# Fluorescent Probes for Cytometry

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## INTRODUCTION

Fluorescent probes provide a sensitive method with which to obtain information about the structure, function, and health of cells. In fact, the method can be sensitive in two ways. First, it is possible to detect very few fluorescent molecules in a cell. Fluorescent chromophores for labeling antibodies, DNA, and lipids are designed for high detection sensitivity [26]. The ideal labels have large extinction coefficients and high quantum yields and are insensitive to pH and the polarity of their local molecular environments.

The second way fluorescence can be sensitive is in the ability of probe molecules to respond spectroscopically to subtle changes in their molecular environment. The dependence of absorption wavelength, extinction, emission wavelength, quantum yield, excited state lifetime, and emission polarization on fluorescent probe microenvironment provides a powerful method for understanding the behavior of cells. One of the key requirements for exploiting the sensitivity of probes microenvironment is the careful design of the fluorophore structure so that it can be targeted to the site of interest in the cell, e.g., plasma membrane versus endoplasmic reticulum, or mitochondria versus cytoplasm, or one class of lysosomes versus another. Therefore, the skills of the organic chemist must be blended with those of the spectroscopist, biochemist, and cell biologist to exploit this potential of fluorescent probes optimally.

## OPTICAL PROPERTIES OF IDEAL FLUORESCENT PROBES

### Large Extinction Coefficient ($\epsilon$) at the Wavelength of Excitation

The value of $\epsilon$, which characterizes the light-absorbing power of the fluorophore, should be as large as possible. Extinction coefficients and other spectroscopic parameters for a variety of fluorescent probes are listed in Table 1. Fluorescein has an $\epsilon \sim 67{,}000$ liters/mole · cm at its wavelength of maximum absorption (pH 7.0). The multichromophore phycobiliproteins have exceedingly large extinction coefficients ($\sim 2 \times 10^6$ liters/mole · cm), which contributes to their value as very fluorescent antibody-labeling reagents. There are useful fluorescent probes with lower excitation coefficients but, to be detected by flow cytometry, they must have high quantum yields and be at high concentrations within cells.

### High Quantum Yield ($\phi$)

$\phi$ should be large when the probe is bound to the target and is in the solvent environment where the fluorescence measurement is to be made. Fluorescein-labeled proteins can have quantum yields near 0.5 to 0.7 at pH 8, but $\phi$ drops rapidly with decreasing pH and is lower when more than one fluorescein is bound to the protein. Rhodamine is much less sensitive to pH and is still fluorescent in acidic compartments of cells or in the presence of acidic fixatives. If a large amount of a probe is associated with each cell, e.g., with propidium staining of DNA, the limitations due to small $\epsilon$ and $\phi$ are not as severe. The fluorescence intensity from a fluorophore is proportional to the product of $\epsilon$ and $\phi$.

### Optimal Excitation Wavelength

Cells excited at wavelengths below 500 nm produce considerable autofluorescence that arises mainly from flavins, flavoporteins, and NADH [3,8]. In situations in which autofluorescence can swamp the probe fluorescence, it is useful to have the probe absorb at wavelengths above 500 nm.

### Photostability

Fluorescein can survive between $10^4$ and $10^5$ excitations before decomposing [57]. As Mathies and Stryer discuss, photostability is important for detecting a small number of probes in solutions. Photostability is also a concern for visualization of tagged materials with conventional fluorescence microscopes. For example, the fading of fluorescein fluorescence under intense microscopic illumination is well known, and numerous chemical approaches have been taken with various degrees of success to reduce fading [10,60]. Removal of oxygen is the most effective step that can be taken but this is impossible in studies of most living cells. Photofading is generally not a problem in flow cytometry because the stained sample remains in the laser beam only a short time.

Flow Cytometry and Sorting, Second Edition, pages 209–225

Table 1. Spectral Properties of Selected Fluorescent Probes[a]

| PARAMETER | PROBE (a) | ABSORPTION MAXIMUM (b) | EXTINCTION MAX. (c) | EMISSION MAXIMUM (b) | QUANTUM YIELD | MEASURMENT CONDITIONS | REFERENCES |
|---|---|---|---|---|---|---|---|
| Covalent labeling reagents | FITC-NH-CH3 | 490 | 67 | 520 | 0.71 | pH7, PBS | [32], W, MP |
| | FITC-NH-Ab | 490 | 67 | 520 | 0.1-0.4 (d) | pH7, PBS | W |
| | TRITC-amines | 554 | 85 | 573 | 0.28 | pH7, PBS | [32], MP |
| | XRITC-NHCH3 | 582 | 79 | 601 | 0.26 | pH7, PBS | W |
| | XRITC-NH-Ab | 580 | | 604 | 0.08 (h) | pH7, PBS | W |
| | Texas Red-amines | 596 | 85 | 620 | 0.51 (w) | pH7, PBS | [94], W, MP |
| | Texas Red-NH-Ab | 596 | 85 | 620 | 0.01 (g) | pH7, PBS | W |
| | Phycoerythrin-R | 480-565 | 1960 | 578 | 0.68 | pH 7, PBS | [67] |
| | Allophycocyanine | 650 | 700 | 660 | 0.68 | pH7, PBS | [67] |
| DNA-RNA content | Hoechst 33342 | 340 | 120 | 450 | 0.83 | +DNA | W |
| | DAPI | 350 | | 470 | | +DNA | W |
| | Ethidium Bromide | 510 | 3.2 | 595 | | +DNA | [71] |
| | Propidium Iodide | 536 | 6.4 | 623 | 0.09 | +DNA | W |
| | Acridine Orange | 480 | | 520 | | +DNA | [43, 78] |
| | | 440-470 | | 650 | | +RNA | [43, 78] |
| | Pyronine Y | 549-561 | 67-84 | 567-574 | 0.04-0.26 | +ds DNA (e) | [17, 42] |
| | | 560-562 | 70-90 | 565-574 | 0.05-0.21 | +ds RNA (e) | [17, 42] |
| | | 497 | 42 | 563 | LOW | + ss RNA | [17, 42] |
| | Thiazole Orange | 453 | 26 | 480 | 0.08 | RNA | [52] |
| Membrane potential | diO-Cn-(3) | 485 | 149 | 505 | 0.05 | MeOH | [82], W |
| | diI-Cn-(3) | 548 | 126 | 567 | 0.07 | MeOH | [82], W |

## Excited State Lifetime ($\tau$)

In flow cytometry the greatest sensitivity can be achieved with a combination of high laser power and probes with short fluorescence lifetimes. Under saturation conditions, the strong illumination of a focused laser spot can excite dye molecules immediately after they have fluoresced or relaxed from a previous excitation. Thus, as a cell passes through the beam the greatest number of excitations can occur when a dye has a very short $\tau$. The maximum number of excitations (and photons emitted) that are possible with a saturating laser beam is approximately given by the time the fluorescent molecule is in the beam divided by $\tau$. Most highly fluorescent molecules do not have lifetimes much shorter than a nanosecond. Fluroescein and pyrene have emission lifetimes of ~4 and 100 nsec, respectively.

In addition to the ideal optical properties listed, there are other considerations, e.g., solubility, chemical stability, and photostability. Also, the probe should not perturb the function of the cell, organelle, or target molecule by reacting with key groups in active sites or by causing steric perturbations because of its size. The probe should not be phototoxic. Few fluorescent probes have all the ideal properties, but a number have proved to be very valuable and are widely used. They are discussed more fully later.

