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Detecting Enzymes in Living Cells Using Fluorogenic Substrates

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Characteristics of fluorogenic substrates designed for detection of enzyme activity in living cells are reviewed. Improved retention of the fluorescent products in the cell of origin can be achieved by structural modifications to the substrate that result in association with membrane lipids or conjugation to intracellular glutathione. Newly-developed substrates that yield fluorescent precipitates provide the additional advantage of allowing subcellular localization of sites of enzymatic activity. Improved detection sensitivity can also be achieved by targeted delivery of substrates for processing by specific organelles. Substrates designed for monitoring oxidative activity and lipid metabolism provide examples of this approach.

KEY WORDS: Enzyme detection; live cells; fluorescein digalactoside; glutathione; fluorogenic substrates; flow cytometry; oxidative activity; lipid metabolism.

INTRODUCTION

Enzymes in living cells perform many functions including energy transduction, biosynthesis, degradation of toxic substances, active transport, and regulation of metabolic activity. Detection of these enzymes can potentially be used to sort cells, to trace metabolic pathways, and to study cell regulation and proliferation. Because a detectable product can be generated by very low levels of enzymatic activity, fluorogenic substrates can potentially provide the sensitivity required to make these determinations on single cells. Combining fluorogenic substrates with flow cytometry provides a means for classifying and, if required, sorting abnormal cells. Unlike immunofluorescent labels for cell surface antigens, fluorogenic substrates can discriminate cells based on properties that are closely related to cell function. Formation of a fluorescent product is highly enzyme specific and may therefore be used to detect differences between cells that are otherwise difficult to distinguish by immunological differences alone. Furthermore, the amplification inherent in an enzyme-mediated detection process provides much higher fluorescent signals than can be obtained using conjugates of fluorescent dyes.

Many fluorogenic substrates have been described for measuring the activity of isolated enzymes in solutions or in cell homogenates [1,2]. The enzymes for which the most successful substrates have been developed are predominantly hydrolytic enzymes such as glycosidases, peptidases and phosphatases, and oxidative enzymes such as peroxidases and microsomal dealkylases. The reason for this is based on the typical fluorescent properties of the dyes used to prepare the substrates—those that form ethers, amides, or chemically reduced dyes tend to have much shorter-wavelength absorption and very weak fluorescence emission relative to their hydrolysis products, which are phenols, anilines, or the oxidized fluorophore. Thus, the most common fluorogenic substrates are aromatic ethers (including glycosides) or phosphates of various naphthols,

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7-hydroxycoumarins (umbelliferones) or fluoresceins, peptide amides of various naphthylamines or 7-aminocoumarins, or chemically reduced fluoresceins or rhodamines that can be reoxidized to their parent fluorophores.

Several of these fluorogenic substrates have been applied to enzymatic detection in living cells. Unfortunately, however, the fluorescent products are relatively lipophilic organic dyes that are usually not well retained in living cells. For instance L-leucine, 7-amino-4-methylcoumarin amide has little long-wavelength fluorescence when excited at 365 nm but is readily converted to bright blue fluorescent 7-amino-4-methylcoumarin in many cells by leucine aminopeptidase (Fig. 1). However, the dye rapidly leaks out of live cells and washing the cells eliminates the fluorescent staining.

PRODUCT RETENTION IN MEMBRANES

Researchers at Molecular Probes have been actively developing new fluorogenic substrates that yield fluorescent products that are well retained in cells. We have found several solutions, three of which appear to be of broad general utility, especially for hydrolytic substrates. Our initial target was to develop substrates that could monitor cloning of the *lacZ* gene (which codes for the enzyme β-galactosidase) into cells, because of its importance in biology as a traceable marker. We were most interested in substrates based on fluorescein, because they have no long wavelength absorbance or fluorescence until hydrolyzed, and their favorable spectra make them useful for both flow cytometry and confocal microscopy. The first solution was to attach a lipophilic moiety to the known β-galactosidase substrate, fluorescein digalactoside (FDG). Although FDG has been extensively used for *lacZ* detection in live cells [3,4], brief permeabilization by hypoosmotic shock and incubation at $<4°C$ are necessary to give any retention of fluorescein in *lacZ*-positive cells. We have shown that incorporation of a 12-carbon amide into FDG gives a substrate, $C_{12}FDG$, that has certain advantages over FDG: The lipophilic tail facilitates staining of cells under physiological conditions, and more importantly, the fluorescent product, 5-dodecanoylaminofluorescein (Fig. 2), is well retained in the membranes of viable cells, even through cell division [5]. The most difficult problem in using these substrates has been that there is usually endogenous β-galactosidase activity in the lysosomes of cells and this may result in the formation of a fluorescent product in *lacZ*-negative cells. We have extended the membrane retention approach to include lipophilic versions of the shorter-wavelength 7-hydroxycoumarin galactosides, longer-wavelength resorufin galactosides [6], and their corresponding β-D-glucuronides. The latter compounds have been used to monitor incorporation of the GUS gene (which codes for β-D-glucuronidase) into plant and mammalian cells.

