dye fluorescence in *Staphylococcus aureus*, which, while otherwise fairly user-friendly, grows in clumps. This creates even more of a problem than there is with most other bacteria when you look at fluorescence distributions, because fluorescence from a clump of n cells is n times as bright as fluorescence from a single cell. In work with oxacarbocyanines some years back, I had noted that there was a fair amount of fluorescence detectable from these dyes at wavelengths above 580 nm, and Dave followed up on these observations with some rigorous spectrofluorimetric analyses that established that, at very high concentrations, the $DiOC_n(3)$ dyes developed a second emission peak at around 610 nm. The concentration dependence suggested that the long-wavelength fluorescence comes from J-aggregates.

In order to get substantial emission above 600 nm from microorganisms, we had to use much higher dye concentrations than we or anyone else had used previously; the concentration we found suitable for the most tractable of the dyes, $DiOC_2(3)$, was 30 µM. When we loaded bacteria with dye at this concentration, we found that 530 nm fluorescence from the organisms (panel A of Figure 7-32) no longer changed significantly with ΔΨ, but that fluorescence above 600 nm (panel B of Figure 7-32) did. Both 530 nm and >600 nm fluorescence signals appeared to be sensitive to cell (or clump) mass or size, and a dot plot (panel C of Figure 7-32, also shown in color on the back cover) showed high degrees of correlation between the two fluorescence values in clusters of depolarized and control cells, with the two clusters clearly separated. It thus came as no surprise to us that the ratio of

(>600 nm fluorescence)/(530 nm fluorescence) ,

multiplied by an appropriate constant to get it on the display scale, could serve as a measure of ΔΨ. This quantity is plotted in panel D of Figure 7-32, and it is clear that the degree of overlap between values of the ratio measured in depolarized and control cells is extremely small.

By examining valinomycin-treated organisms in buffers containing a range of potassium concentrations (distributions are shown on the back cover), the fluorescence ratio measurement can be calibrated to read out values of ΔΨ; we found that the usable range for *S. aureus* ran from approximately -30 mV to approximately -130 mV.

As far as I know, the ratiometric technique we described remains the most accurate and precise flow cytometric method for measurement (we've gone beyond estimation) of bacterial ΔΨ. The details may be found in reference 2357; I should, however, add a few things we have learned since that paper was published.

The technique can be applied to Gram-negative bacteria if EDTA is used to permeabilize the outer membrane, but, while it did provide the same large degree of separation of clusters of depolarized and control cells as we noted when working with Gram-positive organisms, we were unable to construct a calibration curve because the valinomycin-potassium buffers tended to lyse the EDTA-treated Gram-negative organisms.

We examined a number of indocarbocyanine, indodicarbocyanine, thiacarbocyanine, and thiadicarbocyanine dyes and did not find one that exhibited sufficient long wavelength fluorescence at high concentrations to permit its use for ratiometric measurements. The Molecular Probes dye **JC-9**[2332], a dibenzo homologue of $DiOC_2(3)$ that shares the tendency of JC-1 to form fluorescent J-aggregates, can be used for ratiometric measurements but the fluorescence ratio distributions obtained from control and depolarized bacteria stained with JC-9 are not as well separated as those obtained from bacteria stained with $DiOC_2(3)$. In our hands, JC-1 itself has not been usable for ratiometric measurements of ΔΨ in bacteria.

I suspect that $DiOC_2(3)$ could be used for ratiometric membrane potential measurement in isolated mitochondria, however, at the concentration necessary to produce a substantial fraction of J-aggregates, it is likely to perturb mitochondrial function[2818].

In the paper[235], we reported that we were unable to grow bacteria after they had been exposed even briefly to 30 µM $DiOC_2(3)$. However, Imogen Wilding of GlaxoSmithKline reports (personal communication, 2001) that it is possible to culture organisms that have been exposed to the dye at 30 µM and at higher concentrations. I should have learned from my experience with pyronin Y (p. 324) that growing cells is not my strong point.

It is worth thinking about why this particular ratiometric method works so well in bacteria. I used to use cyanine dye fluorescence for $\Delta\Psi$ estimation, working with an external concentration of no more than 100 nM. Assuming that $\Delta\Psi$ = -122 mV, which would make the intracellular dye concentration 100 times the extracellular concentration according to the Nernst equation, and adding another factor of 10 for non-Nernstian effects that increase intracellular dye concentration, we come up with an intracellular concentration of 100 µM, which sounds very impressive, until we consider two other factors. First, the volume of the bacterial cell is only about 1 fL, and the word from Avogadro is that, given that intracellular concentration, we will only find about 60,000 molecules of dye in the organism. Second, most of those molecules are likely to be quenched, meaning that the quantum efficiency will not be on the order of 0.5, which is not atypical for cyanine dyes in nonpolar solvents, but is more likely to be a tenth of that value or less, so it will take at least ten molecules of intracellular dye to give us one MESF worth of fluorescence, leaving us with no more than 6,000 MESF. If we assume that the Q value (p. 222) for detecting a cyanine dye is similar to that for detecting fluorescein (.012 photoelectrons/MESF), we'll generate one photoelectron for every 80 MESF, or 75 photoelectrons for 6,000 MESF. If photoelectron statistics were the only source of variance in the fluorescence distribution, the CV would be no lower than 11.4 percent. The ratiometric measurement method uses a dye concentration 300 times higher, and, even though we can expect there to be even more quenching of the fluorescence of intracellular dye, the photoelectron statistics should improve substantially.

The ratiometric $\Delta\Psi$ measurement described by Gonzales and Tsien[2456,2808,9] (p. 392), using a labeled fatty acid or tracking dye and an oxonol, could, in principle, be applied to bacteria, but since one would expect there to be only a few thousand molecules of each of the dyes present per cell, it would probably be necessary to use either a slow flow system or a static or scanning cytometer to get good enough photoelectron statistics to make the exercise worthwhile.

$\Delta\Psi$ Measurement: Cautions and Conclusions

Whether you measure cytoplasmic, mitochondrial, or bacterial membrane potential, you will end up working with some extremely lipophilic dyes. Lipophilic implies hydrophobic; these materials are almost insoluble in water and would prefer to be almost anywhere else. That's what accounts for their non-Nernstian uptake into cells, and it also makes them stick to just about any surface to which they may be exposed while in aqueous solution. The dyes, and the ionophores used to prepare controls, which are also lipophilic, will bind to the tubing in your flow cytometer. If you don't flush the system carefully when you are finished, the dyes and ionophores will abandon the tubing for the next batch of cells that comes along. I use both chlorine bleach and alcohol to clean my system, and, when I think it is clean, I run some unstained cells through and see whether they become fluorescent during the run. If they do, I go through another cleaning cycle. As a last resort, I change all the sample tubing. That's easy to do on a Cytomutt; it may not be on your apparatus. If you use a shared instrument, it might be a good idea to check with your fellow users and the operator, if there is one, before you attempt to do ratiometric measurements in bacteria with 30 µM cyanine dye.

When you do immunofluorescence measurements on fixed cells, you don't find that your cells have good days and bad days; when you measure membrane potential, or pH, or calcium, in live cells, you do. If the cells (or at least the control cells) aren't happy, you won't be.

You do have to be fairly obsessive-compulsive about technique to get good results. The primary papers and the protocol books I mentioned in Chapter 2 and at the beginning of this chapter are a good place to start; if you need more detail or clarification, an e-mail to an author or a posting to the Purdue Cytometry Mailing list is likely to get you the information you need.

Optical Probes of Intracellular Calcium

The Bad Old Days

In 1984, when the First Edition of this book was written, it wasn't clear that flow cytometry had much to contribute in the area of intracellular calcium measurements; a lot of people were interested, and the existence and importance of calcium fluxes and redistributions was obvious, but the probes weren't there. Now, they are.

