Histochemical Journal **30**, 123–140 (1998)

REVIEW

Fluorescent probes for living cells

IAIN JOHNSON

Molecular Probes, 4849 Pitchford Av., Eugene, OR 97402–9165, USA

Summary

The functional characteristics of fluorescent probes used for imaging and measuring dynamic processes in living cells are reviewed. Initial consideration is given to general design requirements for delivery, targeting, detectability and fluorescence readout, and current technologies for attaining them. Discussion then proceeds to the more application-specific properties of intracellur ion indicators, membrane potential sensors, probes for proteins and lipids, and cell viability markers.

Introduction

As research in cell biology, neuroscience and immunology has progressed from purely structural characterization of cells towards understanding dynamic processes, such as signalling, excitability, growth and metabolic transport, fixed cell fluorescence detection techniques, such as immunofluorescence, have been augmented by new methodologies suitable for application to living cells. In general, fluorescence instrumentation used for measurements on fixed cells does not require major modification for live cell work, although the study of dynamic processes creates substantial demands for temporal resolution in both photometric and image data. Instead, the primary experimental aspect that requires modification is sample preparation and, in particular, the design of fluorescent probes. Fluorescent probes are molecules designed to localize to a specific region within a biological specimen or to produce a spectroscopically observable response to a specific stimulus. The function of the probe is to identify a target specifically (a molecule, ion, organelle or other entity) in a complex specimen, enabling the target to be detected and either quantitated by photometry or visualized by microscopy. Although endogenous fluorophores, such as NADH, flavins and porphyrins, can be useful indicators of cellular processes (Brandes & Bers, 1996; Masters & Chance, 1993), such intrinsic probes are generally rare. It is the design, synthesis and application of extrinsic probes that transforms fluorescence from a phenomenon of incidental usefulness to the powerful and versatile detection technique that it has become. This article is intended to outline design criteria for live cell fluorescent probes and to examine how these criteria are fulfilled by some probes in current use.

Design criteria for live cell fluorescent probes

Four sets of design criteria must be addressed in the development of fluorescent probes for application to live cell systems:

- Delivery. The probe must be introduced in a way that maintains the physiological and structural integrity of the cell.
- Targeting. The probe must interact selectively with a target ion, molecule, organelle or other structure.
- Detectability. The spectroscopic characteristics of the probe must be compatible with the instrumentation that is to be used for detection. The fluorescence output of the probe must be sufficient to permit the detection of the target at natural abundance levels.
- Fluorescence response. Interaction with the target must be detectable either by imaging

0018–2214

the spatial distribution of fluorescence or via a spectroscopic response, such as a fluorescence intensity change or a spectral shift.

Delivery

Probes with generally lipophilic structural characteristics can often be introduced into cells merely by adding a small amount of water-miscible stock solution to the extracellular medium. Delivery of more polar probes requires intervention using one of several techniques. Bulk loading procedures are necessary for the analysis of populations of cells by flow cytometry and spectrofluorometry. These procedures include loading in the form of membrane-permeant acetate or acetoxymethyl ester derivatives (Tsien, 1981), ATP-induced permeabilization (Steinberg *et al*., 1987) acid loading (particularly applicable to plant cells; Bush and Jones, 1990), hypo-osmotic shock (Nolan *et al*., 1988), scrape loading and electroporation (Bright *et al*., 1996). A recent promising development in this area has been the use of cationic liposomes, a standard tool for DNA transfection, to load the F-actin probe BODIPY FL phallacidin into live neuronal cells (Barber *et al*., 1996). Single-cell loading procedures, such as microinjection and patch pipette perfusion, are primarily applicable to fluorescence imaging applications in which the experimental sample comprises only a few cells. Detailed comparisons of loading techniques have been published elsewhere (McNeil, 1989; Lee *et al*., 1993).

Targeting

In analysing the design of fluorescent probes, it is useful to consider their molecular structure as comprised of two components – a fluorophore and a targeting group. This concept is illustrated in Fig. 1.