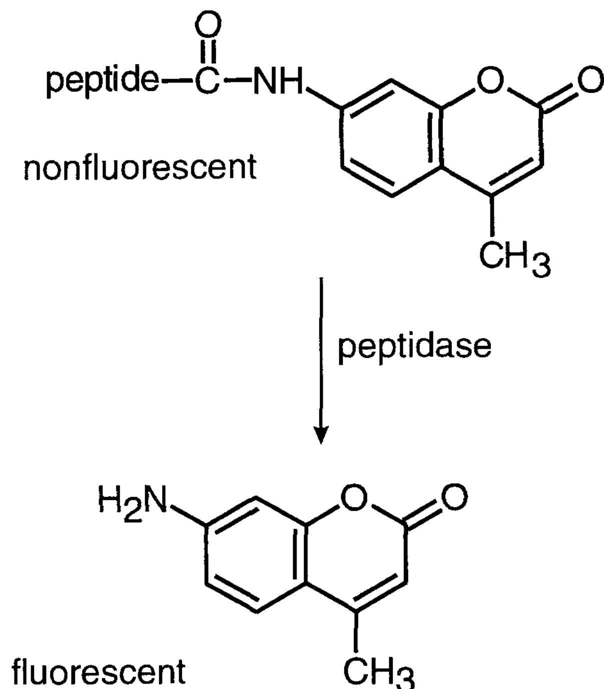

Fig. 1. Principle of fluorogenic aminopeptidase substrates derived from 7-amino-4-methylcoumarin.

PRODUCT RETENTION BY INTRACELLULAR CONJUGATION

For a second solution, we have developed probes that are bifunctional substrates for both glutathione *S*-transferase and the intracellular enzyme whose activity is to be detected. Most cells have a high level of the cysteine-containing tripeptide, glutathione, which is used by the cell as an antioxidant for maintainance of its redox state and is also involved in detoxification processes. We incorporated glutathione-reactive chloromethyl moieties into substrates such as FDG to give a β-D-galactosidase substrate, CMFDG (Fig. 3) In galactosidase-containing cells, this is (in either order) hydrolyzed to fluorescein and conjugated to glutathione. Again the fluorescent adduct is well retained in the cytoplasm of