The older generation of optical and alternative techniques for measurement of intracellular Ca^{++} concentrations were discussed at length in a book by Thomas[502] and in review articles by Blinks et al[503] and Tsien[504]. Among the more classical optical probes of intracellular $[Ca^{++}]$[502,4] are **aequorin**, a luminescent protein now best known because of its connection to GFP, and **arsenazo III**, a metallochromic dye, neither of which enters intact cells. Arsenazo III response to changes in $[Ca^{++}]$ is measured by a spectral shift detected by two-wavelength absorption measurements; this technique could conceivably be adapted to flow cytometry but cells would have to be permeabilized or microinjected to get the dye in before they could be studied.

Chlortetracycline as a Probe of "Membrane-Bound" Calcium in Cells

Caswell and Hutchison[505,6] described the use of the antibiotic **chlortetracycline (CTC)** (not, I hope, likely to be confused with the tetrazolium dye with the same abbrevia-

tion (p. 379)) as a fluorescent probe of intracellular "membrane-bound" Ca^{++}. The concept of **"membrane-bound" Ca^{++}** arose from their finding that the fluorescence of CTC was increased when the compound was bound in hydrophobic regions, and that the fluorescence of Ca^{++} and Mg^{++} chelates of CTC was higher than that of CTC. Measurement of emission at 520 nm with excitation at 390 nm supposedly maximizes the discrimination of fluorescence emitted by molecules of CTC-Ca^{++} chelate bound in hydrophobic regions from fluorescence emitted by free CTC, bound CTC, and other chelates.

When cells equilibrated with 10-200 μM CTC are examined under a fluorescence microscope, fluorescence is observed in the same membranous and lipid-bearing structures that are stained by cyanine dyes. If the cells are then washed and the fluorescence of a cell suspension is measured in a cuvette, a gradual decrease in fluorescence is observed as CTC is released from cells into the medium. Treatment of the cells with **Ca^{++} ionophore A23187** or with **ligands reactive with cell surface receptors** (e.g., chemotactic peptides in the case of neutrophils[457], bethanechol in pancreatic acinar cells[507-8], con A in thymocytes[509]) causes a decrease in fluorescence, observable under the microscope as well as in a fluorometer. This is interpreted as indicating **release of Ca^{++} from hydrophobic (membrane) binding sites.**

Flow cytometry of CTC-stained cells[112], using UV or violet excitation from lasers or arc lamps, yields "haystack" fluorescence distributions with large variances, similar to those obtained from cells stained with dye $\Delta\Psi$ probes. The distributions shift following addition of A23187 or stimulating ligands, but one cannot discriminate subpopulations of stimulated cells. Thus, single cell studies with CTC are probably better done by methods that allow sequential observations of the same cells than by flow cytometry.

Spectral studies by Mathew and Balaram[510] suggest that two-wavelength emission measurements of CTC fluorescence excited in the UV might yield a ratio sensitive to shifts in intracellular Ca^{++}, which should have a narrower distribution than is observed for CTC fluorescence. Since the CTC spectrum is sensitive to pH changes in the physiologic range, however, which may also occur during cell activation, results might be difficult to interpret.

Probes for Free Cytoplasmic Calcium: Quin-2

The first probe to come into vogue for studies of **free cytoplasmic [Ca^{++}]** was **quin-2**, first introduced by Tsien and his associates.[504,511-8]. This material is an extremely selective calcium chelator that is introduced into cells as an inactive ester that yields reactive quin-2 after enzymatic hydrolysis. Quin-2 is UV-excited (334-337 nm is the favored wavelength region) and blue fluorescent (maximum emission from the intracellular Ca^{++} chelate is at about 490 nm). However, the fluorescence emission from the amount of quin-2 that can be gotten into a cell is pretty weak, generally no more than 10 times the level of cellular autofluorescence (in the spectral regions applicable to quin-2 excitation and emission measurement, this is generally pyridine nucleotide autofluorescence). The Ca^{++} chelate is about 7 times as fluorescent as the uncomplexed dye.

Quin-2 has been used for bulk measurements of cells in cuvettes and for single-cell measurements by microphotometry; it has been possible using either method to demonstrate rises in cytoplasmic [Ca^{++}] that occur within seconds following receptor stimulation or ionophore addition and which abate within a few minutes thereafter. The extremely high affinity of quin-2 for Ca^{++} results in considerable perturbation of Ca^{++} metabolism in quin-2-loaded cells and tissues; I have heard of the dye stopping hearts and triggering mitosis in lymphocytes. I also heard that some preparations of quin-2 yielded 10 or 12 spots on chromatography.

Even without its toxicity or impurities, quin-2 is not well suited for use with most flow cytometers because the absorption of the Ca^{++} chelate is extremely low at the wavelengths above 350 nm at which most ion laser and arc lamp sources are operated for UV excitation. Better excitation wavelengths are available from arc lamps, krypton lasers (a very weak line at 337 nm) and from He-Cd lasers. I have used the 325 nm line from a He-Cd laser for quin-2 fluorescence excitation, which allows demonstration of an ionophore-induced [Ca^{++}] transient by a shift in the position of a fluorescence distribution. The distribution itself, however, is a haystack, making it unlikely that a small number of activated cells within a heterogeneous population could be discriminated by flow cytometry using quin-2.

For microphotometric and cinefluorometric studies in which fluorescence from the same cells or tissues was measured repeatedly, quin-2 did not have the same disadvantages as for flow cytometry, and the probe provided investigators with a substantial amount of information about cytoplasmic [Ca^{++}] changes before Roger Tsien and his colleagues cooked up two much better reagents, **fura-2** and **indo-1**[858].

Fura-2 and Indo-l: Ratiometric Ca^{++} Indicators

Like quin-2, fura-2 and indo-1 are selective calcium chelators; since neither compound has as high an affinity for calcium as does quin-2, the newer probes do not perturb cellular calcium metabolism to anywhere near the extent to which quin-2 does. Also like quin-2, both fura-2 and indo-1 are typically introduced into cells in the form of acetoxymethyl (AM) esters, which are enzymatically hydrolyzed in cells to the free dyes. Cells are loaded by incubation with 1-15 μM concentrations of AM ester for 30-90 min at 37 °C; in some cases, an agent such as a Pluronic detergent may be needed to improve penetration of cells by the dye[2358].

Both fura-2 and indo-1 are at least ten times as fluorescent as quin-2, and the newer dyes also share the desirable characteristic, absent in quin-2, of undergoing substantial **spectral shifts** upon binding calcium. In the case of fura-2, the most pronounced change is in the excitation spectrum; while, **for indo-1, the emission spectra of the free dye and the calcium chelate differ substantially**, as shown in Figure 7-9 (p. 296) and Figure 7-33 (next page). Fura-2 and

indo-1 can, therefore, both be used to make **ratiometric** measurements of cytoplasmic [Ca⁺⁺], which cancel out many extraneous factors, including the effect of cell-to-cell variations in dye content.

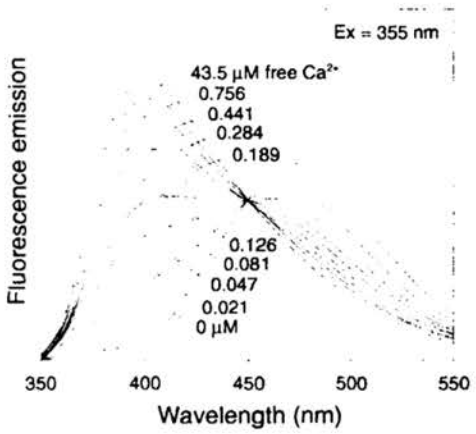


Figure 7-33. Emission spectra of indo-1 in solutions of increasing free calcium ion concentration. The figure was provided by Molecular Probes.

Fura-2 has an emission maximum at 510 nm; the absorption maximum for the calcium chelate is at about 335 nm and that for the free dye is at about 362 nm. If fluorescence at 510 nm is measured, first using 340 nm excitation and then using 380 nm excitation, the ratio ($F_{340\rightarrow510}/F_{380\rightarrow510}$) provides a measure of cytoplasmic [Ca⁺⁺]. The excitation wavelengths used for fura-2 measurements are readily available from xenon arc lamp sources, which do not have sharp spectral peaks in the near UV and visible regions, and fura-2 has been used for single cell [Ca⁺⁺] studies employing microspectrophotometers and video microscopes[859-61]. Fura-2 is less well suited for use in flow cytometers, because the excitation wavelengths are not readily obtained from the standard laser or arc sources.