## FLUORESCENT LABELS

Reactive fluorescent chromophores can be used to tag proteins, polynucleotides, lipids, or other biological molecules, which can in turn be used as biological probes. The structures of common covalent labeling reagents are shown in Fig. 1 and their optical properties are given in Table 1. Ideal labeling reagents generally have appropriate selectivity and modest reactivity. If too reactive, the tagging reagent hydrolyzes before binding to the protein. Isothiocyanates, chlorotriazinyl derivatives, and succinimide active esters are the most common functional groups that permit chromophores to be attached covalently to primary amino groups of proteins. Generally, the reactions are carried out in aqueous solutions at pH 8.9 to 9.5. Iodoacetamido and malemido functional groups on chromophores can be used to form linkages to protein sulfhydryl groups. This chemistry has been reviewed by Haugland [32].

TABLE 1. *Continued*

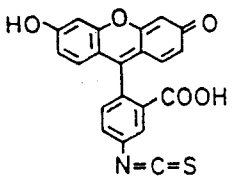
| PARAMETER | PROBE (a) | ABSORPTION MAXIMUM (b) | EXTINCTION MAX. (c) | EMISSION MAXIMUM (b) | QUANTUM YIELD | MEASURMENT CONDITIONS | REFERENCES |
|---|---|---|---|---|---|---|---|
| | di-Cn-(5) | 646 | 200 | 668 | 0.4 | MeOH | [82], W |
| | diBA-isopr-(3) | 493 | 130 | 517 | 0.03 | MeOH | [82], W |
| | Rhodamine 123 | 511 | 85 | | | EtOH | EK |
| Lipid content and fluidity | Nile Red | 485 | | 525 | | Heptane | [30] |
| | | 530 | | 605 | | Acetone | [30] |
| | Diphenylhexatriene (DPH) | 330, 351, 370 | 77 (351nm) | 430 | | Hexane | MP |
| | dil-C18-(3) | 546 | 126 | 565 | 0.07 | MeOH | W |
| | NBD phosphatidylethanolamine | 450 | 24 (f) | 530 | | Lipid | [85] |
| | Anthroyl stearate | 361, 381 | 8.4, 7.5 | 446 | | MeOH | [99] |
| pH | 6-Carboxyfluorescein | 495 | | 520 | | High pH | MP |
| | | 450 | | | | Low pH | MP |
| | BCECF (i) | 505 | | 530 | | High pH | MP |
| | | 460 | | | | Low pH | MP |
| | DCDHB (j) | 340-360 | | 500-580 | | High pH | [97], MP |
| | | 340-360 | | 420-440 | | Low pH | [97], MP |
| Calcium | Fura 2 | 335 | 33 | 512-518 | 0.23 | Low Calcium | [31] |
| | | 360 | 27 | 505-510 | 0.49 | High Calcium | [31] |
| | Indo 1 | 330 | 34 | 390-410 | 0.56 | High Calcium | [31] |
| | | 350 | 34 | 482-485 | 0.38 | Low Calcium | [31] |
| Enzyme substrates | Rhodamine-di-arg-CBZ substr. | - | Low at 495nm | 532 | 0.09 | Hepes pH7.5+15% EtOH | [53] |
| | Product of rxn. (rhodamine) | 495 | 67 | 523 | 0.91 | Hepes pH7.5+15%EtOH | [53] |
| | Coumarin-glucoside substr. | 316 | 13 | 395 | | Acetate pH 5.5+1%Lubrol | W |
| | Product of rxn (hydroxy coumarin) | 370 | 17 | 450 | | Glycine pH10+1%Lubrol | W |

[a]Key: EK = Eastman Kodak Chemical Catalog; MP = Molecular Probes, Inc catalog (or personal communication); W = Waggoner laboratory determination; (a) Ab = antibody; (b) nanometers; (c) in thousands (liters/mol · cm); (d) dye–antibody ratio 2–5; (e) base pair dependent; (f) value for NBD–ethanolamine in MeOH, which has an abs. max at 470 nm and an emission max at 550 nm [4]; (g) dye–antibody ratio 1.2; (h) dye–antibody ratio 2.5; (i) BCECF = 2',7'-bis(2-carboxyethyl)-5(and 6)carboxyfluorescein; (j) DCDHB = 2,3-dicyano-1,4-dihydroxybenzene.

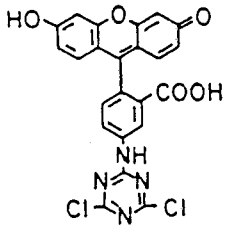
Some tagging reagents, such as fluorescein, are soluble in aqueous solutions and are easily conjugated to proteins. Others, such as the hydrophobic rhodamines, are less soluble and not only can they precipitate from the labeling reaction mixture but they can also cause precipitation of more heavily labeled antibodies (even with only 2 to 3 fluorochrome molecules per protein molecule).

Certainly the most commonly used fluorescent tag is fluorescein isothiocyanate. It has been attached to antibodies, lectins, avidins, hormones, lipids, protein analogues, and other biological molecules [32]. Properties that make fluorescein popular are its reasonably high extinction coefficient and quantum yield, its water solubility, ideal reactivity, and emission at a perfect wavelength (515 nm) for detection by the human eye and by photodetectors. Antibodies, avidin and lectins can be labeled with 2 to 8 fluoresceins before fluorescence quenching, which occurs when fluorophores are in close proximity, reduces the brightness of the labeled species. The disadvantages of fluorescein are its relative photoinstability [57], its loss of fluorescence when the pH is below 8, and its wavelength of excitation, which is in a region that produces autofluorescence.

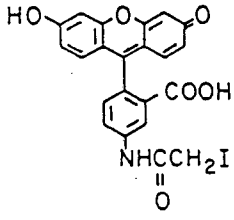
Other fluorescent labels have been developed either to improve on the properties of fluorescein or to provide additional reagents that absorb and emit at different wavelengths and can therefore be detected simultaneously. Rhodamines, for example, are generally more photostable than fluorescein, are pH insensitive under physiological conditions, and excite in the 500- to 600-nm range, where less autofluorescence is generated. However, the quantum yield of rhodamine on labeled antibodies is generally less than that of fluorescein at similar dye/protein ratios and, as mentioned earlier, problems with the limited water solubility of rhodamines make these reagents less attractive as labeling reagents. Lissamine rhodamine and Texas Red are more soluble rhodamines and are excited at longer wavelengths.



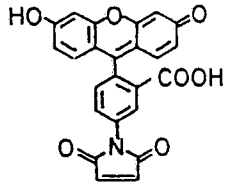

I – FITC



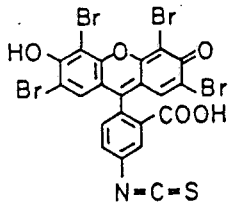

II – DTAF



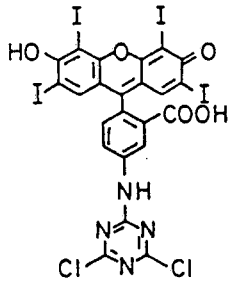

III – IAF



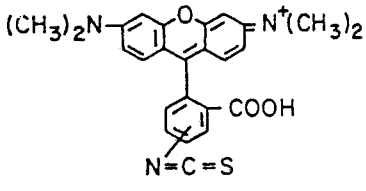

IV – MALF



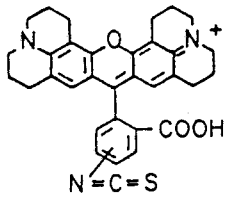

V – EYITC



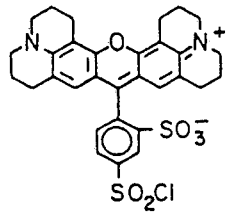

VI – DTAEry




VII – TRITC




VIII – XRITC




IX – TEXAS RED

Fig. 1. Fluorescent labeling probes. I, Fluorescein isothiocyanate; II, Dichlorotriazinylamino-fluorescein; III, Iodoacetamidofluorescein; IV, Maleimidofluorescein; V, Eosin Y isothiocyanate; VI, Dichlorotriazinylaminoerythrosin; VII, Tetramethylrhodamine isothiocyanate; VIII, Rhodamine isothiocyanate analog; IX, Texas Red is a trademark of Molecular Probes, Inc.