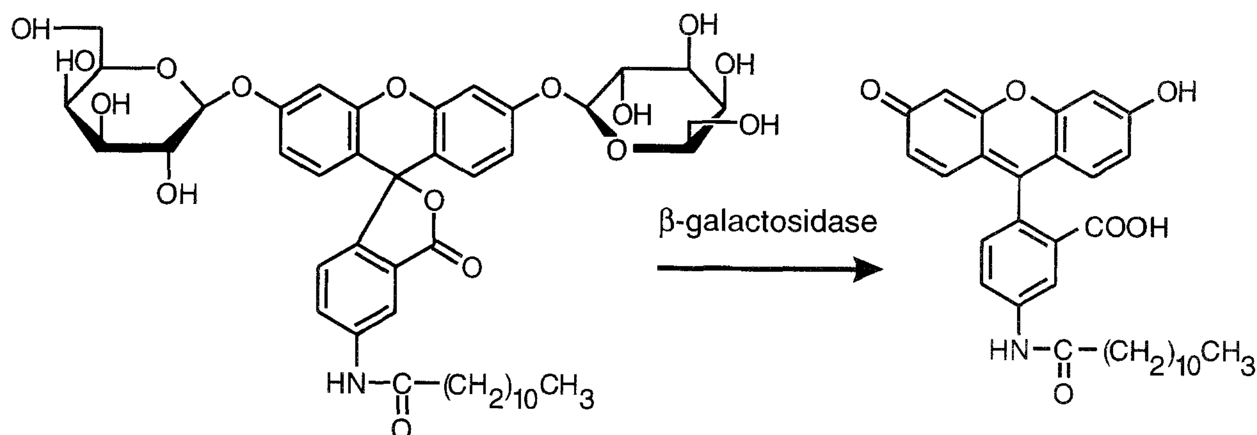

Fig. 2. Principle of β-galactosidase detection via conversion of nonfluorescent 5-dodecanoylaminofluorescein di-β-D-galactopyranoside ($C_{12}FDG$) to 5-dodecanoylaminofluorescein.

cells through several cell divisions. Like FDG, it is usually loaded into cells by brief hypoosmotic shock.

More recently, we have applied the glutathione transferase-mediated trapping mechanism to peptidase substrates based on 7-amino-4-methylcoumarin. By substituting a chloro atom on the methyl group to give 7-amino-4-chloromethylcoumarin peptide amides (Fig. 4), we have achieved long-term labeling of cells with a blue fluorescent product. Use of a substrate of this type for fluorescence imaging of intracellular calpain protease activity has recently been described [7]. Previous research on detecting peptidase activity in single cells has usually depended on capturing the fluorescent product as its adduct with 5-nitrosalicylaldehyde [8]. Recently Mangel, Valet, and co-workers have described the use of peptidase substrates derived from rhodamine 110 that are retained by live cells for some time following hydrolysis [9,10]. Substrates derived from this dye yield fluorescent products with spectra similar to that of fluorescein (Fig. 5).

A very recent development of a similar nature is the preparation of new "MitoTracker" dyes such as 4-chloromethyldihydrotetramethylrosamine (CMTMRos-H_2; Fig. 6). This colorless double substrate requires metabolic oxidation inside the cell to 4-chloromethyltetramethylrosamine (CMTMRos). The positive charge on the dye and its glutathione conjugate causes it to localize selectively in the mitochondria of metabolically active cells. The mitochondrial staining produced by CMTMRos, unlike that of probes such as rhodamine 123, persists through cell fixation with aldehyde fixatives. This permits subsequent introduction of a second probe, such as an antibody, directed to a different target within the cell.

SUBSTRATES YIELDING LOCALIZED PRODUCT DEPOSITION

Although the above approaches yield fluorescent products that stay associated with cells, they do not permit the exact localization of the enzymatic activity in cells because the products are free to diffuse from their site of formation. To obtain specific localization, one must form a precipitate of the fluorescent product at the site of enzymatic activity immediately following its formation. Very few substrates of this type are available, and those that have been available have required the use of a "capture reagent" to form a fluorescent precipitate [11,12]. The kinetics of this capture step may permit diffusion of the fluorescent product with considerable loss of optical resolution. We have found a 2-hydroxyphenylquinoxazolinone fluorophore that does not have long-wavelength fluorescence when it is blocked by formation of an ether or an ester (Fig. 7) but that immediately forms a fluorescent precipitate when hydrolyzed by the enzyme, in the same way as does the common chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). So far our studies have concentrated on the phosphate derivative because of its high intrinsic solubility. Because of their low permeability in live cells, the substrates have been useful for detection of endogenous phosphatase activity mostly in fixed cell or tissue