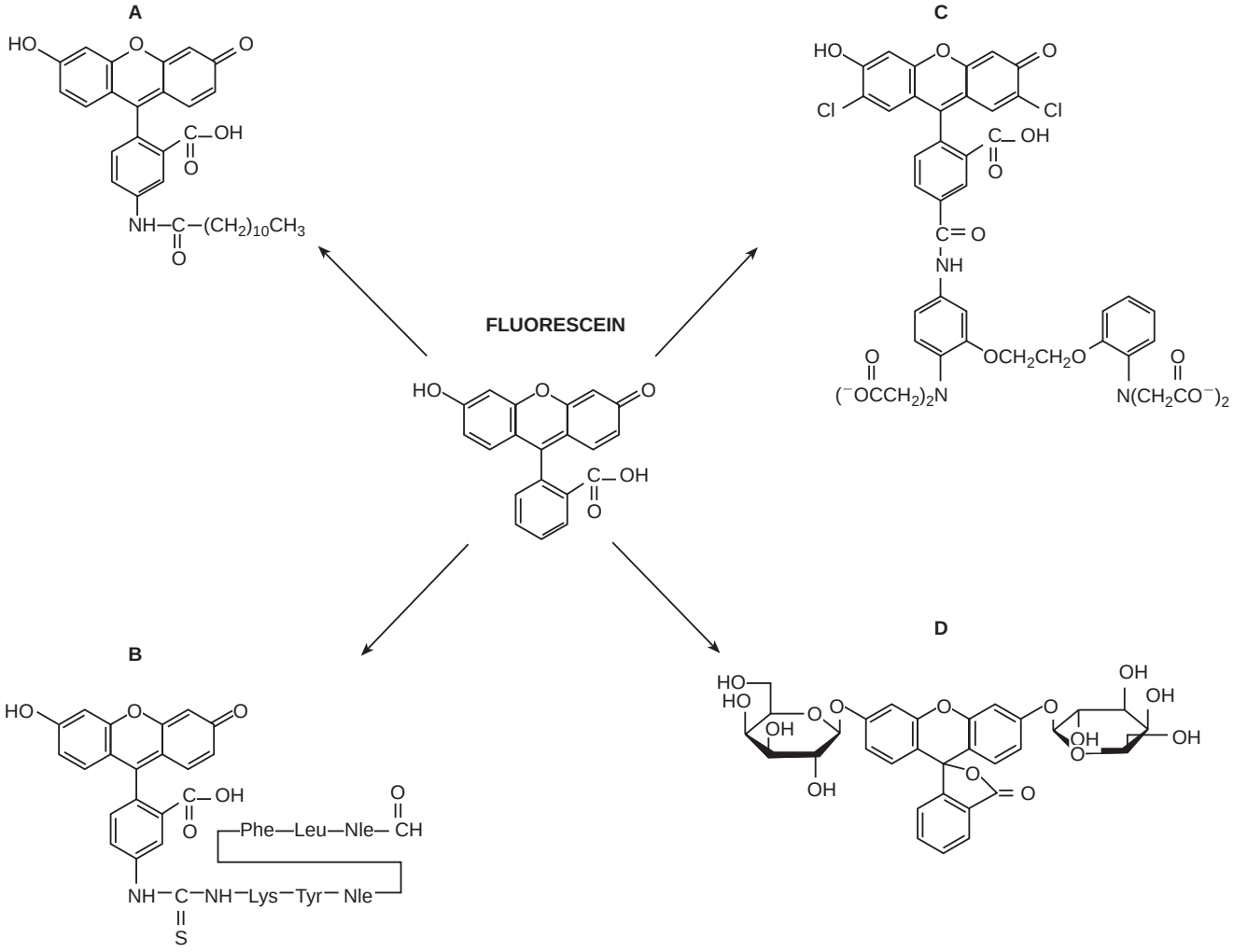

Fig. 1. Fluorescent probes derived from fluorescein. (A) 5-Dodecanoylaminofluorescein, a lipophilic membrane probe; (B) fluorescein coupled to the hexapeptide formyl-Nle-Leu-Phe-Nle-Tyr-Lys, a fluorescent agonist for the chemotactic peptide receptor of neutrophils; (C) Calcium Green-1, consisting of dichlorofluorescein coupled to the Ca^{2+} chelator BAPTA; (D) fluorescein di-β-D-galactopyranoside (FDG), a fluorogenic substrate for β-galactosidase.