The phycobiliproteins are unique in their strong absorbing and fluorescing capabilities [29,57,67]. Methods were developed for coupling these large protein–chromophore complexes to antibodies, and the phycobiliprotein-tagged antibodies have become very popular for flow cytometric studies of cell surface antigens. Of particular value are phycoerythrin-labeled antibodies, which can be used simultaneously with fluorescein-labeled antibodies for two-color analysis of cells [45]. Both tags can be excited at 488 nm, but the phycoerythrin has a large Stokes shift and its orange emission can be detected independently of the yellow green fluorescein emission.

More labeling reagents need to be developed. For example, it would be valuable to have a substitute for fluorescein that is as easy to couple to antibodies as fluorescein isothiocyanate but which is more photostable, less sensitive to pH, and has a higher $\epsilon$ and $\phi$ when bound to proteins. Probes that absorb and emit further to the red (even into the near-infrared) than those available now would be valuable for a number of reasons. Excitation of such dyes would produce far less autofluorescence, and inexpensive HeNe lasers could be used for excitation sources. It may be possible to develop fluorescent labels that can be excited in the 670- to 820-nm range and can be used in flow cytometers "built on chips" that incorporate laser diodes for excitation and solid-state detectors. Cyanine, merocyanine, and oxonol dyes are being exploited as labeling reagents in the laboratory of the author. These chromophores have very large extinction coefficients

and can be synthesized with many different structures, charges, and wavelengths. The cyanines appear particularly promising as antibody tags.

Fluorescent labels can also be used as probes to localize and monitor interesting biological processes in living cells. Endocytic processing of receptors and ligands, e.g., insulin and EGF, which have been tagged with fluorescein and other markers, have been studied [64,65].

Detection of specific genes in situ with biotin derivatized DNA probes and fluorescein-tagged avidin first occurred in 1981 [51]. Subsequently, hapten molecules were attached to DNA probes so that they could be detected with fluorescent monoclonal antibodies. The result is a powerful technology for detection of infectious agents, oncogenes, and genetic defects in tissues, cells and fluids [12]. Efforts are being made in a number of laboratories to develop procedure for in situ hybridization with cells and chromosomes to be analyzed by flow cytometry. For analysis of cells by this method it is likely that multiple gene copies will have to be present or else new fluorescence amplification methods will have to be devised to provide sufficient signal to overcome auto fluorescence and other sources of noise. For flow cytometric analysis of chromosomes, hybridization methods will have to be developed that preserve chromosome morphology so that flow karyotyping and gene detection can be done simultaneously.

Another important advance was the development of fluorescein-tagged antibodies to quantify incorporation of bromodeoxyuridine into DNA [23] (see also Chapter 23). This reagent is useful in identifying S-phase cells in cell kinetic analysis. A complete volume of Cytometry [Vol. 6, No. 6, 1985] is devoted to this subject.

There is no doubt that there is a bright future for the application of fluorescent probes for analysis of antigens, genes, and biochemical processes in living and fixed cells and tissues. Still needed is further development of fluorescent chromophores with extended spectroscopic properties that can be covalently attached to biological molecules without interfering with their function. Interested organic and inorganic [34] chemists: take note!

## NONCOVALENTLY ASSOCIATING FLUORESCENT PROBES

Because of their particular molecular composition, fluorescent probes in this class associate noncovalently with special structures in cells. Structures that can be visualized with these probes include DNA, RNA, lipid, electrically negative or positive compartments such as mitochondria, and compartments with a low pH.

### Probes of DNA and RNA Content

A wide variety of fluorescent probes have been developed for quantification of the DNA and RNA contents of cells. The structures of the more commonly used probes are shown in Figure 2. The major applications involve cell cycle analysis (see Chapter 24), chromosome analysis (see Chapter 25), and detection of aneuploid cells in tumor samples (see Chapter 37). Other applications can be found in other reviews [46,63,78,84].

There are a number of important factors to consider when selecting a DNA or RNA content probe. The first is specificity. Some probes, such as Hoechst 33342 and the Feulgen stain, interact preferentially with DNA, whereas intercalating fluorophores such as propidium and acridine orange bind to double-stranded RNA as well as DNA [46,63,78,84]. In order to use one of the nonselective probes to measure only DNA content, prior treatment of the sample with ribonuclease is generally required to remove RNA.

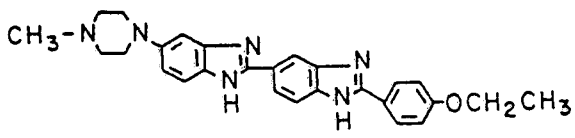
It is interesting that the DNA-intercalating probe, acridine orange, will also bind to single stranded RNA, but shows a red-shifted emission spectrum when this occurs. Darzynkiewicz, Traganos, and Melamed [18] and their colleagues have taken advantage of the green DNA fluorescence and the red RNA fluorescence from acridine orange to develop an important method for simultaneously quantifying DNA and RNA in cells by flow cytometry (see Chapter 15). Kapuscinski et al. [43] have elucidated the mechanism for the spectral shift. This group has also recently analyzed the interaction of pyronin Y with DNA and RNA, and has been able to account for absorption and fluorescence properties of these complexes [17,42].

Shapiro showed that it is possible to combine probes, Hoechst 33342 and pyronin Y, which have differential selectivity for DNA and RNA to measure DNA and RNA simultaneously on single cells [79]. Thioflavin T [73] and $diOC_1$ (3) [38] and thiazole orange [52] have been used to estimate RNA content of reticulocytes.

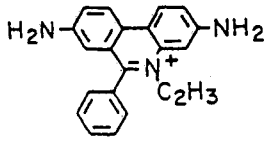
Some probes have selectivity for AT-rich or GC-rich regions of DNA [46,63,78]. Hoechst 33342, Hoechst 33258, DAPI, and DIPI (4-6-bis[2-imidazxolinyl-4H,5H]-2-phenylindol) prefer AT-rich regions, but mithramycin, chromomycin A3, and olivomycin select GC-rich regions of DNA. This selectivity is useful for analysis of bacterial samples which have a wide range of AT/GC ratios. Measurement of this parameter using appropriate AT/GC-selective probes can provide a method for bacterial classification by flow cytometry [98] (see also Chapter 29). Two-color analysis using probes selective for AT-rich or GC-rich regions is also valuable for karyotype analysis of chromosomes (see Chapter 25). Chromosomes that have near-identical total DNA content and that are not separable in a single-parameter DNA histogram often are sufficiently different in AT/GC base content to be discriminated by appropriate base-selective DNA stains used in dual parameter histograms.

The second factor to be considered in DNA and RNA probe selection is plasma membrane and nuclear permeability. Hoechst 33342 is the only commonly used DNA probe that will stain living cells. Thus, living cells in different stages of the cell cycle can be sorted and analyzed on the basis of DNA content determined with the use of Hoechst 33342 [2]. Other stains, such as propidium, are too highly charged or too polar to cross the membrane of a living cell. (Propidium readily crosses the membranes of "dead" cells to make them highly fluorescent. This property is useful in immunology for cell-killing assays [63].) Darzynkiewicz and colleagues [19] found significant differences in the accessibility of cellular DNA to different fluorochromes. Studies of these differences may lead to a better understanding of chromatin structure.