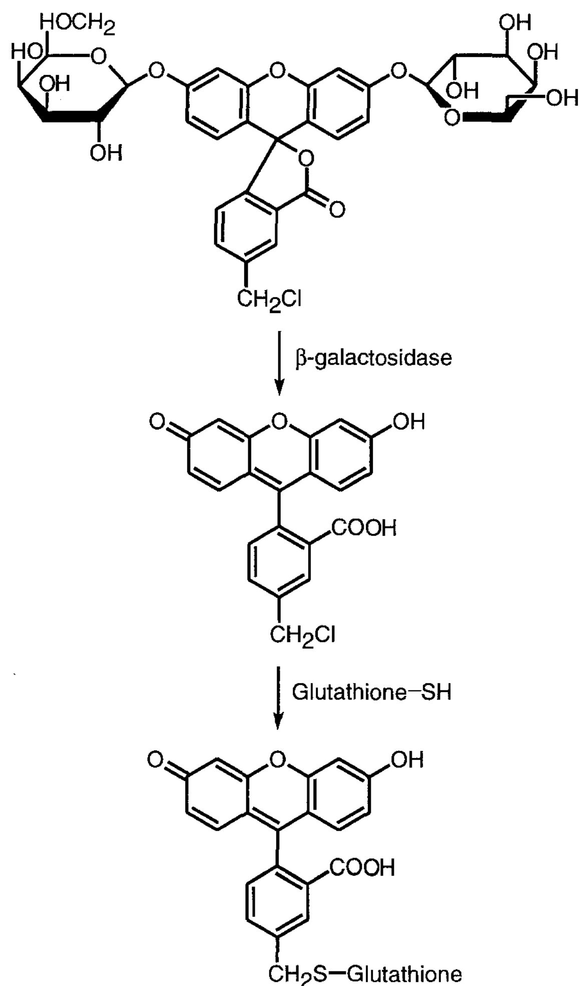

Fig. 3. Sequential hydrolysis and glutathione conjugate formation from nonfluorescent 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG).

preparations. These substrates can detect either acid or alkaline phosphatases. Furthermore, the fluorescent products—which are excited in the ultraviolet but which have large Stokes shifts and bright blue, green, or yellow fluorescence—form exceedingly stable precipitates that can be illuminated in a microscope under full power for over an hour and still have detectable fluorescence. Our recent studies have centered on using these enzyme-labeled fluorescence (ELF) substrates for enzyme amplified detection of cell staining, immunofluorescence, and fluorescence *in situ* hybridization (FISH). We have shown that under carefully controlled conditions, we can obtain site-specific staining that is below the resolution of light, and that the enzyme-amplified formation of a fluorescent precipitate permits one to obtain significantly more signal than is possible with any other technique involving soluble fluorescent dyes. These studies are continuing.

DETECTION OF OXIDATIVE ACTIVITY

Several fluorogenic substrates have been developed for measuring oxidative activity in single cells. Dichlorodihydrofluorescein diacetate (H_2-DCF-DA) is a colorless dye that is freely membrane permeant. Inside the cytoplasm of cells, hydrolytic cleavage of the acetate groups by nonspecific esterases yields colorless, reduced dichlorofluorescein (H_2-DCF). This can be oxidized to fluorescent dichlorofluorescein by several types of cells—in particular, by neutrophils following stimulation. In a collaboration with Dr. Elizabeth Simons [13], we prepared a covalent immune complex of this dye that is internalized into the phagovacuole of neutrophils by an F_c receptor-mediated mechanism (Fig. 8). Because superoxide-mediated oxidation occurs within the phagovacuole rather than in the cytoplasm, the fluorescence development using this immune complex is more rapid and specific than is observed with H_2-DCF-DA. This is an example of an organelle-targeted fluorogenic substrate. Chemical coupling of H_2-DCF to bacteria and yeast is a general approach for following the phagocytic and oxidative degradation processes of cells.

Analogous to the new MitoTracker dye CMTMRos-H_2 are several dihydrorhodamines and dihydrorosamines, including dihydrorhodamine 123 (DHR 123). DHR 123 has been used by Rothe and Valet to detect superoxide production during respiratory bursts in neutrophils [14,15]. Following oxidation, these probes localize in mitochondria, but they diffuse from the cell when the mitochondrial membrane potential is lost. On the other hand, dihydroethidium is oxidized by cells to ethidium, which is retained in single cells as its nucleic acid complex [16].

Microsomal dealkylases have a broad substrate specificity for oxidative metabolism of xenobiotics. Enzyme activity induced by suspected carcinogens such as polycyclic aromatic hydrocarbons and drugs such as phenobarbital is potentially useful as an indicator of these species. Fluorogenic ethoxycoumarin and ethoxyresorufin substrates have been widely used to measure microsomal dealkylase activity [17]. However since the turnover rates of these enzymes are very low, fluorescent products that are well retained in cells are essential for monitoring activity on a single-cell basis. To address this need, we have recently developed coumarin-, fluorescein-, and resorufin-based substrates that are dealkylated

Fig. 4. Conversion of nonfluorescent amide derivatives of 7-amino-4-chloromethylcoumarin (A) to fluorescent glutathione conjugates (B) by sequential action of peptidase and glutathione *S*-transferase in either order.