Attaching fluorescent dyes to biological target molecules yields probes that duplicate the transport and metabolism of the native molecule, enabling the cellular distribution of the target molecule or its receptor to be mapped by fluorescence imaging. An extensive array of reactive fluorophores is available for probe synthesis (Table 1), usually via amine or thiol derivatization (Haugland, 1996a,b; Waggoner, 1995). Fluorescent lipid analogues and receptor ligands are examples of this approach, which has been termed fluorescent analogue cytochemistry (Wang, 1989). A second approach involves coupling targeting groups and fluorophores, so that interaction with a target ion, molecule or structure produces a specific change in the fluorescence intensity or spectra of the probe. These probes are highly selective environmental sensors and are typified by Ca^{2+} indicators and fluorogenic enzyme substrates (Fig. 1). However, it is not always practicable to synthesize a targeting group chemically for every species of biological interest. An alternative is to use proteins as transducers. The major practical challenges in the design of fluorescent protein biosensors are to attach the fluorophore(s) to the protein in such a way that (a) the modification does not significantly perturb the interaction with the target molecule or ion and (b) the interaction is transmitted to the fluorophore resulting in a measurable fluorescence change (Giuliano *et al*., 1995). Cyclic AMP (Adams *et al*., 1991), inorganic phosphate (Brune *et al*., 1994) and fatty acids (Richieri *et al*., 1992) are among the growing number of species for which protein-based fluorescent sensors have been developed (Giuliano *et al*., 1995). Limitations on the intracellular use of protein-based sensors on account of the need for microinjection seem likely to diminish with the advent of green fluorescent protein (GFP) expression techniques (see below). Fluorescent calcium indicators consisting of fusions of GFP with Ca^{2+}-binding proteins have been described recently (Miyawaki *et al*., 1997; Persechini *et al*., 1997).

Of course, the utility of fluorescent probes is not restricted to the detection of a target ion or molecule. If a permeability barrier impedes the interaction of a probe with its target, then the measured fluorescence will be determined by the

Table 1. Spectroscopic parameters of selected fluorophores

Fluorophore	*Applications*[a]	λ_A[b]	$\varepsilon \times 10^{-3}$[c]	λ_F[b]	$\lambda_F - \lambda_A$[d]	*QY*[e]	*Solvent*[f]	*Notes*
AMC	Enzyme substrates	351	18	430	79	0.75	Methanol	1
BODIPY FL	Reactive label, lipid analogues	505	91	511	6	0.94	Methanol	
BODIPY TR	Reactive label	588	68	616	28	0.84	Methanol	
Calcein	Cytoplasmic label	494	81	516	22	0.78	pH 9.0	2
Cy3	Reactive label	560	150	575	15	0.09	Ethanol	3
Cy5	Reactive label	658	250	677	19	0.40	Ethanol	3
DiI	Membrane label	555	148	569	14	0.20	Lipid	
Fluorescein	Reactive label, enzyme substrates	490	88	514	24	0.92	pH 9.0	4
FM 1–43	Membrane endocytosis sensor	479	40	598	119	0.30	Lipid	5
fura-2	Ca^{2+} indicator	335	34	505	170	0.49	pH 7.2 + Ca^{2+}	6
GFP (wild type)	Protein label	395	21	508	113	0.77	pH 7.4	7
GFP (S65T)	Protein label	489	39	511	22	0.66	pH 7.4	7
indo-1	Ca^{2+} indicator	330	33	401	71	0.56	pH 7.2 + Ca^{2+}	6
C_6-NBD-PC	Lipid analogue	475	20	540	65	0.31	Lipid	
Oregon Green 514	Reactive label	506	88	526	20	0.96	pH 9.0	2
Rhodamine 110	Enzyme substrates	496	80	520	24	0.89	pH 7.0	
Carboxy SNARF-1	pH indicator	576	48	635	59	0.09	pH 10.0	4
Texas Red	Reactive label	586	108	605	19	0.77	pH 7.0	
TMR	Reactive label	540	95	565	25	0.68	Methanol	8

[a]This is not an exhaustive list and refers mainly to applications discussed in this article. [b]λ_A = absorption maximum (in nm); λ_F = fluorescence emission maximum (in nm). [c]Molar extinction coefficient; multiply listed values by 1000 to convert to units of $cm^{-1}\,M^{-1}$. [d]Fluorescence Stokes shift (nm). [e]Fluorescence quantum yield; values listed are for the free fluorophore in the solvent specified and often change substantially in other environments, e.g. upon coupling to proteins. [f]Spectroscopic parameters are independent of pH in the physiological range, unless otherwise specified.