Indo-1 fluorescence is excited by UV light; the free dye shows maximum emission at about 480 nm, and the calcium chelate emits maximally at about 405 nm. Any of the UV excitation sources commonly employed in flow cytometry can be used to excite indo-1, including He-Cd (325 nm), argon (351/363 nm) and krypton (350/356 nm) lasers and Hg arc lamps (366 nm). Fluorescence is measured through 10-20 nm bandpass filters centered near 405 and 480 nm, although I have been told by various people that they prefer 500 or even 520 nm filters to 480 nm filters for the longer wavelength measurement. The ratio, i.e., ($F_{UV\rightarrow405}/F_{UV\rightarrow480}$), is taken as a measure of cytoplasmic [Ca⁺⁺][862]. An example of flow cytometric measurement of cytoplasmic [Ca⁺⁺] changes in cell activation appears in Figure 7-21 (p. 365).

Since quin-2 provided only poor resolution of responding and nonresponding cell subpopulations[864], it was not widely used in flow cytometry. Indo-1, however, came into considerable vogue in a relatively short time after its properties were first described[853,865]. A particularly useful technique described by Rabinovitch et al[866-7] employs **indo-1 in combination with fluorescent antibodies** to follow cytoplasmic [Ca⁺⁺] responses in cell populations defined by the presence of a specific surface antigen.

While ratiometric [Ca⁺⁺] measurements made with either fura-2 or indo-1 eliminate the effect of cell-to-cell variations in dye content, measurements made with indo-1 have an additional advantage in that the effects of uneven illumination and of light source noise also are eliminated by virtue of their equal influences on the numerator and denominator of the ratio. This advantage, it should be noted, is common to other flow cytometric ratio measurements in which both parameters used in the ratio are measured at the same time in the same beam.

If aliquots of fura-2 or indo-1 loaded cells are placed in solutions with various known Ca⁺⁺ concentrations and treated with a **calcium ionophore** such as **A23187** or **ionomycin**, it is possible to calibrate the fluorescence ratio measurement to yield accurate molar values of cytoplasmic [Ca⁺⁺]. Kachel et al[1713] have described a general method for calibration of flow cytometric wavelength shift fluorescence measurements based on pulse modulation of the excitation source in a flow cytometer; it is not clear how readily this can be implemented in existing apparatus.

Indo-1 is widely used, at least by people with UV excitation sources in their flow cytometers[1714-8]. Since there are probably more than 10,000 fluorescence flow cytometers out there that don't have UV sources, that's small comfort. Luckily, there are alternatives.

Fluo-3 and Other Visible-Excited Ca⁺⁺ Probes

In 1989, Minta, Kao, and Tsien[1719] described a series of fluorescein- and rhodamine-based calcium indicators suitable for use with 488 nm excitation. The most widely used of these is **fluo-3**, which has the spectral characteristics of fluorescein, but which is almost nonfluorescent unless bound to calcium. The emission spectrum of fluo-3 at various values of [Ca⁺⁺] is shown in Figure 7-34 (next page).

Unlike fura-2 and indo-1, fluo-3 does not exhibit a spectral shift with changes in calcium concentration. A fluo-3 fluorescence distribution is a haystack; if you're stimulating a cell population, the haystack moves to the right when the cytoplasmic [Ca⁺⁺] goes up and back to the left when it goes back down. In 1990, Rijkers et al[1720] came up with a dodge that enables fluo-3 to be used in a more or less ratiometric mode; they load cells simultaneously with fluo-3 and with SNARF-1, a pH-sensitive dye that will be discussed in the next section. The amounts of the two dyes taken up by cells are roughly similar. The emission of SNARF-1 at 600 nm does not change appreciably during activation; as a result, the ratio of fluo-3/SNARF-1 fluorescence (520 nm emission to 600 nm emission) provides a measure of cytoplasmic [Ca⁺⁺] with considerably less variance than there is in the fluo-3 histogram.

In 1994, Novak and Rabinovitch[2833] introduced an improved ratiometric method combining fluo-3 with another

calcium probe, **Fura Red**. Fura Red is excitable at 488 nm, emits maximally at 660 nm, and becomes less fluorescent on binding calcium; the ratio of fluo-3 to Fura Red fluorescence is therefore relatively sensitive to small changes in cytoplasmic [Ca^{++}].

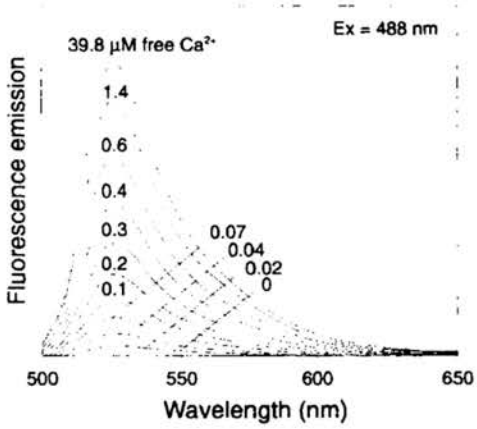


Figure 7-34. Emission spectrum of fluo-3 in solutions of increasing free Ca^{++} concentration. The figure was provided by Molecular Probes.

Molecular Probes (see the *Handbook*[2332]), which offers quin-2, indo-1, fluo-3, and Fura Red, has some other visible excited calcium indicators, such as the **Calcium Green** series.

Akkaya and Lakowicz[1722] reported they were developing a series of styryl-based, visible-excited, ratiometric indicators, but these have apparently not hit the market yet.

I am told that, while it might be possible to make an indo-1-like calcium probe that could be excited by a UV (370 nm) diode laser, it would be difficult, if not impossible, to tweak a similar molecule sufficiently to permit excitation by a violet diode.

AM esters and dyes loaded as AM esters may be pumped out of cells; Baus et al[2834] reported that the anion transport inhibitor sulfinpyrazone blocked the efflux of Fluo-3 from transformed murine T cells.

Flow Cytometric Probes of Intracellular pH

Since we have all been exposed, generally starting in junior high school (that might be middle school for some of you young ones), to dyes that change color with changes in pH, it should come as no surprise that some such materials can be used for single cell analysis. If a dye changes color with pH, one can find two wavelengths at which absorption, excitation, or emission will change differently as pH changes; the pH can then be estimated from the ratio of measurements made at those two wavelengths. As we have just noted while discussing the use of fura-2 and indo-1 as calcium probes, ratiometric measurements are highly desirable, especially in flow cytometry; as we shall shortly see, ratiometric measurements of intracellular pH by flow cytometry antedate ratio measurements of cell calcium.

The first description of a flow cytometric method for estimation of intracellular pH was published in 1979 by Visser, Jongeling and Tanke[519]; they measured the fluorescence of intracellular **fluorescein** produced by enzymatic hydrolysis of FDA. It is the excitation spectrum, rather than the emission spectrum, of fluorescein that shows the greatest dependence upon pH[202,4,369,376]. Since Visser et al did not have two-wavelength excitation capability in their flow cytometer, they estimated pH changes from changes in emission above 530 nm of cells excited at a single wavelength (488 nm).

When wavelengths above 465 nm are used for fluorescein excitation, emission intensity increases with pH; at excitation wavelengths below 465 nm, emission intensity decreases with pH. When excitation is at 465 nm, an **isosbestic point**, emission intensity is independent of pH. Visser et al noted that the average emission intensity of cells equilibrated with buffers of increasing pH increased, as would be expected, when 488 nm excitation was used; decreases in intensity with increasing pH were observed when the excitation wavelength was changed to 458 nm.

Valet et al[200] and Gerson[201] described ratiometric techniques for flow cytometric pH estimation in which the ratio of intensities of green and blue fluorescence emission from UV-excited dyes was used as a measure of pH. This ratio increases with pH. The probe used by Valet et al is **1,4-diacetoxy-2,3-dicyanobenzene (ADB)**, also called **2,3-dicyano-1,4-hydroquinone diacetate**; it is available from **Sigma**. ADB is enzymatically hydrolyzed inside cells to yield **2,3-dicyanohydroquinone (DCH)**. Gerson used the coumarin **4-methylumbelliferone (4-MU)** in free and esterified forms. These probes have an advantage over fluorescein derivatives in that they can be used with single beam excitation; 4-MU, however, also has the disadvantage of being able to cross cell membranes freely, and therefore tends to leak out of cells rapidly.