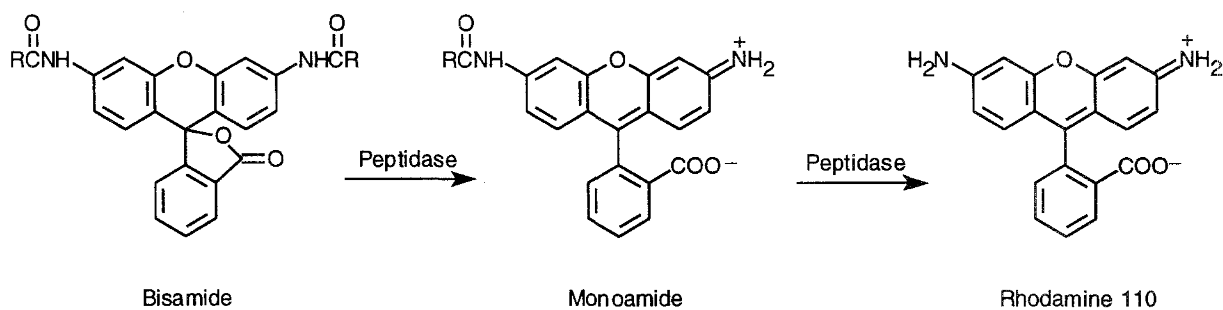

Fig. 5. Bisamide peptidase substrates derived from rhodamine 110 incorporate two benzyloxycarbonyl (CBZ)-terminated peptides, represented by R. Sequential peptidase cleavage of these groups produces a fluorescent monoamide followed by the more highly fluorescent parent dye, rhodamine 110.

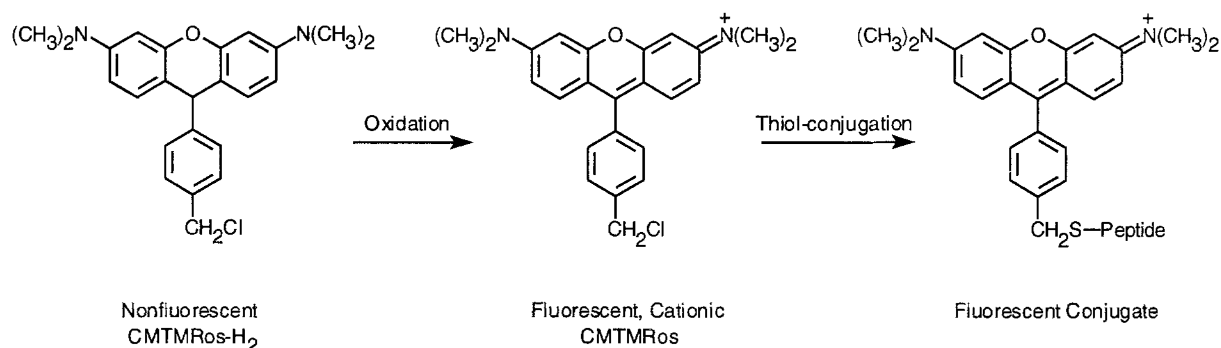

Fig. 6. Intracellular reactions of 4-chloromethyldihydrotetramethylrosamine (CMTMRos-H_2). Oxidation to the cationic product CMTMRos results in potential-dependent sequestration in mitochondria, where reaction with peptide thiol groups yields an aldehyde-fixable conjugate.