Notes

1. AMC = 7-amino-4-methylcoumarin.
2. Parameters are not significantly pH dependent at pH > 5.
3. *Bioconjug. Chem*. **4**, 105–11 (1993).
4. Parameters are pH dependent in the pH 5–9 range.
5. ε and QY estimated based on data reported in *Biochemistry* **24**, 5749–55 (1985).
6. Parameters are dependent on intracellular Ca^{2+} concentration.
7. *Curr. Biol*. **6**, 178–82 (1996).
8. TMR = tetramethylrhodamine.

integrity of the barrier as well as by the abundance of the target. Thus, fluorescent nucleic acid stains, which, with some notable exceptions (Knowles *et al*., 1996), are not membrane permeant, can be used as indicators of membrane integrity and, therefore, cell viability (Dive *et al*., 1990).

Detectability

Optimal detection of fluorescence involves several factors relating to the probe, the sample and the instrument being used.

Loading. Attempts to increase fluorescence signal levels by raising the intracellular probe concentration are often counterproductive. Perturbation of the physiological system being studied and cytotoxic effects of the probe or its degradation products generally dictate that the intracellular probe concentration be as low as possible. It is usually more effective to focus on maintaining the probe concentration after loading by minimizing leakage (see Fig. 8).

Extinction coefficient and fluorescence quantum yield. The efficiency with which a fluorophore absorbs and emits photons is represented by the molar extinction coefficient (ε) for absorption and the quantum yield (QY) for fluorescence. Fluorescence intensity per fluorophore is proportional to the product of ε and QY. The range of these parameters among fluorophores of current practical importance is approximately 10 000 to 200 000 $cm^{-1}\,M^{-1}$ for ε and 0.05 to 1.0 for QY (Table 1). Fluorescence quantum yields are often very sensitive to environmental factors; extinction coefficients typically are not.

Photobleaching. Under the high-intensity illumination conditions used for fluorescence microscopy, the irreversible destruction of the excited fluorophore (photobleaching) often becomes the factor limiting fluorescence detectability. Recent investigations have provided a detailed description of the multiple photochemical reaction pathways responsible for photobleaching of fluorescein (Song *et al*., 1995, 1996). Some pathways involve reactions between adjacent dye molecules, making the process considerably more complex in labelled biological specimens than in dilute solutions of free dye. Although effective 'antifade' reagents are available, they are usually incompatible with living cells; an exception is the water-soluble vitamin E analogue Trolox (Scheenen *et al*., 1996). Alternatively, a more intrinsically photostable probe may be used (Fig. 2).

Excitation wavelength. To maximize fluorescence signals, the absorption maximum of the probe should be matched closely to the output wavelength of the excitation source. Flow cytometers and laser-scanning microscopes commonly require probes that are excitable by the 488-nm line of the argon-ion laser. Excitation at longer wavelengths (>500 nm) typically results in lower levels of background autofluorescence (Beumer *et al*., 1995) and decreased photochemically induced damage to the cell (Bloom & Webb, 1984). Furthermore, at longer wavelengths, light scattering by dense media is much reduced, resulting in deeper penetration of excitation light into thick tissue samples.

Stokes shift. When the fluorescence Stokes shift (the wavelength separation of the absorption and fluorescence maxima) is small, it is difficult to maximize