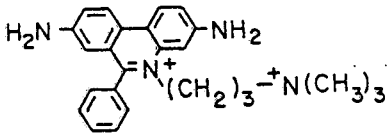
The third factor is the spectral properties of the probe. Hoechst 33342, DAPI, and a number of other DNA/RNA probes are optimally excited in the ultraviolet (UV) region of the spectrum. This is an advantage when dual laser instruments are used to quantify DNA with UV laser excitation and simultaneously to measure immunological properties of the cells using a laser emitting visible light. In other cases, it is preferable to excite DNA probe fluorescence in the visible spectrum because less autofluorescence is produced and because laser tubes operating in the visible range generally last longer. Propidium is popular for blue light excitation at 488 nm. Shapiro and Stephens [81] discovered several helium neon-laser-excitable (633-nm) dyes that may be useful for



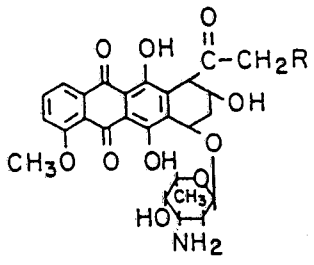
I-HOECHST 33342



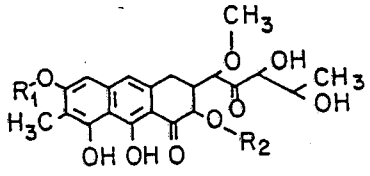
II- ETHIDIUM



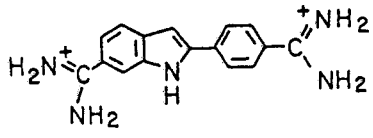
III PROPIDIUM



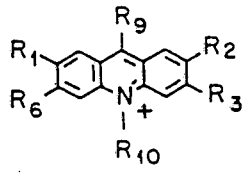
IV ANTHRACYCLINE DYES



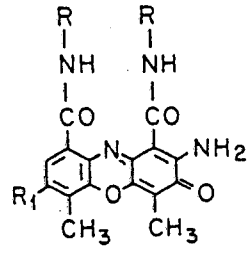
V MITHRAMYCIN



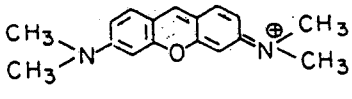
VI DAPI



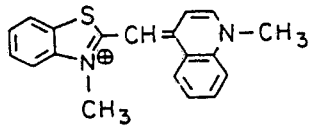
VII ACRIDINE DYES



VIII ACTINOMYCINS



PYRONIN Y



THIAZOLE ORANGE

Fig. 2. Fluorescent probes that bind noncovalently to DNA or RNA. For the anthracycline dyes (IV) the R group is -H on Daunomycin and -OH on Adriamycin. The R groups of mithramycin (V) are two- and three-unit oligosaccharides. The acridines (VII) have a wide variety of R-groups including alkyl, amino, alkylamino, methoxy, and halogen substituents. The R groups on Actinomycin D (VIII) are cyclic polypeptide chains and $R_1$ is a hydrogen.

quantifying DNA. This advance would leave available the shorter regions of the spectrum for measurement of other cell parameters with flow cytometers.

## Membrane Potential Probes

Membrane potential is an important property of cells and some organelles. The electrical potential difference across the plasma membrane is directly involved in the transport of ions and nutrients into and out of the cell. Modulation of the cell membrane potential is the mechanism of nerve conduction and a part of sensory transduction. The electrical potential difference works together with the pH gradient across the mitochondrial membrane to couple energy released during electron transport to the phosphorylation of ADP to form ATP. Quantification of membrane potentials can be accomplished with microelectrodes in large cells, but in small cells and organelles, membrane potentials are estimated from the transmembrane distribution of membrane permeant cations and anions that are either radioactive or fluorescent. Cyanine dyes (Fig. 3) were the first fluorescent membrane potential probes to have had their mechanism of action explained [82], and they have been used extensively since [100,101]. The cationic cyanine dyes are accumulated in compartments with a negative potential, such as cell interiors and mitochondria. Depolarization of the cell membrane or uncoupling of mitochondria leads to a loss of fluorescence as cyanine dye leaks out. Rhodamine 123 is also a membrane potential stain, exploited by Chen and colleagues for visualization of mitochondria in living cells under the fluorescence microscope [39]. Rhodamine 123 has been termed a mitochondrial specific dye because the mitochondria are so brightly stained. Cyanine dyes also yield bright mitochondria in healthy cells. Undoubtedly, the high fluorescence of mitochondria arises because they have a negative membrane potential. Uncoupling agents and inhibitors reduce mitochondrial fluorescence. The large membrane surface area in the mitochondrial matrix may contribute to the staining by binding large amounts of accumulated probe. Rhodamine 123 retention by tumor cell lines has been studied and related to anticancer drug sensitivity [50]. Other uses of this mitochondrial stain have been reviewed recently [102].

Cyanine dyes and other membrane potential probes have been used in flow cytometry experiments to study changes in neutrophils [76,77] and lymphocytes [60,61,80,103,104] following stimulation of the cells with formylated peptides and mitogens, respectively. The fluorescent changes are substantial, but the mechanisms are not well understood [14,91]. A major complication is the possible contribution of mitochondria to the total cell fluorescence change [11,60, 61,101–104]. Chused et al. [15] show that membrane-permeant anionic oxonol dyes may provide answers to this question. Mitochondria tend to exclude anionic dye, decreasing the contribution by this organelle. Anionic dyes also distribute across the plasma membrane according to the membrane potential, but the total cell fluorescence will be small because these dyes are excluded from the negatively charged (−50 to −60 mV) cells.

The large fluorescence changes that occur when these blood cells are stimulated in the presence of these probes undoubtedly reflect important physiological changes taking place in cells. It will be interesting to determine the nature of these changes eventually as well as their relationship to probe fluorescence alterations.

## Probes for Visualization of Membranes and Lipid Compartments

Fluorescent probes for lipid bilayers (Fig. 4) have been available for some time [99]. Many are uncharged hydrocarbons like diphenyl hexatriene (DPH). Others are charged but have long hydrocarbon chains attached (e.g., di$C_{18}$[3]). Since not all lipid membranes in a cell are alike, it is not surprising that fluorescent lipid probes with different structures partition between the different membranes in interesting ways. For example, Pagano and Sleight [68] made extensive use of fluorescent lipid analogues to trace pathways of membrane movement during endocytosis and recycling. Unfortunately, flow cytometry gives little information about the spatial distribution of these probes within cells. However, lipid probes are taken up differently by cells that differ in physiology, and flow cytometry can be used to quantify the uptake. For example, the hydrophobic dye Nile Red has been shown to be highly specific for lipid oil droplets in cells [30]. With 488 nm excitation, fluorescence measurements in the 515- to 560-nm range give good discrimination of acylated low-density lipoprotein uptake by macrophages.

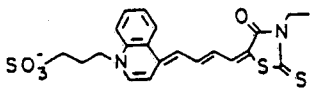
## pH Probes That Partition Between Compartments with Different pH

There are two classes of pH probes. One class of fluorophores have spectral properties that change with pH (e.g., fluorescein). These fluorophores are discussed in the next section, which centers on environment-sensitive fluorescent probes.