There exist **distributional radiolabel and dye probes of intracellular pH**. These are weak acids and bases that partition across membranes as a function of transmembrane pH gradients; 9-aminoacridine is an example. While they are usable for studies of cells in bulk, there is absolutely no reason to choose distributional pH probes for single cell studies, given the existence of dyes that can be used for ratiometric measurements.

The **calibration of ratiometric pH probes** is straightforward; fluorescence measurements are made of aliquots of cells suspended in high-potassium buffers at different, known pH's, and the cytoplasmic pH is made equal to the buffer pH by addition of the **proton ionophore nigericin** prior to flow cytometric measurement of emission or excitation ratios. This allows construction of a calibration curve. As is the case with the college chemistry variety of pH indicator techniques, cytometric measurements of pH will be most precise in the region of the pK_a of the indicator used.

An alternative calibration procedure was described by Chow et al[2835], who adapted a method described by Eisner et al[2836]. Cells are suspended in solutions containing mixtures of

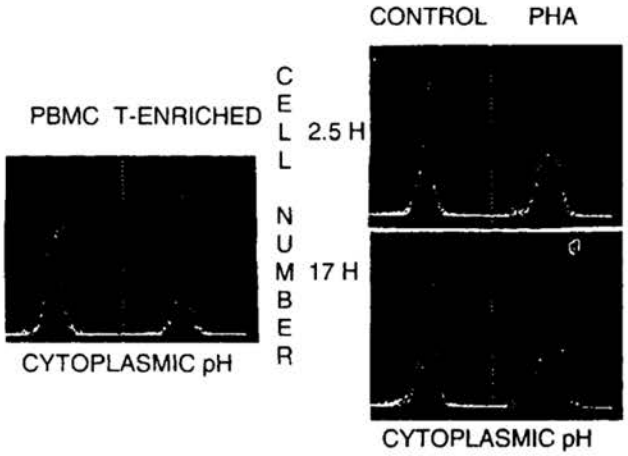


Figure 7-35. Estimation of cytoplasmic pH in human lymphocytes from carboxyfluorescein fluorescence ratio (520 nm emission; 488 and 441 nm excitation from argon and He-Cd lasers). The panel at left shows unstimulated peripheral blood mononuclear cells (PBMC) and a T cell enriched fraction. The panel at right shows control T-enriched cells and T-enriched cells exposed to PHA, 2.5 (upper pair) and 17 (lower pair) hours after exposure. PHA appears to increase pH in some cells and decrease it in others.

a weak acid (butyric acid) and a weak base (trimethylamine) in fixed ratios but different molar concentrations, allowing determination of a "pseudo null point" pH. The calibration curve is different for each cell type.

Valet et al[200] used ADB (DCH) primarily to demonstrate pH heterogeneity in tumor cell populations; Alabaster[520] employed the same probe for the same purpose. Gerson reported increases in pH within a short time following lymphocyte stimulation[427] and also with progression of stimulated lymphocytes through the cell cycle[521]; he has used bulk distributional measurements as well as flow cytometry with 4-MU and derivatives[201] for his analyses.

Musgrove, Rugg and Hedley[868] published a comparative study of **4-MU, DCH, and 2′,7′-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF)**[869] as flow cytometric pH probes; this provides a good reference on the use of these dyes and on the calibration procedure as well. UV excitation was used for 4-MU and DCH, with pH being estimated for 4-MU from the ratio $F_{UV\rightarrow450}/F_{UV\rightarrow560}$ and for DCH from the ratio $F_{UV\rightarrow425}/F_{UV\rightarrow540}$. BCECF was excited at 488 nm, and pH was estimated from the ratio $F_{488\rightarrow520}/F_{488\rightarrow620}$. 4-MU was poorly retained by cells; both DCH and BCECF were found to be usable over a pH range from 6.0 to 7.5. DCH gave the most sensitive indication of pH changes; BCECF was retained longest by cells. However, BCECF may be better suited to ratiometric measurements using dual-wavelength excitation than to emission ratio measurements.

Years ago, when I played the pH game briefly, I chose to pursue a flow cytometric pH estimation technique based on **fluorescein derivatives**, in preference to the probes described by Valet et al and Gerson, for several reasons. First, I wanted to be able to make **correlated measurements of pH and cell cycle position** in cells stained with Hoechst 33342, and, if possible, to measure pH and [Ca^{++}] together in quin-2 (or indo-1) loaded cells. Both Hoechst 33342 and the calcium probes are UV-excited and blue fluorescent, and thus cannot be used with ADB or 4-MU, which share their spectral characteristics. There are no substitutes presently available for either Hoechst 33342 or indo-1 for their respective purposes. However, I already knew[8] that it was possible to measure Hoechst dye fluorescence and pH independently in a three-beam flow cytometer if fluorescein derivatives were used for pH estimation.

Carboxyfluorescein (COF), at the time, was the probe best suited for such studies; while its spectrum is nearly identical to that of fluorescein[203,4], COF offers two significant advantages. The free dye produced intracellularly by enzymatic hydrolysis of COFDA is lost from cells much more slowly than is FDA-derived fluorescein. More important, **free COF does not enter the mitochondrial compartment**, and thus provides a good estimate of **cytoplasmic pH** rather than a composite of cytoplasmic and mitochondrial pH; BCECF shares this characteristic. In order for COFDA to enter cells, the buffer pH during loading must be kept relatively low (≤ 7.0) to keep most of the material in an unionized form. Thereafter, pH can be restored to the physiologic (7.3-7.4) range used for most experiments. **BCECF is preferable to COF in that cells can be loaded at physiologic pH.**

The pH estimate obtained from **COF or BCECF fluorescence** is derived from the ratio of emission intensities at 520-550 nm obtained using **two excitation wavelengths** above and below 465 nm. The 488 nm argon ion laser line is nearly optimal as the longer excitation wavelength. For maximum sensitivity of the emission intensity ratio to pH, the shorter excitation wavelength used should be in the 420-450 nm range; the 441 nm He-Cd laser line and the 436 nm Hg arc lamp line are both suitable for this purpose. The ratio $F_{488\rightarrow530}/F_{441\rightarrow530}$ increases with increasing pH.

Nancy Allbritton and I (unpublished) used 488 and 441 nm excitation to make estimates of pH in human peripheral blood mononuclear cells, and in samples enriched in T cells, before and after PHA stimulation. Some of our results are shown as Figure 7-35.

The advantages of a ratio measurement are immediately apparent from the figure; this was the first "needle" distribution not representing DNA content that I had ever seen emerge from flow cytometry of real cells. The CVs of pH distributions are on the order of 5%; the displacement of peaks after lectin stimulation is at least 10%, suggesting that this parameter could be used to discriminate small subpopulations of activated lymphocytes within a few hours follow-

ing stimulation. There remains some controversy[870-1] as to whether pH change can serve as a litmus test for lymphocyte activation. The indicators, however, keep getting better.

Figure 7-36 shows the emission spectra of another item from Molecular Probes' copious catalog[2332], the ratiometric pH indicator **carboxy SNARF-1**, which is a current favorite. This dye is well excited at 488 nm; the ratio of emission intensities at 640 and 580 nm provides a measure of pH.

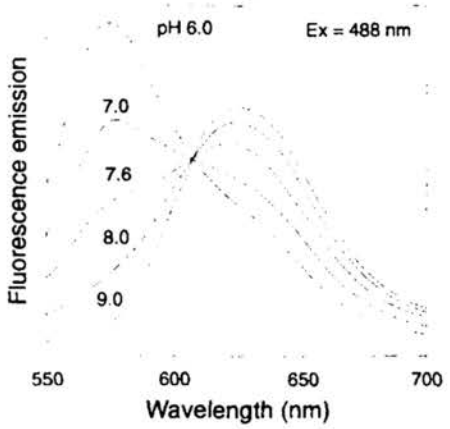


Figure 7-36. The pH-dependent emission spectra of carboxy SNARF-1 excited at 488 nm. The figure was provided by Molecular Probes.