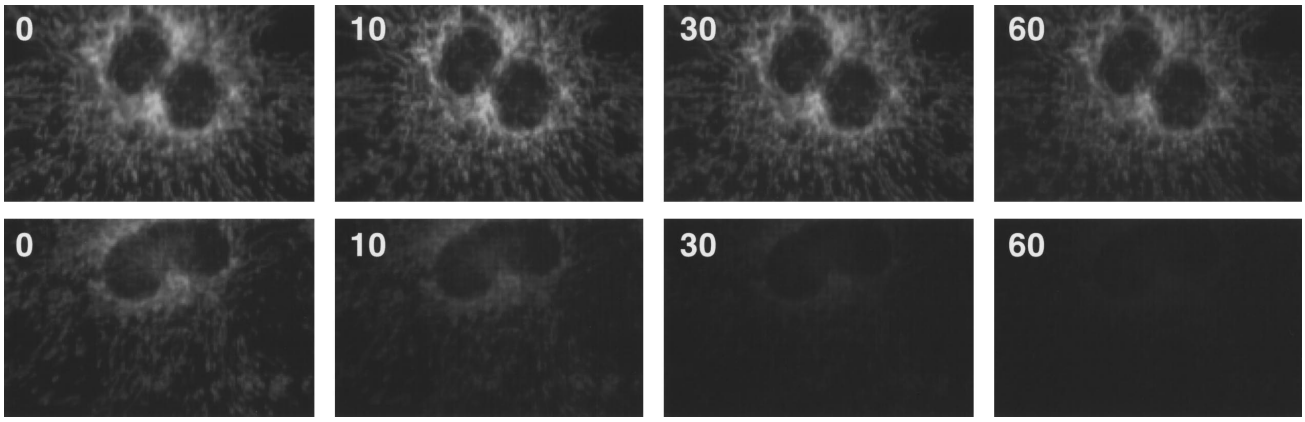

Fig. 2. Photostability comparison of mitochondrial staining by MitoFluor Green (upper series) and Rhodamine 123 (lower series). HeLa cells were stained with 100 nM MitoFluor Green or 500 nM Rhodamine 123 in growth medium for 20 min at 37°C. Cells were then rinsed and mounted in Hanks' balanced salt solution (HBSS) with 10% calf serum. Samples were continuously illuminated and viewed on a fluorescence microscope equipped with a fluorescein longpass filter set (Omega Optical), a Star 1 CCD camera (Photometrics) and Image-1 software (Universal Imaging Corp.). Images acquired 0, 10, 30 and 60 s after the start of illumination (as indicated in the top lefthand corner of each image) illustrate the superior image persistence produced by MitoFluor Green staining.

excitation and detection of the fluorophore while at the same time isolating the fluorescence emission signal from scattered excitation light. For multiparameter fluorescence applications, in which two or more physiological parameters are monitored simultaneously (Chacon *et al.*, 1994), an ideal combination of probes would have strong absorption at a coincident excitation wavelength and well-separated emission spectra. Unfortunately, it is not easy to find fluorophores with the requisite combination of a large extinction coefficient (ε) and a large Stokes shift (Haugland, 1990).

Fluorescence response

In some cases, a lack of fluorescence sensitivity to environmental factors is advantageous, making the representation of the intracellular distribution of a probe obtained from fluorescence imaging relatively unbiased. However, it is more often the case that environmental sensitivity can be exploited to generate a change of fluorescence intensity or wavelength in response to the interaction of the probe with its target. Some examples are given below.

(A) The binding of Ca^{2+} to the BAPTA targeting group of fura-2, Calcium Green-1 (Fig. 1) and other calcium indicators reconfigures the nitrogen lone pair electrons, resulting in spectral shifts and increased fluorescence quantum yields (Fig. 3).

(B) Membrane probes, such as FM 1-43 (see below), are often effectively non-fluorescent in aqueous solution, resulting in high intrinsic sensitivity, because the fluorescence background resulting from unbound probe is very low. Fluorogenic enzyme substrates, which are non-fluorescent until they are converted to fluorescent products by enzymatic cleavage, are advantageous for the same reason.

(C) Fluorophores, such as fluorescein and 7-hydroxycoumarin that have strongly pH-dependent absorption and fluorescence characteristics, can be adapted for use as physiological pH indicators. Furthermore, derivatization of the 3′- and 6′-hydroxyl groups causes fluorescein to adopt the non-fluorescent lactone configuration that is also prevalent at low pH, yielding fluorogenic substrates for β-galactosidase (FDG; Fig. 1), alkaline phosphatase and other enzymes (see below).

(D) Fluorescence resonance energy transfer (FRET) is a strongly distance-dependent electronic interaction in which emission of one fluorophore is coupled to the excitation of another. FRET can be exploited for monitoring a wide array of molecular assembly or fragmentation processes, such as membrane fusion (Uster, 1993), nucleic acid hybridization (Sixou *et al.*, 1994) and protein complexation (Bastiaens and Jovin, 1996), and has been used to generate the fluorescence response of sensors for