A second class of pH probes partition differently into cell compartments with different internal pH. The pH of each compartment can be estimated by the amount of fluorescent weak acid or weak base that distributes into that compartment. Weak bases cross membranes as the conjugate base (usually the whole molecule is neutral) but become trapped in acidic compartments when protonated. The converse is true for weak acids. Acridine orange is accumulated in neutrophil granules [1] and acidic lysomal compartments presumably for this reason. These authors showed that the kinetics of degranulation following stimulation of the neutrophils with the calcium ionophore A23187 could be followed by flow cytometry. Degranulation leads to loss of red fluorescence as the acridine orange (in an aggregated state?) in the lysosomes is released into the extracellular space. Apparently, the lysomal fluorescence is not masked or complicated by acridine orange fluorescence from cellular RNA in the live cell. The probe 9-amino acridine also is accumulated in acidic compartments [20] but this probe has evidently not been useful in flow cytometry experiments. Unless this probe is used at extremely low concentrations, the fluorescence of accumulated 9-amino acridine molecules is actually reduced as a result of concentration quenching inside the compartment. It would be useful if there were specific fluorescent probes for acidic and basic compartments. This should provide another area for collaboration of organic chemists and spectroscopists.

# FLUORESCENT PROBES THAT ARE SENSITIVE TO THEIR MICROENVIRONMENT

Certain fluorescent chromophores can be used to estimate the following properties of their local environment: pH, calcium concentration redox potential, polarity, fluidity, and the presence of other ions or molecules that can deactivate the excited state of the chromophore by energy transfer or



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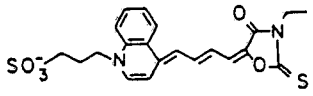
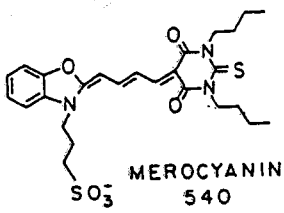
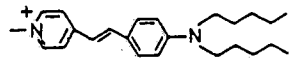


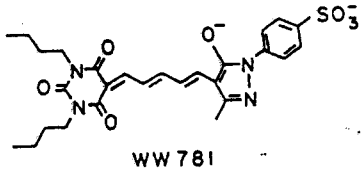
NK2367



MEROCYANINE 540



di-$C_5$-ASP



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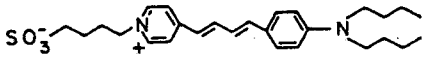
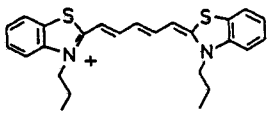
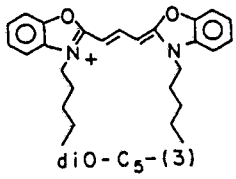


RH 160

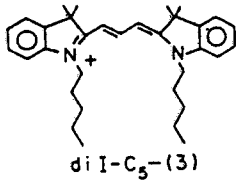
Poly-methine



diS-$C_3$-(5)

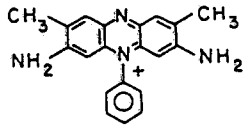


diO-$C_5$-(3)

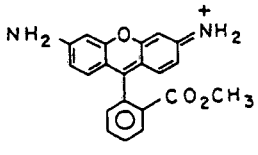


diI-$C_5$-(3)

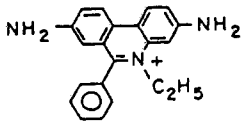
For NRBC



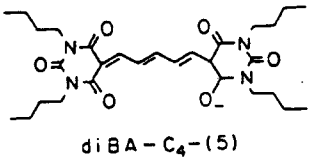
SAFRANIN O



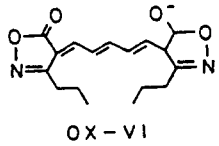
RHODAMINE 123



ETHIDIUM



diBA-$C_4$-(5)



OX-VI

Fig. 3.

charge transfer. The major challenge in making use of these dyes is in finding a method, by synthetic modification of the probe or otherwise, to target the probe to the region of interest within the cell.

## pH Probes That Respond with Spectral Changes

Hydrogen ion or metal ion binding changes the electronic structure of a number of dyes. The absorption properties, the fluorescence properties, or both, of the molecule can be affected, depending on whether the hydrogen or metal ion concentration is near the dissociation constant of the ground state or the excited state, respectively [75]. The rates of complexation and decomplexation relative to the excited state lifetime of the probe also play a role in the sensitivity of fluorescence to pH or to pION [−log(cation or anion)]. An example of a probe with an absorption spectrum sensitive to pH changes in the physiological range is 6-carboxyfluorescein, which has a ground-state pKa near 6.5. The absorption spectrum shifts from the 440 to 450 region at more acid pH to near 490 as the pH is raised [92]. Emission is not especially affected for reasons discussed by Martin and Lindquist [56]. Therefore, pH measurements are made by calculating the ratio of the emission produced with 496 excitation and the emission produced with 452 excitation. Ohkuma and Poole [66] used a standard fluorometer to measure pH in endosomes. Heiple and Taylor [33] and Tanasugarn et al [90] demonstrated how this method can be used to determine cellular pH with a fluorescence microscope. Murphy et al. [64] used an interesting variation of this technique to measure endosomal pH of single cells with a dual-laser excitation flow cytometer. Instead of exciting fluorescein at two wavelengths, a mixture of endocytosed fluorescein and rhodamine labeled insulin molecules were excited. PH-insensitive rhodamine fluorescence provided a measure of the amount of both ligands in each cell. The ratio of fluorescein emission to rhodamine emission gave the pH of the cell. (See Chapter 18 for further discussion of pH measurements by flow cytometry.)

There is also a useful pH probe that changes its *emission* properties in the physiological pH range. Valet et al. [97] showed that 2,3-dicyano-1,4-dihydroxybenzene can be excited at a single wavelength, and the emission spectrum shifts from 450 to 384 nm as pH increases [47]. The excited state pKa of this molecule is in the physiological range, and proton exchange can occur within its excited-state lifetime. The probe is loaded into cells in the diacetyl form. Nonspecific esterases expose the pH-sensitive hydroxyl groups. Thus, this probe is expected to report the pH of esterase-rich regions, including the cytoplasmic compartment. The ester-loading trick can be used with 6-carboxyfluorescein diacetate [92]. Unfortunately, these probes may not be limited to any one compartment of most cells.

Fig. 3. Membrane potential probes. The top six structures are fast dyes that respond to membrane potential changes that take place in microseconds. These probes are membrane impermeant and are useful for detecting action potentials in excitable membranes. The work of London et al. [55] illustrates the use of these probes. The bottom eight structures are slow dyes that redistribute across membranes over a period of milliseconds to seconds and accumulate in cells or cellular compartments according to the membrane potential difference. Chused et al. [15] discuss the application of several of these probes, as does Waggoner [100,101].

Hydroxypyrene trisulfonate is pH sensitive in the physiological range [16]. The molecule has the advantage that it does not leak out of cells but has the disadvantage that loading methods [59] are required to get the probe into cells. The relevance of cellular pH to function has been reviewed [13].