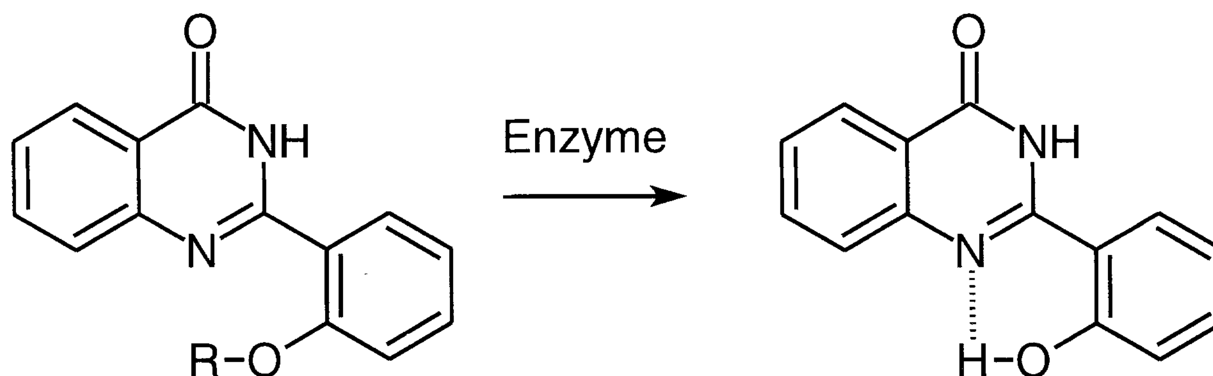

Fig. 7. Principle of water-soluble substrates that yield fluorescent precipitates upon enzymatic cleavage of a blocking group (R), resulting in the formation of the intramolecularly hydrogen-bonded 2-hydroxyphenylquinoxazolinone fluorophore.

to glutathione-reactive or lipophilic products. The practical utility of these new substrates has yet to be tested.

MONITORING LIPID METABOLISM

Following lipid metabolism in single cells has not been easy because the chemistry of the process commonly does not result in the type of optical change observed with the hydrolytic or oxidative substrates. However, we have developed some substrates based on the 4,4-difluoro-4-bora-3a,4a-diazaindacene (BODIPY) fluorophore that permit one to detect and even quantitate lipid metabolism in living cells. Substrates such as NBD ceramide and BODIPY ceramide are intrinsically fluorescent in cells, where they are metabolized to sphingomyelin and glucosylceramide within the Golgi apparatus. This represents another example of targeting a substrate to an organelle. A particularly useful characteristic of BODIPY ceramide is that its accumulation at the site of metabolic conversion in the Golgi complex results in a shift of the emission maximum from about 515 nm (green) to 620 nm (red), allowing its distribution to be visualized by ratio imaging techniques [18]. We have also observed this phenomenon, ascribed to excimer formation, for BODIPY-labeled phospholipids when their concentration exceeds a few percent of the total lipid in liposomes or low-density lipoproteins (Fig. 9).

Metabolic incorporation of green fluorscent BODIPY fatty acids into cells produces red fluorescent spherical structures, identified as cytoplasmic lipid droplets [19]. This concentration-dependent emission shift has the same origin as that of BODIPY ceramide. Analysis by thin-layer chromatography and HPLC of the metabolic products of exogenous BODIPY dodecanoic acid shows incorporation predominantly into phosphatidylcholines and diacyl- and triacyl-glycerols [19].

In collaboration with Dr. Elizabeth Simons, we have used a phosphatidylcholine analog, in which both fatty acyl substituents are labeled with BODIPY fluorophores