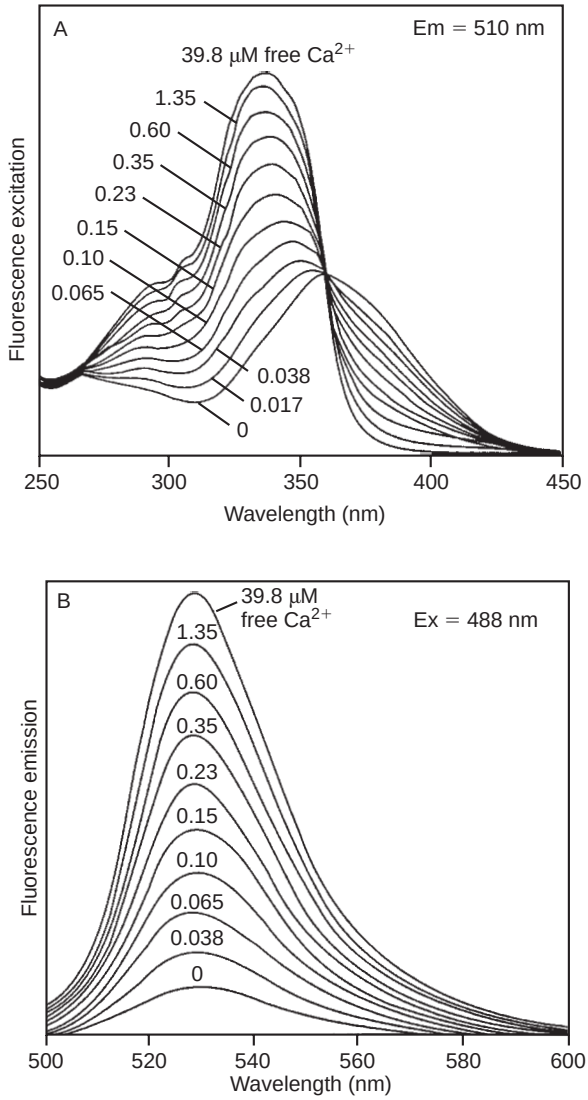

Fig. 3. (A) The Ca^{2+}-dependent fluorescence excitation spectral shift of fura-2 (emission detected at 510 nm). (B) The Ca^{2+}-dependent fluorescence quantum yield increase of Calcium Green-1 (excited at 488 nm) is not accompanied by a spectral shift.

Zn^{2+} (Godwin & Berg, 1996), cyclic AMP (Adams *et al.*, 1991) and membrane potential (González & Tsien, 1995).

Live cell probes

PROBES FOR INTRACELLULAR IONS

Ca^{2+} indicators

Fluorescent calcium indicators represent the largest single application of fluorescence detection techniques in living cells. The development of these indicators by Roger Tsien and colleagues in the early 1980s involved three major technical accomplishments: (1) the synthesis of the chelating group

BAPTA with Ca^{2+} binding affinity matching typical intracellular concentrations, high selectivity for Ca^{2+} over Mg^{2+} and low pH sensitivity (Tsien, 1980); (2) the use of cell-permeant acetoxymethyl ester derivatives of the anionic indicators for the bulk loading of cells (Fig. 4); and (3) the fusion of the BAPTA chelator to a fluorophore producing a fluorescence spectral shift in response to ion binding without substantially diminishing the binding affinity (Grynkiewicz *et al*., 1985). The Ca^{2+} response of spectrally sensitive indicators can be calibrated in terms of a ratio of fluorescence intensities measured at two different wavelengths, resulting in the cancellation of artifactual fluorescence signal variations that might otherwise be misinterpreted as changes in Ca^{2+} concentration. Sources of these artifacts include photobleaching, leakage of the intracellular indicator, variable cell thickness and non-uniform indicator distribution within cells. Unfortunately, indicators with long-wavelength excitation and emission characteristics suitable for confocal microscopy and flow cytometry do not usually exhibit spectral shifts upon binding Ca^{2+} (Fig. 3). Simultaneous use of indicator pairs has provided a solution to this problem in some cases (Lipp & Niggli, 1993; Novak & Rabinovitch, 1994). More recent developments in intracellular Ca^{2+} indicators have included conjugation of indicators to dextran carriers, reducing problems of leakage and compartmentalization associated with acetoxymethyl ester loading (Read *et al*., 1992) and targeting of indicators to particular intracellular locations, such as the plasma membrane (Lloyd *et al*. 1995; Etter *et al*., 1996), mitochondria (Hajnóczky *et al*., 1995) or the nucleus (Allbritton *et al*., 1994).