## Calcium Probes

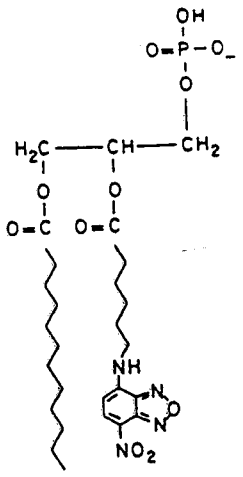
Because of its important role in regulating many cellular processes, calcium has received considerable attention in recent years [14,96] (see Chapter 32). The indicator Quin II, developed by Tsien [95], provided the first fluorescence method for detecting changes in cytoplasmic calcium [96]. The structure of Quin II is shown in Figure 6. Its acetoxy ester precursor is membrane premeant. Cytoplasmic esterase activity liberates the carboxylate groups, which limit the escape of the probe into the medium and provide chelation groups for binding the calcium. Bound calcium shifts the absorption peak toward the red and measurements are usually made with excitation near 340 nm. Calcium determinations with Quin II and its improved analogue fura-2 [31] are best made by a ratio technique whereby emission is measured at the two optimal excitation wavelengths for probe with and without bound calcium. Quin II is sufficiently nonfluorescent, so that cells must be loaded with 0.1- to 1.0-mM probe. The large Quin II concentration has worried some investigators because the probe is such a strong calcium buffer. The dissociation constant of the probe calcium complex is below $10^{-6}$ M. Thus, makeup calcium must enter the cytoplasm, which normally contains less than $10^{-6}$ M calcium, before the cytoplasmic Quin II is at equilibrium and calcium measurements can begin. Makeup calcium is provided in the medium during experiments. Several recent reports describing internal calcium concentration changes in platelets [40] and 3T3 fibroblasts [58,62] suggest that Quin II signals may not accurately indicate the magnitude or kinetics of the changes. Thus Quin II may be best for qualitatively detecting calcium level changes. The improved probe from Tsien's laboratory, fura-2, is more sensitive and can be used at lower concentrations [31]. Another advance is the synthesis of indo-1 which shows an emission shift upon calcium binding [31]. In flow cytometry, this is a definitive advantage since indo-1-stained cells can be excited with a single laser line and calcium concentration determined from the ratio at two emission wavelengths [15]. Care must be exercised in applying and interpreting the fluorescence signals of the new generation of fluorescent calcium indicators.

## Fluorescent Redox Potential Probes

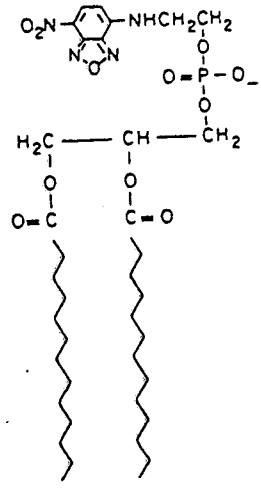
Neutrophils release superoxide and hydrogen peroxide when they are stimulated with chemotactic factors. Several fluorescent probes have been used to monitor the kinetics of this process in normal and diseased cells. Dichlorofluorescin (Fig. 7) can be loaded into cells in its acetylated form and trapped as a result of esterase activity. Bass and associates [7] showed that $H_2O_2$ released from stimulated neutrophils converts the nonfluorescent reduced form into dichlorofluorescein thereby making stimulated cells fluorescent. Cellular NADH and NADPH are intrinsic fluorescent probes of the redox state of cells and have also been used to monitor the energy state of cells and tissues [93].

## Fluorescent Probes for Measuring Enzyme Activities

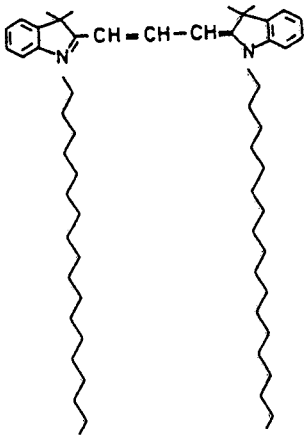
Fluorogenic enzyme substrates are chromophores converted by specific enzymes into products that have either



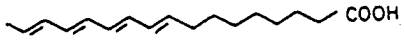
$C_6$ NBD PA



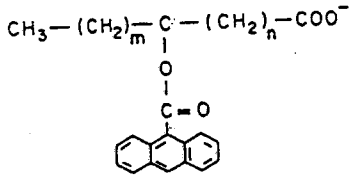
NBD PE



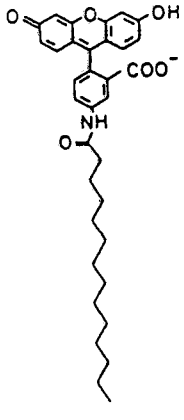
diI $C_{18}$-(3)



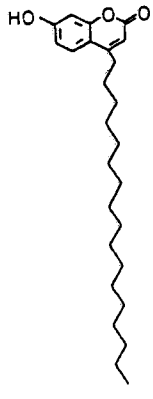
TRANS-PARANARIC ACID



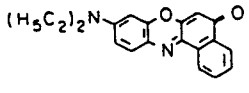
(m,n)-ANTHROYLSTEARATE



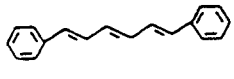
ACYL AMINOFLUORESCEIN



4-ALKYL UMBELLIFERONE



NILE RED



DPH

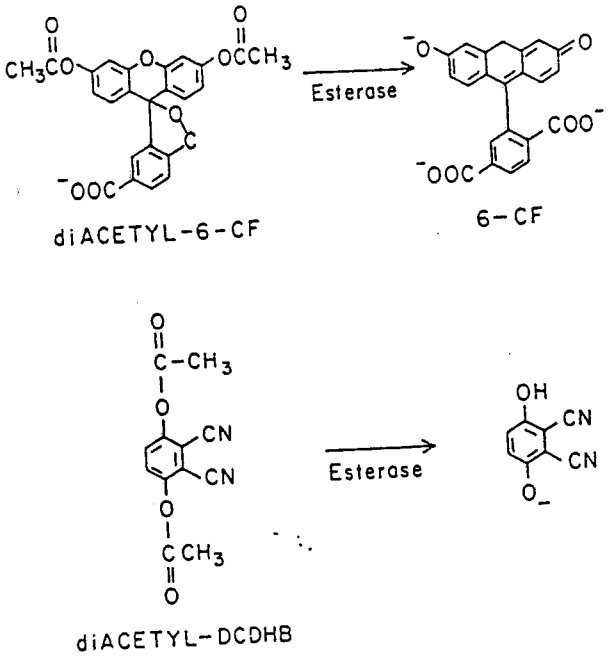



Fig. 5. pH probes. Two reactions illustrate the conversion, by cellular esterase enzymes, of membrane permeant ester molecules into fluorescent pH probes that are membrane impermeant because of their increased charge. The trapped products are 6-carboxyfluorescein (top) and 2,3-dicyano-1,4-dihydroxybenzene (bottom).

increased fluorescence or shifted spectra. These substrates can therefore be said to be sensitive to the presence of certain enzymes in their microenvironment. Dolbeare [21,22] provided excellent reviews of the use of fluorogenic substrates in flow cytometry.

Hydrolase enzymes play crucial roles in cell function. Lysosomal proteases for example are responsible for breakdown of intracellular proteins and peptides as well as polypeptide material that has been endocytosed [6,36]. Other proteases are bound to the external surfaces of cells or are secreted in soluble form. The latter enzymes breakdown extracellular protein molecules [70,72,86,107]. The breakdown of extracellular macromolecules is required for movement of phagocytic cells like macrophages and neutrophils through tissues [35,105]. It is also thought that the extracellular proteases play a significant role in tumor invasion, rheumatoid arthritis, and other disease states [86]. Thus, because of their significant roles in cell function, it is important to be able to measure activity and localization of enzymes

Fig. 4. Lipid probes. The top two probes have been exploited by Pagano and colleagues [68]. Barak and Webb [5] used $diIC_{18}$-(3) to study the low-density lipoprotein receptor in cultured cells. Parinaric acid is discussed by Hudson et al. [37]. The anthroyl stearates have been used to study lipid fluidity and organization as a function of depth in bilayer membranes [9]. The pH-sensitive fluorescein and umbelliferone chromophores with long alkyl chains can be used to study the effects of membrane surface potential on the pH of aqueous regions close to the membrane surface [28]. Nile red is a lipid droplet probe [30]. Diphenylhexatriene, DPH, is a membrane lipid fluidity probe discussed by Lakowicz [48,49].

such as proteases, glycosidases, esterases, phosphodiesterases, phosphatases, and sulfatases, in tissues, cells, organelles, and fluids.