Although some people got reasonably good results with ADB[1722-3], by the late 1980's, BCECF had become the indicator of choice. Hedley and Jorgensen[1724] found an 8% CV in the BCECF fluorescence ratio obtained from B16 melanoma cells, which would correspond to 0.4 pH units; they felt, however, that much of the variance was due to instrumental factors rather than pH variation. Musgrove, Seaman, and Hedley[1725] used BCECF to measure pH in samples enriched for different cell cycle phases; higher pH values were associated with S, G_2, and M phases of the cell cycle, with a corresponding increase in the percentage of G_1 cells at the lower pH range, suggesting cell-cycle dependence of pH.

Wang et al[1726] measured intracellular pH by the distribution of the weak acid, 5,5-dimethyl-2,4-oxazolidinedione-2-14C (^{14}C-DMO), a classical method, and by fluorescence flow cytometry with BCECF. The pH determined for CHO cells by the FCM method at external pH values of 6.0-8.1 agreed within 0.1 pH unit with that determined by the ^{14}C-DMO method.

Carboxy SNARF-1 is said to be more sensitive than either BCECF or ADB over the pH range 7.0-8.0[1727]; the dye is also better retained than ADB[1728]. Simultaneous detection of conjugate formation between cytotoxic and target cells during the cytotoxic process and determination of cytoplasmic [Ca^{++}] and pH are possible if one cell type is labeled with Fluo-3, and the other with SNARF-1; events positive for both dyes are identified as conjugates[1729].

In studies of rat basophil leukemia cells, Lee et al[1730] observed that external bicarbonate was necessary for pH regulation in these cells; it is thus advisable to have bicarbonate in your buffer when doing pH measurements.

Carboxy SNARF-1 and other dyes, e.g., BCECF and indo-1, are typically loaded into cells as acetoxymethyl (AM) esters; the hydrolysis of these esters results in a net acidification, and probe-induced perturbations, problematic in [Ca^{++}] studies with quin-2 (p. 403), may also need to be considered in critical analyses of pH[1731].

Measurement of pH in intracellular compartments is feasible if you can localize the pH indicator to those compartments. McNamara et al[2837] coated small polystyrene beads with phospholipids covalently labeled with fluorescein (pH-sensitive) and tetramethylrhodamine (pH-insensitive). The lipobeads are ingested by macrophages and directed to lysosomes; fluorescence measurements by flow or image cytometry can, respectively, provide values of average lysosomal pH or of the pH in an individual lysosome. Levitz et al[2838] covalently labeled the fungus *Cryptococcus neoformans* with fluorescein and 2',7'-difluorofluorescein (Oregon Green), which, respectively, have pK_as of 6.4 and 4.7, respectively, allowing sensitive pH detection over a broad range. They used the labeled fungi to study phagosomal pH.

Chitarra et al[2839] used ratiometric flow cytometric measurements of CFSE fluorescence to estimate internal pH in the bacterium *Clavibacter michiganensis*, taking the ability of cells to maintain a high pH in media of lower pH as an indicator of viability. This is, in effect, a rather fancy way of doing a dye exclusion test. It should not come as a surprise that the rather broad distributions of CFSE fluorescence ratio did not completely distinguish viable and killed cell populations; as was mentioned on pp. 256 and 402, if you only have a few thousand molecules of dye to work with, you probably won't get enough photoelectrons to do a precise measurement. The method would almost certainly work better in a slow flow, scanning or imaging system; others have reported making reasonably precise measurements of bacterial pH with the latter[2840-1].

The Hat Trick: Multiparameter Approaches to Ion Flux Measurements in Cell Activation

It has been my experience that you can use good parameters in multiparameter measurements to help make sense of bad ones. When I started working with membrane potential and immunofluorescence, using only forward scatter measurements for gating, I always suspected that I was measuring garbage along with my cells. I feel much more comfortable when I gate on Hoechst dye fluorescence, because I'm sure there's really a cell there.

Until I got ratiometric pH measurements working, I had no way of improving my conception of what was going on in flow cytometric estimation of $\Delta\Psi$ with cyanine dyes, [Ca^{++}] measurements with quin-2, etc. I thought I might be able to clarify things a little bit if I correlated signals from these probes with measurements of the amount of labeled ligand bound to the cell surface, but I couldn't work up

much enthusiasm about this because I'd be looking at haystack vs. haystack. When ratiometric pH and [Ca^{++}] measurements became available, the picture changed considerably, and it became logical to reformulate some of the questions I originally tried to ask when there was finally some hope of answering them.

Since I wanted to look at stimulation of cells by ligands that react with cell surface receptors, I envisioned ways of getting some kind of dose-response curve, for which I would need a labeled ligand. If the ligand were bound covalently to a fluorescent bead, I could count the number of beads bound to a cell to determine the "dose" of ligand, and follow the time courses of [Ca^{++}], pH, and/or $\Delta\Psi$ changes in cells with various numbers of beads to determine the "response."

I figured that if I had the right dyes and the right excitation beams, I would to be able to get measurements of [Ca^{++}], pH, membrane potential, and ligand binding all at once, assuming that probe interactions wouldn't cause big problems. Since I and others[872, 1718,1729,1737-9] had done simultaneous measurements of multiple functional parameters, I thought (and think) the assumption was (and is) fairly safe. And, of course, I'd have at least one or two fluorescence channels available to gate out cell subpopulations

This is still about as fancy a flow cytometric experiment as I can conjure up; not surprisingly, it takes a fairly elaborate flow cytometer, with four beams. As it happens, all four beams (UV, violet, 488 nm, and red) can be derived from small, air-cooled lasers that plug into the wall. Nicer still, once we put the beast together, we can use it to do almost all of the other measurements that have been discussed in this chapter (I said all this in the previous edition, in 1994; in 2003, as you will find in Chapter 8, you can buy such an instrument).

I puckishly referred to the experiment described above as the hat trick, because it had three goals and might net me some valuable information about early events in cell activation if I could stick to it. However, it's on ice for now, and maybe for good. The reason is simple. A flow cytometer, no matter how fancy, is not very good at looking at individual cells over time, and it has become obvious that we need to do more of that in functional studies.

Wacholtz and Lipsky[1730] used single cell image analysis of changes in cytoplasmic [Ca^{++}] after mitogen stimulation of individual human T cells to determine the relation between the [Ca^{++}] signal and subsequent functional changes. Marked heterogeneity was observed in the magnitude of increase in [Ca^{++}], in the lag time of responses, and in the percentage of T cells that responded to anti-CD3 and to PHA. However, mitogenic stimuli that induced IL-2 production or DNA synthesis consistently generated increases in [Ca^{++}] that were sustained for 1 to 2 hours; elevations as small as 50 to 100 nM above control were associated with evidence of activation. Functional activation of T cells by PHA and anti-CD3 is thus correlated with the induction of small, but sustained increases in [Ca^{++}] that might or might not have been detectable by flow cytometry. Image cytometry worked fine.

And, these days, while you're doing the image cytometry, you can use the patch clamp technique and other modern electrophysiological methods to follow fast membrane potential changes and dissect the roles of the various calcium, chloride, and potassium channels in activation processes in lymphocytes and other cells[2842 69]. However, people who do that seem to use cell sorters to pick out interesting cells to work on, so you might want to finish the book before you order the new equipment.

NOsing Around for Nitric Oxide

Rao et al[1617] described a method for detection of the production of **Nitric Oxide ("Just Say NO")**, now recognized as a key intermediate in cellular signal transduction, by neutrophils, in which DCFH-DA fluorescence was measured in cells to which calmodulin inhibitors had been added. There are a number of probes for NO; Several authors[2870-2] have used 4,5-diaminofluorescein; Molecular Probes[2332] recommends DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein because it is unaffected by pH above 5.5 and more sensitive than other probes. These probes are all loaded into cells as "diacetates," i.e., as acetyl esters, in the manner of FDA.