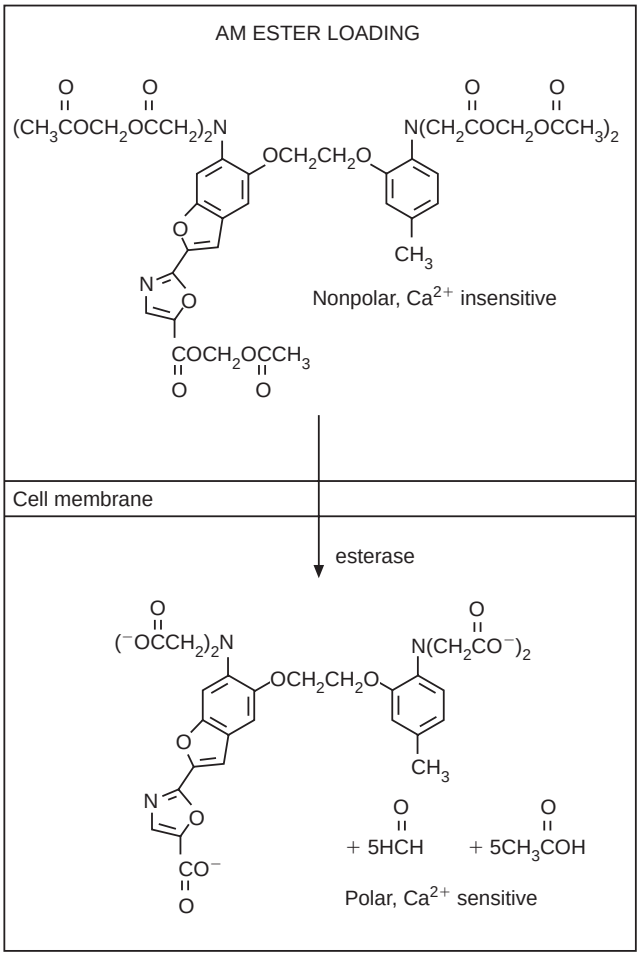

Fig. 4. Schematic diagram of the processes involved in loading cells using membrane-permeant acetoxymethyl ester derivatives of polar fluorescent indicators, exemplified by fura-2. Note the generation of potentially toxic byproducts (formaldehyde and acetic acid).

Selection of a fluorescent calcium indicator for a particular application involves the consideration of three main criteria:

1. The form of indicator (acid, acetoxymethyl ester or dextran). This choice is dictated by the method of cell loading and the requirements for intracellular distribution and retention.
2. The measurement mode. This choice is dictated primarily by whether quantitative (ratiometric indicators preferred) or qualitative ion concentration data is required. Excitation and emission wavelength preferences depend on the type of instrumentation being used, requirements for the avoidance of autofluorescence and the spectral properties of any other probes being used simultaneously.
3. Dissociation constant (K_d). This choice is dictated by the Ca^{2+} concentration range of interest. Indicators have a detectable response in the concentration range from approximately $0.1 \times K_d$ to $10 \times K_d$.

Variations of these criteria among currently available indicators are summarized in Table 2.

Indicators for other ions (H^+, Mg^{2+}, Na^+, K^+, Zn^{2+} and Cl^-)

The development of indicators for intracellular Mg^{2+}, Na^+ and K^+ has largely paralleled that of Ca^{2+} indicators. Indicators with excitation wavelengths in the ultraviolet range (mag-fura-2 also known as furaptra for Mg^{2+}, SBFI and PBFI for Na^+ and K^+ respectively) (Minta & Tsien, 1989; Raju *et al*., 1989) have been followed by indicators excited in the visible range (Magnesium Green and Sodium Green; Amorino & Fox, 1995; Van der Wolk *et al*., 1995). Magnesium indicators, such as mag-fura-2, are also useful as low-affinity Ca^{2+} indicators (Hofer & Machen, 1993; Tse *et al*., 1994; Table 2). Furthermore, many Ca^{2+} and Mg^{2+} indicators have higher binding affinities for ions such as Ni^{2+}, Zn^{2+} and Cd^{2+} than

Table 2. Properties and forms of commercially available fluorescent Ca^{2+} indicators