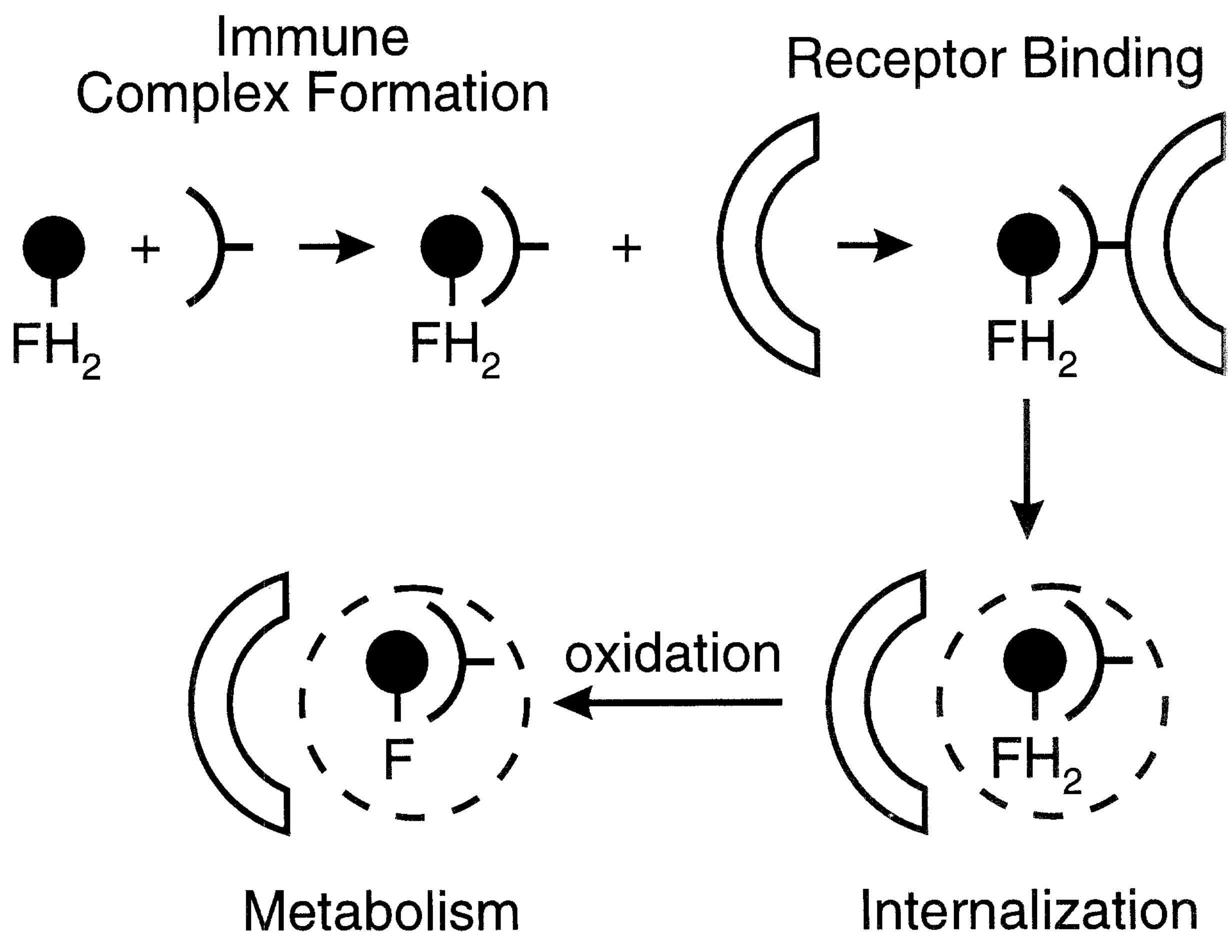

Fig. 8. Schematic diagram of the use of a reduced fluorescent dye (FH_2) conjugated to an antigen (●) to generate an immune complex that is internalized by F_c receptor-mediated phagocytosis. Formation of the fluorescent product (F) provides highly sensitive detection of the oxidative burst that is activated by internalization of the immune complex.

(Fig. 10), to monitor the activity of phospholipase A in single cells by flow cytometry [20]. In this case, metabolism results in relief of the self-quenching of the adjacent fluorophores following hydrolysis of a fatty acyl chain. Free fatty acids and lysophospholipids derived from the substrate following phospholipase activation appear to be rapidly metabolized to phosphatidylcholine, neutral triacylglycerols, and cholesteryl esters.

SUMMARY

Chemical synthetic techniques have resulted in the availability of fluorogenic enzyme substrates with superior cell targeting and product retention characteristics. Improvements in these characteristics directly impact the ability to specifically localize enzyme activity within single cells and among mixed cell populations. Continued development of fluorogenic substrates for use in live cells promises to be of considerable importance for both research and diagnostics.