Other Ions in the Fire

Amorino and Fox[2875] describe measurement of intracellular **sodium** ion concentration using **Sodium Green**. Balkay et al[2874] measured intracellular **potassium** ion, deriving the parameter from measurements of pH (using **BCECF**) made in the presence of **nigericin**, which keeps the ratio of potassium and hydrogen ions constant on both sides of the cell membrane. Pilas and Durack[2875] used **6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ)**, a UV-excited, blue-green fluorescent probe quenched by halides, to measure intracellular **chloride** ion concentration. All of the probes mentioned come from – guess where – Molecular Probes, and the *Handbook*[2332] has much more information on them.

7.14 REPORTER GENES

Somebody Cloned My Gal: Enzymes as Reporter Genes

In this era of molecular biology, flow cytometric detection of enzyme activity has come into increasing use as a method for detecting and selecting cells expressing **reporter genes**. Reporter genes are genes that encode protein products that are relatively readily detectable; they are linked by standard recombinant DNA technology to a gene or genes of interest to an investigator, and the resulting construct is introduced into cells. Those cells in which the gene of interest is expressed can then be identified by the presence of the reporter gene product.

In the 1980's, genes encoding **surface antigens** not normally present on the cells of interest were used as reporter genes; expression of these genes was detected by immunofluorescence. This was unsatisfactory; what was gener-

ally observed were very broad distributions of relatively weak signals, and successful isolation of cells bearing the genes of real interest to investigators generally required multiple cycles of cell sorting, in which the brightest cells were sorted and clones, and the brightest progeny were resorted, etc.

In 1988, the Herzenberg lab[1642] reported the development of a new method, called **FACS-Gal**, in which the *lacZ* gene from *E. coli* was used as the reporter gene. This gene encodes the enzyme **β-D-galactosidase**, which is not normally present in many eukaryotic cell types. The activity of this enzyme can be detected by flow cytometry using the fluorogenic substrate **fluorescein di-β-D-galactoside (FDG)**, available from Molecular Probes, which is hydrolyzed to fluorescein by the enzyme. Since a few molecules of enzyme can produce a lot of molecules of fluorescein, the FACS-Gal technique made it much simpler to detect and sort cells expressing the reporter gene; the stronger signals also made it possible to distinguish cells expressing different amounts of the reporter gene and, therefore, of the gene(s) of interest, and to sort these separately for further analyses.

The FACS-Gal assay as originally published, although demonstrably usable (e.g., reference 1643) had some problems; loading the cells with FDG was difficult, and there were interferences from rare autofluorescent cells and from endogenous enzyme activity. Finally, it was difficult to do long sorting experiments because the enzymatic reaction continued over time. In 1991, several improvements were described by Fiering et al[1644]. Optimal loading conditions were determined, two-color fluorescence was found to identify interfering autofluorescent cells, chloroquine was used to inhibit endogenous mammalian enzyme, and **phenylethyl-β-D-thiogalactoside**, a competitive inhibitor, was added to stop the enzymatic reaction before sorting.

Jasin and Zalamea[1645] reported isolation of galactosidase-bearing sperm cells from transgenic mice using **5-dodecanoylaminofluorescein di-β-D-galactopyranoside**, a substrate that is more lipophilic than FDG; it may also be possible to use other substrates, e.g., **resorufin galactoside**[1646], which has been used for analyses of galactosidase activity in yeast.

Genes encoding enzymes other than β-D-galactosidase may be used as reporter genes; Puchalski et al[1646] transfected COS monkey kidney cells with a construct incorporating a recombinant **glutathione S-transferase (GST)** gene, which was demonstrable using **monochlorobimane**, using procedures that were discussed in the section on analysis of thiol groups and glutathione (p. 381). More recently, Lorincz et al[2876] developed a reporter system using *E. coli* **β-glucuronidase** as the enzyme and **fluorescein-di-β-D-glucuronide (FDGlcu)** as the fluorogenic substrate. Because selective inhibitors are available for β-glucuronidase and β-galactosidase, it is possible to detect both enzymes in transfected cells.

Puchalski and Fahl[1647] compared gene transfer by electroporation, lipofection, and DEAE-dextran transfection for compatibility with cell-sorting, with the requirements that the procedure used should generate morphologically homogeneous populations with at least a 5% colony-forming ability in which at least 10% of the cells expressed recombinant GST. Of the transfection techniques tested, only electroporation satisfied all requirements.

At this point, I'm supposed to say "But that's all ancient history..." because you can see GFP and its friends and relatives just a little bit down the page. I will, instead, point out that enzymes are still being used as reporter genes. One of the newer wrinkles was developed by Roger Tsien et al[2877], and made available by his company, **Aurora Biosciences**, which is now merged with **PanVera**. The enzyme is a **β-lactamase**, produced by the ampicillin resistance gene of *E. coli*; it is not found in untransfected mammalian or avian cells. The fluorogenic substrate used to detect it is called **CCF2**; it is loaded into cells as the acetoxymethyl ester, **CCF2-AM**. CCF2 contains a coumarin donor and a fluorescein acceptor attached to a cephalosporin β-lactam ring. When the ring is intact, the coumarin and fluorescein moieties of the probe are close enough together for energy transfer to occur, and excitation (the excitation maximum is 409 nm) produces green (530 nm) fluorescence. When the ring is cleaved by β-lactamase, the distance between the coumarin and the fluorescein is increased sufficiently to greatly reduce the efficiency of energy transfer, and the probe emits blue (450 nm) fluorescence. The intensity of blue emission from the hydrolyzed probe is about twice the intensity of green emission from the intact probe. CCF2 was not of great interest to the flow community when it was only feasible to excite it with a violet krypton laser; it may be a lot more interesting now that relatively inexpensive 405 nm violet diode lasers are available. Cavrois et al[2878] used the β-lactamase/CCF2 system to develop an assay for HIV-1 virion fusion with target cells.

Green Fluorescent Protein (GFP) et al

In early 1994, Chalfie et al[1648] described the use of a gene encoding an intrinsically fluorescent protein from the bioluminescent jellyfish *Aequorea victoria* as a reporter gene. The native **green fluorescent protein (GFP)** absorbs maximally at 395 nm, but can be excited moderately effectively at 488 nm, where its absorption is about one-third maximum; the emission spectrum has a sharp peak at 510 nm with a shoulder at 540 nm. I included this information in the previous edition; at the time, GFP expression in prokaryotic (*E. coli*) and eukaryotic (*Caenorhabditis elegans*) cells had been shown to render those cells fluorescent. I opined that "GFP should work for cell sorting, provided the cells used don't exhibit a lot of autofluorescence." Boy, did it. As I noted on p. 48, what I described in 1994 as a "mild mannered reporter" turned into Supermolecule. The first book[2376] and one of the big review articles[2377] are distinctly dated (1998), and it's hard to keep up with the literature. I won't even try. I don't do much GFP work, so I'll just try to cover some points that I think are important and point you elsewhere for more details.

Most of the probes we use in flow cytometry are things you add to cells after the fact. DNA dyes work on any cell that contains DNA, provided you can get the dye in. True, you wouldn't want to waste highly specific reagents, such as fluorescent antibodies and nucleic acid probes, on cells that you didn't think were carrying any detectable target material, but, in principle, you could throw any probe at any cell that comes through the door. Reporter genes, and fluorescent proteins in particular, are different; they have to be in or on the cells before they come through the door.

The protocols for labeling cells with fluorescent protein reporter genes are molecular biology protocols, not cytometry protocols; if your focus is on cytometry, what you need to know are the excitation and emission spectral characteristics of the proteins you're looking for and how much of them you expect to find in the cells. Figure 7-9 (p. 296) includes the spectra of four fluorescent proteins, **ECFP** (cyan; excitation maximum 434 nm, emission maximum 477 nm), **EGFP** (green; excitation maximum 489 nm, emission maximum 508 nm), **EYFP** (yellow-green, excitation maximum 514 nm, emission maximum 527 nm), and **DsRed** (orange; excitation maximum 558 nm, emission maximum 583 nm). They are all available, or, more accurately, the constructs you need to get the appropriate genes into cells are available, from **Clontech**, a division of **BD Biosciences**. Hawley et al[2879] recently described flow cytometric detection of all four at once using 458 nm excitation from an argon laser for ECFP, EGFP, and EYFP and 568 nm excitation from a krypton laser for DsRed.