Antibodies against proteases have come into wide use during the past 5 years for localizing and quantifying the enzymes in fixed tissues and cells and in serum. However, antibodies do not indicate whether the enzymes they bind to are functional (since the epitope on the enzyme is usually not associated with the active site of the enzyme) nor are antibodies sensitive to features involved in allosteric regulation of enzyme activity.

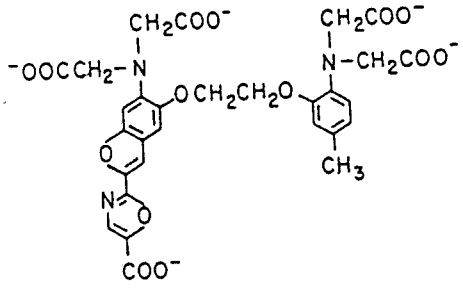
Most fluorogenic substrates have been constructed from chromophores that have either an amino or a hydroxyl group that participates in the electronic conjugation (Fig. 8). When an appropriate amino acid sequence is attached by an amide bond to an aminochromophore, the molecule usually becomes much less fluorescent. Cleavage of the amide bond of the substrate by a peptidase that will act upon the particular chromophore–peptide complex releases the more fluorescent primary amine chromophore. Naphthylamine, amino coumarins, amino quinolines, and rhodamine have served as chromophore bases for fluorogenic peptidase substrates [21,53].

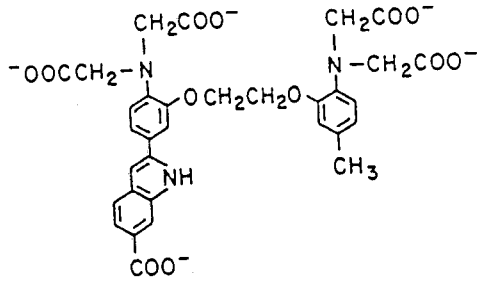
Esterase, glycosidase, sulfatase, phosphatase, and phosphodiesterase substrates can be made by attaching the appropriate functional group to a hydroxyl-containing chromophore, as shown in Figure 8. Hydroxy coumarins and fluorescein have most often been used for construction of these substrates.

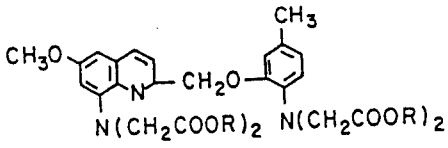
Fluorogenic substrates are now routinely used to measure activities of enzymes in such fluids as blood plasma [83]. However, these substrates have found little use in studies of enzyme activities in intact cells because they do not usually diffuse readily into cells and the fluorescent products rapidly leak out of the cells [21,25]. A variety of coupling reagents have been developed that convert products into insoluble colored substances that can be measured by absorbance techniques, but only one coupling reaction is available that results in an insoluble fluorescent product that stays within the cell. The latter involves coupling of nitrosalicylaldehyde to the methoxynaphthylamine product that is liberated by peptidase activity. This reaction, developed by Dolbeare and Smith [24] is illustrated in Figure 8. It is surprising that little use has been made of the coupling reaction, since it provides a way to produce fluorescence in cells that is proportional to enzyme activity.

A second disadvantage of available substrates is that most need to be excited with UV light, so their signals may be obscured by cell autofluorescence. The most commonly used peptidase substrates are based on the coumarin and naphthylamine chromophores that are excited near 365 nm. A recent and significant improvement was made when peptidase substrates based on the rhodamine chromophore were developed by Leytus et al. [53]. These substrates are excited at wavelengths near 490 nm. A limitation of presently available fluorogenic substrates is that none have been attached to peptide hormones or to phagocytosable particles like viruses, bacteria, and parasites so that the kinetics of lysosomal processing of these materials by living cells could be followed by sensitive fluorescent methods.

Certainly if the technical problems with fluorogenic substrates can be overcome there is potential for their wide use. Many hydrolases exit in cells and substrates need to be developed for the measurement of their activities in fluids, tissues, and cells.



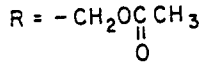




Fig. 6. Calcium probes. Fura-2 and indo-1 can be obtained as acetoxyester derivatives, similar to the derivatized form of Quin II shown on the bottom left. The ester derivatives are membrane permeant. Cellular esterases convert the derivatives into the free-acid forms of the probes, which are trapped inside cells and are able to chelate calcium.

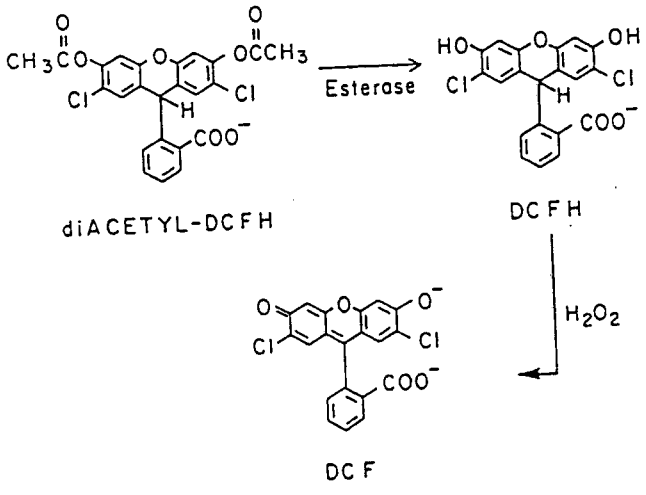



Fig. 7. Peroxide probe. Membrane permeant diacetyl-dichlorofluorescin is converted by cellular esterases to dichlorofluorescin. Peroxides released upon neutrophil stimulation oxidize the nonfluorescent dichlorofluorescin to the fluorescent dichlorofluorescein. Details can be found in Bass et al. [7].

## Energy Transfer

Fluorescent chromophores in the excited state are sensitive to the presence of nearby chromophores that can act as acceptors for resonance energy transfer. Energy transfer can occur between two chromophores separated by distances of tens of Angstroms provided that the donor fluorescence spectrum overlaps significantly with the acceptor absorption spectrum. The relative orientation of the optical transition moments of the two chromophores also affects the efficiency of transfer. Energy transfer is a nonradiative process. That is to say, there is not absorption by the acceptor of a photon emitted by the donor. If the acceptor is fluorescent, however, it can emit a photon as a result of excitation of the donor. The theory and early uses of energy transfer to study protein and membrane structure have been reviewed elsewhere [68,106]. More recently, the technique has been used to detect aggregation of cell surface components which were separately labeled with fluorescein (donor) and rhodamine (acceptor) [27,89]. Energy transfer might also be used to look at self-assembly or binding events even inside living cells [91], since large numbers of cells can be loaded with fluorescent analogs by bulk loading methods [59].

Energy transfer can be a nuisance as well. For example, in experiments designed to quantify cell-surface markers with antibodies tagged with different-colored fluorophores, extensive capping or patching may, depending on the extent of tagging and the size of the antibody–antigen complex, result in energy transfer. The quantity of marker tagged with the energy donor would be underestimated in this circumstance. This possibility should not be overlooked in multicolor flow cytometry experiments.