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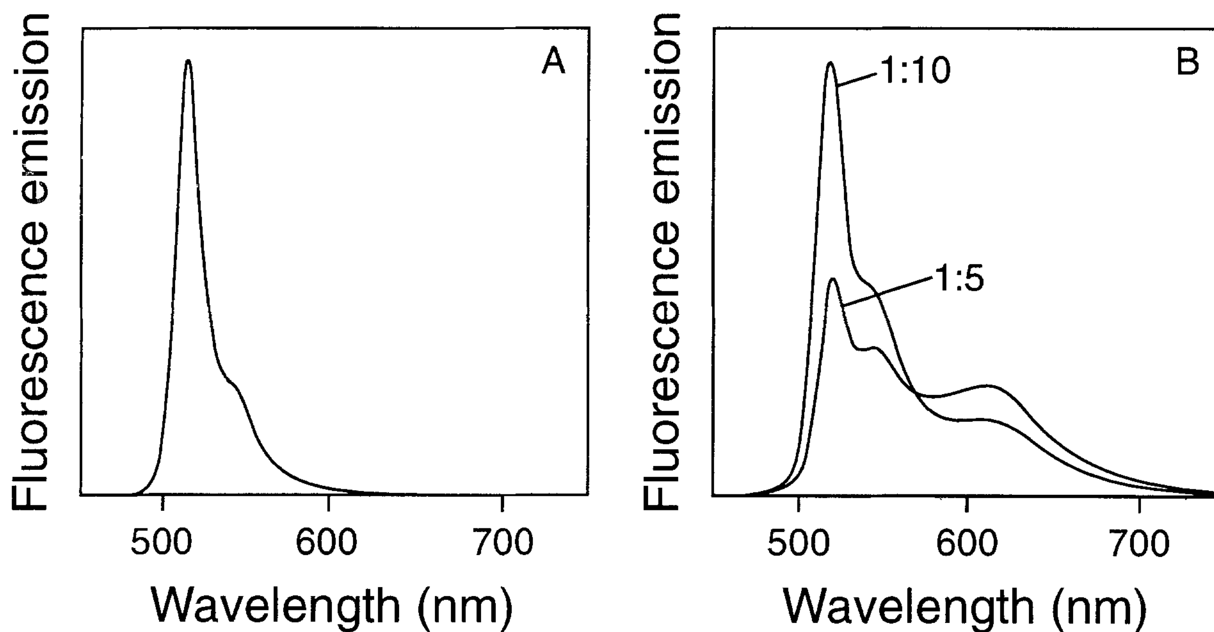

Fig. 9. (A) Fluorescence emission spectrum of a BODIPY-labeled phospholipid analogue imcorporated in DOPC (dioctadecenoylglycerophosphocholine) liposomes at 1:100 mol:mol (labeled:unlabeled phospholipids). (B) Spectra at higher incorporation levels, 1:10 and 1:5 mol:mol.

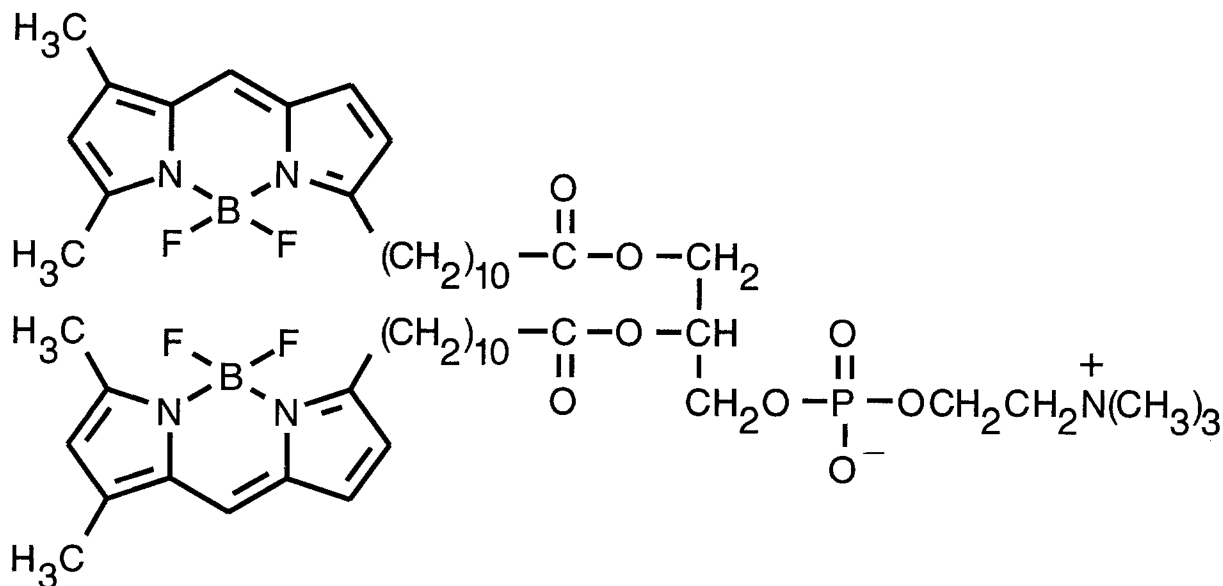

Fig. 10. Structure of a fluorogenic phospholipase A substrate in which BODIPY fluorophores are incorporated in adjacent phospholipid acyl chains.

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