All of these proteins have been engineered to give them more desirable fluorescence characteristics. ECFP, EGFP, and EYFP, produced by mutants of the *Aequorea* GFP gene[2877], are well-developed, as GFP variants go, while dsRed[2459], derived from a coral of the species *Discosoma*, is more of a work in progress. There are several variants, including dsRed Express[2884], which matures more rapidly and apparently yields more fluorescence than the original.

The longest wavelength fluorescent protein available from Clontech is HcRed, from the reef coral *Heteractis crispa*[2880]; its excitation and emission maxima are at 588 and 618 nm. HcRed could be excited by a 532 nm YAG laser, but the 568 nm krypton line, 590 nm from a dye laser, or 594 nm from a He-Ne laser (assuming you could get a He-Ne laser with enough power) would be a better bet.

As exploration of the animal kingdom[2881] and protein engineering[2877,2450,2882] have produced new and improved better fluorescent proteins, investigators have worked out optimal schemes to detect them by flow cytometry and put them to use[2886-96]. We can expect that to continue.

The brief review by Matz et al[2881] on GFPs packs more information into 7 pages than just about any other paper I have ever read; I heartily recommend it. Among other things, I learned that members of the GFP family are unique among pigment proteins in that they act as enzymes and synthesize their fluorophores from amino acids in their own polypeptide chains. Other pigment proteins, such as the phycobiliproteins, make their chromophores or fluorophores from small molecules and usually require several enzymes to get the job done. Thus, it would be a much harder job to make a phycobiliprotein reporter than it is to make a GFP reporter. The self-contained palette of GFP proteins also makes it much easier to modify their spectra by site-directed mutagenesis than it would be to change the spectrum of another type of pigment protein.

With a little help from their friends, GFPs themselves have "evolved" far beyond the point of merely marking transfection; there are fluorescent protein variants that are sensitive to pH[2897-9], calcium ion concentration (shades of aequorin!)[2900-2], and membrane potential[2903], and others that monitor cell surface receptor interactions[2378] and kinase activities[2904]. There is also a "timer" protein[2905] that gradually changes color from green to red. Of course, you do have to put genes into your cells to take advantage of all these benefits; I can't help you much there.

Minority Report(er)?

With fluorescent proteins and enzymes around, why does anybody still want to bother using proteins expressed on cell surfaces as reporters? Well, if your system works well enough so that only the cells expressing the reporter gene carry the product on their surfaces, you can then use antibodies on magnetic beads or a column to separate your transfectants instead of, or in addition to, a cell sorter. Christine et al[2906] describe a system for detecting recombinant switch activity in B cells based on expression of CD4 or a histocompatibility antigen normally not found on the cells.

Well, that does it for parameters and probes. If you decide to quit at this point and get into image analysis or confocal microscopy, you'll be using many of the same probes, detectors, electronics, computers, etc., so you haven't wasted too much time. If not, stick with me, and I'll tell you how to acquire a flow cytometer, and then go on to a survey of some of the applications of the technology.

FACSCalibur. The acoustic sorter, however, unlike the mechanism used by B-D, only diverts fluid into the sort stream when cells are being sorted, meaning that rare cells can be sorted without being diluted by a large volume of extraneous fluid.

8.12 THIRD-PARTY SOFTWARE

The adoption of the FCS file format made it feasible for various third-party developers to write and sell, or, in some cases, give away, programs for analysis of flow cytometric data. A market for such software existed, and exists, because peoples' preferences in software tend to be idiosyncratic, and because the third-party programs generally have at least a few features which are not included, or not as well implemented, in software from the manufacturers.

This is not to knock the manufacturers' software; manufacturers, however, tend to have more people writing software than do the third-party companies, and breaking software projects into chunks, while sometimes necessary, almost always results in chinks between the chunks when the overall program is put together. If one good programmer can write the whole program, it's apt to run more smoothly.

I know most of the people who write third-party software, and almost all of them have a lot of experience with and knowledge of flow cytometry. A third-party program is generally written by one such person, and its features and performance characteristics are, as a result, tailored to meet the needs of a demanding user. The programmers writing for the larger companies may know a fair amount about flow cytometry, and may actually be better computer programmers than the third-party developers, but, even if they are working to specifications set by sophisticated, demanding users, it's not quite the same.

Third-party software can get pretty elaborate, especially with hardware added. Both Applied Cytometry Systems and Cytomation, for example, reverse engineered the relatively tight interface between the B-D FACScan and the Consort 32 computer system, which was normally essential for the cytometer's operation, and came up with replacement computer systems and software using Intel processors.

As flow cytometers tend increasingly toward digital signal processing and multiprocessor operation, and as hardware and software combinations replace hardware in the innards of the machines, there will be an increasing amount of software that will, of necessity, remain in the domain of the instrument manufacturers. For everything else, and, certainly, for any manipulation of data already collected in standard file formats, there will be third-party software. If you're satisfied with the software your manufacturer supplies, you may never need anything else. As it happens, though, even the manufacturers are beginning to realize that it takes time and costs them money to reinvent the wheel, and they are making deals with the third-party developers for versions of many of the better software packages which will interface smoothly with the manufacturers' software.

In the area of clinical instruments, the Food and Drug Administration (FDA, not to be confused with fluorescein diacetate) has regulatory authority over software; the software itself has to meet certain specifications. It is to be hoped that the FDA will recognize the benefits of the cooperative approach to software development mentioned above.

You can pretty much get the same data analysis capabilities in third-party software as are available from the manufacturers, and more. A list of third-party developers and some of their offerings appears in Chapter 11. Almost all of the producers offer demos, or at least showcase their products' capabilities on their web sites, so it's fairly easy to find out both whether a particular piece of software does the manipulation(s) you need to do and whether the way in which it does it/them fits with the way you think.

The committee that keeps up the FCS standard has been threatening to release some code for reading and writing FCS files, which may be (certainly is, when I wear my programmer's hat) the hardest part of a flow cytometry data analysis program to code. It would be kind of nice to have a public domain toolkit of program building blocks with which moderately sophisticated users could put together their own *ad hoc* programs. The DOS-based Forth software I wrote had some of this capability, but, at least in my limited experience, it has not been possible to duplicate the critical features in either a Windows or Macintosh GUI.

8.13 THE SELLING OF FLOW CYTOMETERS: HYPE AND REALITY

If you listen to the people who sell flow cytometers, you'll be forced to conclude that none of them works. Each manufacturer's people will tell you that the system they sell is the only one that really does work; a chorus from all the other manufacturers will deny this is so. The manufacturers may unite in denunciation of laboratory-built instruments.

If you listen to the people who use flow cytometers, you'll find that all of them work, and that none of them always works. You'll also find that one group of people thinks that one manufacturer's product is easiest to use and most trouble-free, and other groups argue just as passionately in favor of products from other manufacturers.

For example, people with a lot of experience with a B-D instrument always seem to be able to coax better performance out of it than can somebody who is used to other flow cytometers. I had an Ortho System 50 in my lab for a while; the Ortho people, or some of them, who worked on it could always get it to produce sharper DNA content distributions than I could, and I was always able to do better with my Cytomutt than they could. I look at the manufacturers' published precision specifications with a jaundiced eye, because my inquiries indicate that there is a substantial difference between the best that an instrument can do and its level of performance in routine use by the average operator.

If there weren't so many half-truths and misconceptions flying around as a result of manufacturers' and partisan users' efforts on behalf of their machines, it would be easier for

people to choose the flow cytometers, bought or built, which they needed for their particular applications.

If there is one thing about flow cytometers that isn't the same the whole world over, it probably relates to user preferences in light sources. Arc source instruments have done much better in Europe than in the United States, where people prefer lasers. I would have to ascribe this to the fact that many users and potential users in the United States aren't as well trained in the use of fluorescence microscopes as are their European counterparts, and thus can't get the same good results with arc source systems.