In addition to energy transfer, it is possible for certain molecules and ions to quench fluorescence directly by deactivating the excited state. Direct quenching of probe fluorescence can occur in the presence of oxygen, heavy atoms, and molecules that form charge transfer complexes [49]. The latter processes can in principle be used to learn something about biophysical properties of cells, such as accessibility of dissolved oxygen to structures containing fluorescent probes. This potential has not been exploited with flow cytometry.

## Solvent Polarity

Almost all fluorescent molecules are sensitive to some degree to the dynamic polarity of their solvent environment. It has been known for decades that the quantum yield of anilino-naphthaline sulfonate (ANS) is high in nonpolar solvents but more than 30-fold lower in water [87]. A large red shift in the emission wavelength of ANS also occurs in nonviscous polar solvents. This happens because energy of the

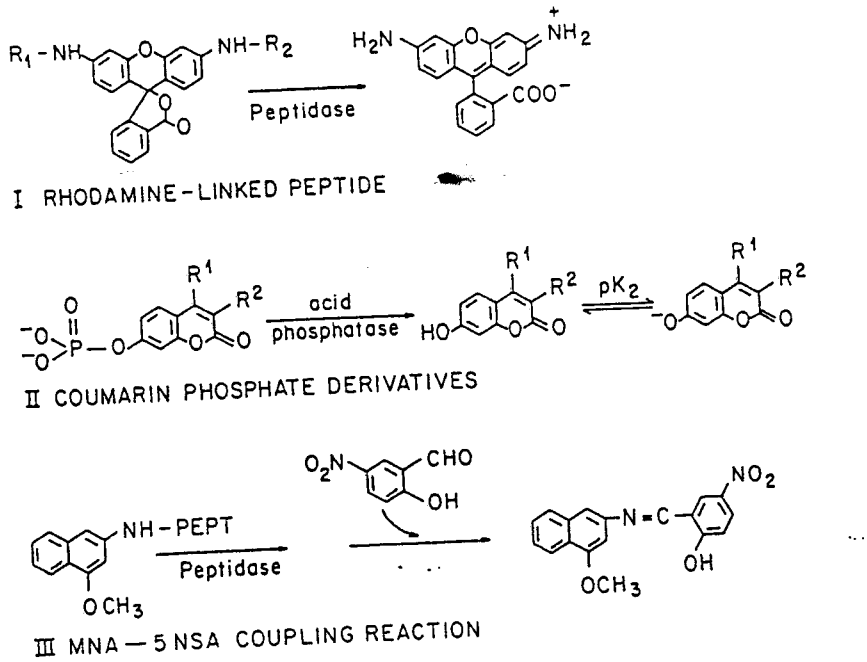



Fig. 8. Fluorogenic enzyme substrates. I, Rhodamine-linked peptides developed by Leytus et al. [53]. The R groups are peptides specific for the enzyme of interest. II, Acid phosphatase derivatives based on the coumarin structure. The R groups are chosen to shift the fluorescence of these derivatives toward the visible region of the spectrum [44]. For I and II, the products are more fluorescent than the substrates. III, The coupling reaction devised by Dolbeare and Smith [24]. The free amino group, which is made available when the peptide bond of peptidylmethoxynaphthylamine is hydrolyzed by a peptidase, couples with 5-nitrosalicylaldehyde, which is present during the reaction, to form the insoluble red-orange-fluorescing product.

excited state is lost to solvent when polar solvent molecules reorient around the more dipolar structure of excited ANS shortly after excitation. Reduction of the distance between the excited and ground state means that emitted photons will have lower energy, i.e., be red-shifted.

Sensitivity to solvent polarity is an important component of the mechanism of certain probes. For example, propidium is more fluorescent when protected from water by DNA intercalation. Also, many potential sensitive dyes are more fluorescent in hydrocarbon environments such as membranes and therefore show fluorescence changes when driven by membrane potential changes to compartments of different polarity [101].

Sometimes it is better if a probe is not sensitive to solvent polarity. Quantification of the binding of fluorescent labeled antibodies may be difficult if the probe is sensitive to the polarity of the cellular environment near the antigen or to different aqueous and nonaqueous preserving agents or to air drying. It complicates physiological interpretations when pH probes, calcium probes, energy-transfer probes, and distribution-type membrane potential probes also change their spectral properties because of their solvent environment changes rather than because of a change in the property they are intended to monitor.

## Probes of Lipid Microviscosity

There is a direction defined on each chromophore—the transition moment—for which polarized light will be optimally absorbed or emitted. Absorption and emission transition moments are not always in the same direction but often they are. This property can be used to find the orientation of probes bound to biological structures illuminated with polarized light. The property can also be used to determine the rotational mobility of probes and therefore determine the microviscosity of their environment or the binding of the probe. The light emitted from a population of fixed fluorophores that have been excited with a beam of polarized light is highly polarized. However, if the fluorophores are rapidly rotating relative to their excited state lifetime, the emitted light will be depolarized. The rate of rotation can be determined by measuring the rate of depolarization of fluorescence after pulse excitation [106], by phase-modulation techniques [49], and by measuring the steady-state fluorescence polarization of probe molecules excited with continuous polarized light [106].

The theory and instrumentation for flow cytometric measurements of fluorescence polarization have been available for a number of years [41,54,108]. However, relatively few biological experiments have been carried out with single cell polarization measurements. Schaap et al. [74] determined the fluorescence polarization of 6 membrane probes associated with embryonic carcinoma cells after differentiation. Diphenylhexatriene (DPH) (see Fig. 4) and a charged analogue of DPH showed polarization increases upon differentiation, but anthroyl stearate analogues (see Fig. 4) gave no polarization change. Fox and Delohery [108] recently detected a difference in the DPH fluorescence from CHO cells grown in different media.

The value of fluorescence polarization measurements ultimately depends on having probes that selectively bind to cell structures or components in which rotational motion or microviscosity is involved in an interesting cell function. As

often is the case in flow cytometry, the instrumentation capabilities outstrip the availability of ideal probes.

## FUTURE FLUORESCENT PROBES FOR FLOW CYTOMETRY

The availability of new specific and sensitive fluorescent probes is the key to advances in the power of flow cytometry. New labeling reagents that fluoresce in all regions of the spectrum from the UV to the near-infrared (IR) have been developed that match new excitation wavelengths from lasers and laser diodes. These advances increase the flexibility a researcher has in choosing combinations of probes and provide additional capabilities for multicolor analysis of single cells. Furthermore, new probes with extended Stokes shifts will permit simultaneous excitation of two or more probes simultaneously with one laser, just as is done presently with the phycoerythrin-fluorescein combination. All these new labeling reagents to come will not only be used for tagging monoclonal antibodies but will also be used in conjunction with DNA and RNA hybridization probes both to detect sequences in individual chromosomes and to quantify the RNA content of single cells.

Fluorescent ion indicators are another class of probes certain to expand because of the driving need of biologists to detect ion concentration changes in single living cells. Besides long-wavelength excitable calcium indicators, sodium, potassium, and magnesium indicators are needed. Anion and trace metal indicators would also be valuable.

Additional and improved fluorogenic enzyme substrates for detection of enzyme activities are needed to study growth control and other functions of single living cells. Organic chemists who understand the needs of the biologists and the capabilities of the flow cytometer will need to overcome the disadvantages of available substrates that were mentioned earlier.

Altogether, the co-development of fluorescent probes, instrumentation, monoclonal antibodies, and nucleic acid hybridization technologies promises an exciting and fruitful future for flow cytometry.

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