If somebody gave me as much money as I wanted, and insisted that I spend it on one commercial flow cytometer, I'd have a problem. My decision would probably be influenced by the same factors that influence my purchases of bicycles, cars, computers, and stereo equipment. I always study the products in great detail, evaluate their performance features, and discover that none of them has all the features I want. I can't build cars; I end up buying the one that feels right. It's the same way with most software; I write my own software for flow cytometry data analysis, but, when it comes to word processors, spreadsheets, drawing packages, etc., I have to buy the one that feels right. With bicycles, computers, stereo equipment, and flow cytometers, I usually have more freedom of choice. I wouldn't like to spend a over a half a million dollars of taxpayers' or investors' money on a piece of equipment because it felt right, but, under the circumstances I just described, I guess I'd have to.

Over the years, we have seen convergent evolution in the development of commercial flow cytometers. If you can get some bell or whistle from one manufacturer, you can get its equivalent from another. Buying decisions tend to hinge on how well you like the user interface, but are also influenced by how well you like the salespeople and/or the local service representatives. Clear edges in performance get harder and harder to find, and manufacturers tend to bring instruments up to spec. That's nothing to complain about.

Over the next few years, there will probably be as many developments in the area of data analysis as in flow cytometric hardware. As more third parties become involved in the generation of hardware and software products for data analysis, users will have more options and more opportunities to tailor the user interface to their own tastes. That's also nothing to complain about. If you're not writing your own data analysis software, you definitely need to find software that feels right. That's why I haven't devoted more space and time to the particulars of this and that software package; what's important is it's not what the program does, but how comfortable you feel with the interface.

The one rule which is most important in deciding which flow cytometer to buy hasn't got anything to do with hardware *per se*. It is, instead,

Shapiro's Eighth Law of Flow Cytometry:
Know Thy Cells!

You really don't want to lay out a lot of money, even if you, as a taxpayer, only contribute a minuscule share of it, for an instrument that won't let you do the analyses and/or experiments you need to do. The manufacturers will, almost without exception, let you run your samples through one of their instruments when you're in the process of making a buying decision. You shouldn't be making a buying decision unless you know enough about flow cytometry to know what cells you're going to be looking at and how they'll be prepared, and what kinds of data analysis you'll need to do. This is especially important if you're looking at something out of the ordinary. If you're trying to sort very big stuff, or very small stuff, be sure the machine can do it. If you're looking for very faint signals, try before you buy.

I have summarized the characteristics of a lot of general- and special-purpose flow cytometric equipment in the previous sections of this chapter; some of the manufacturers provided more information than others. I'd be surprised if I didn't make at least a few mistakes. As most of the manufacturers know, I have always been and still am willing to make corrections in public, in speech, prose, and/or verse. And, for this edition, the corrections should be posted on the web site.

8.14 APPLYING FOR A GRANT FOR A CYTOMETER

Unless you're a very wealthy amateur, have access to lots of industrial money, or hit the lottery, you're going to have to ask somebody for the wherewithal to purchase a flow cytometer. If you work in the United States, that somebody is usually the National Center for Research Resources at the National Institutes of Health, and the mechanism by which you ask for the cash is most often an application for a Shared Instrumentation Grant. To qualify, three or more NIH-funded Principal Investigators have to demonstrate a need for, a capability to use, and an institutional commitment to supporting a "single, commercially available instrument or integrated instrument system" that costs at least $100,000 (the maximum award is $500,000). The funding mechanism covers a lot of high-ticket gadgetry in addition to flow cytometers, e.g., NMR equipment, confocal microscopes, and electron microscopes, so you're not just competing with flow cytometry people.

As you might expect, I occasionally sit on panels that review applications requesting funding for cell sorters and flow cytometers; from what I hear from colleagues who sit on similar panels covering the other high-priced toys, the things that weigh for you and against you are pretty much the same there as well. In the rest of this chapter, I'll try to save you some grief if you're writing a grant application for an instrument, to NIH or elsewhere.

There are usually a few dozen applications in any given cycle, of which the four or five given highest priority, and maybe not even those, will get the money. All of the applications, as I mentioned above, are coming from investigators who have already been through the peer review process, and

have gotten the funds to do their research. The review panel is not allowed to consider or question the underlying science; what is at issue is not whether the applicants know immunology, or genetics, or cell biology, or whatever their primary fields of interest may be, but whether they demonstrate an understanding of what to do, and how best to do it, with the equipment for which they are seeking funds. All of the applications are meritorious; however, they can't all be funded. What happens is that those that survive are those with the fewest obvious faults. I can tell you what some of those faults might be, and if you are writing or assisting in the writing of a grant application, you can at least try to avoid them.

The grant, if you get it, pays for the instrument; it doesn't pay for service contracts, operators' salaries, or any modifications to the site needed to install the instrument (it may cost thousands of dollars to bring in the power and cooling water connections for a big laser). Almost everybody who runs a shared instrument facility charges user fees, which are supposed to defray some of those costs. Almost everybody who has run a shared instrument facility knows that the facility is likely to run at a loss. If the administration at your institution doesn't come across as wildly enthusiastic about providing all funds necessary to make up the difference, you're apt to lose points, because somebody else's administration will look better.

It's advisable for the Principal Investigator (P.I.) on a cell sorter grant to be a person who knows a lot about flow cytometry. It is not required that he or she have an NIH grant, as long as there are three potential users who do have NIH grants and are Principal Investigators on those grants. NIH Policy is that the Principal Investigator on a Shared Instrumentation Grant doesn't even have to be a user of the instrument. However, in my experience, reviewers take a dim view of applications in which people with little or no expertise with a sophisticated instrument are listed as Principal Investigators.

In the old days, when there weren't user-friendly benchtop systems around, the graduate students and postdocs had to hang around the sorter lab, even to do simple analyses, and, at least on paper, it was easy to look knowledgeable. Now, a lot of people do a lot of respectable flow cytometry on benchtop systems, which is fine. However, if the P.I. on an application asking for a big sorter with four lasers is somebody whose experience appears limited to benchtop systems, it's a sure bet that that application won't look too strong in comparison with others.

The manufacturers are all too happy to write quotations according to which they will sell you a big sorter and a benchtop instrument (which will, of course, work together as an "integrated instrument system") for just under $500,000. I personally wouldn't ask for two instruments. You'll be very lucky if you get one instrument funded; leave some money for somebody else. It won't help your application if any of the reviewers gets even a fleeting impression that you're a pig.

If you have an instrument and are requesting an additional instrument, it helps to be able to show that what you've got is used a lot, which probably means nights and weekends; if you want to replace an old, obsolete instrument, bear in mind that most of us don't think a three year old cell sorter is obsolete because a new model has come out. Also, don't ask for more – or less – instrument than you need. If you're requesting an argon laser, a mixed gas laser, and a dye laser, it helps to include some details of the parameters that will be measured and the probes that will be used, because those lasers are expensive. The same goes for other add-ons. If you don't explicitly justify the need for them, they'll get axed from the budget. If you do get funded, you'll be short of cash, but, let's face it, if you do a poor job of justifying what you've asked for, you're less likely to get funded.

If you announce your intention to do experiments with Hoechst dyes, DAPI, or indo-1, but you have only asked for a single 488 nm argon laser, which doesn't emit the UV light needed to excite these dyes, you don't win points for trying to save money; it simply looks as if you don't know what you're doing.

Some instrument manufacturers will be happy to sell you various computer peripherals at substantially higher prices than you'd pay at a computer store. Having these items in your quote probably won't reduce your overall chance of getting funding if your application is otherwise perfect, but the budget is very likely to get cut, as it should be.

These days, applications are getting so thick, what with biographical sketches and information on other projects for all of the investigators, that many people seem to be skimping on the details of what will be done with instruments. Being concise can be a virtue; being concise to the point at which it becomes unclear that you need or can competently use the apparatus is a vice.

Finally, if you don't get funded the first time, and decide to resubmit the next time around, pay attention to the critique of your application. It's likely to call attention to some of the faults I have just mentioned. If you don't fix everything, your next application won't do any better than your first one. If you do, at least you've got a fighting chance. Good luck.