	ACID[a]	*AM*[b]	*DEX*[c]	*Mode*[d]	K_d (nM)[e]	*Notes*
fura-2	✔	✔(+)	✔	EX 340/380	145	
bis-fura-2	✔			EX 340/380	370	1
fura-PE3	✔	✔(+)		EX 340/380	270	2
indo-1	✔	✔(+)	✔	EM 405/485	230	
quin-2	✔	✔(+)		EM 495	60	
BTC	✔	✔(+)		EX 400/480	7 000	3
mag-fura-2	✔	✔(+)		EX 340/380	25 000	
mag-fura-5	✔	✔(+)		EX 340/380	28 000	
mag-indo-1	✔	✔(+)	✔	EX 405/485	35 000	
fluo-3	✔	✔(−)		EM 525	390	4
Calcium Green-1	✔	✔(−)	✔	EM 530	190	4
Calcium Green-2	✔	✔(−)		EM 535	550	
Calcium Green-5N	✔	✔(−)		EM 530	14 000	
Oregon Green 488 BAPTA-1	✔	✔(−)	✔	EM 520	170	5
Oregon Green 488 BAPTA-2	✔	✔(−)		EM 520	580	5
Oregon Green 488 BAPTA-5N	✔	✔(−)		EM 520	20 000	5
Magnesium Green	✔	✔(−)		EM 530	6 000	
rhod-2	✔	✔(+)		EM 570	570	
Calcium Orange	✔	✔(+)		EM 575	185	
Calcium Orange-5N	✔	✔(+)		EM 580	20 000	
Calcium Crimson	✔	✔(+)	✔	EM 615	185	
Fura Red	✔	✔(+)		EX 420/480	140	6, 7
Texas Red + Calcium Green			✔	EM 535/615	370	8

[a]Available in cell-impermeant acid form. [b]Available in cell-permeant acetoxymethyl ester form. Acetoxymethyl esters marked (+) are fluorescent; those marked (−) are non-fluorescent (fluorescence of acetoxymethyl esters is a major source of error in Ca^{2+} measurements). [c]Available as dextran conjugate. [d]Measurement wavelengths (in nm). EX, fluorescence excitation; EM, fluorescence emission. Indicators for which a pair of wavelengths are listed have dual-wavelength ratio measurement capability. [e]Ca^{2+} dissociation constant. Units: nanomolar (10^{-9}M). Generally measured *in vitro* at 22°C in 100 mM KCl, 10 mM MOPS, pH 7.2. Values depend on temperature, ionic strength, pH and other factors and are usually higher *in vivo*.

Notes

1. Similar Ca^{2+}-dependent fluorescence response to fura-2 but 75% greater molar absorptivity.
2. Zwitterionic analogue of fura-2 that is reported to exhibit less leakage and compartmentalization [*Biophys. J.* **69**, 2112–24 (1995)].
3. See [*Cell Calcium* **15**, 190–8 (1994)].
4. Calcium Green-1 is more fluorescent than fluo-3 in both Ca^{2+}-bound and Ca^{2+}-free forms. Magnitude of Ca^{2+}-dependent fluorescence increase is greater for fluo-3.
5. Molar absorptivity at 488 nm is approximately twice that of the corresponding Calcium Green indicator.
6. Can also be used in combination with fluo-3 for dual-wavelength ratio measurements; EX = 488 nm, EM = 530/670 nm [*Cell Calcium* **14**, 359–72 (1993); *Cytometry* **17**, 135–41 (1994)].
7. Mag-Fura Red has similar spectral properties with K_d (Ca^{2+}) = 17 000 nM.
8. This indicator consists of Ca^{2+}-sensitive Calcium Green-1 and Ca^{2+}-insensitive Texas Red linked to the same dextran.

for their primary target ions; fortunately, the normal intracellular concentrations of transition metal ions are usually too low to cause distortions in Ca^{2+} and Mg^{2+} measurements. On the other hand, elevated intracellular concentrations of Ni^{2+}, Zn^{2+}, Cd^{2+} and other related cations resulting from channel mediated influx can be readily detected using indicators such as fura-2 and mag-fura-2 (Shibuya & Douglas, 1992; Simons, 1993; Atar *et al*., 1995). A protein-based Zn^{2+} sensor has been developed recently that exploits the conformational change induced by Zn^{2+} binding to a zinc finger peptide to produce increased fluorescence energy transfer from a fluorescein label to a rhodamine label attached on the other side of the metal binding site (Godwin & Berg, 1996). For measuring intracellular chloride, a series of methoxyquinolinium dyes have been developed by Alan Verkman and colleagues (Verkman, 1990). One such indicator (MEQ) can be converted to a transiently cell-permeant form by *in situ* reduction (Biwersi & Verkman, 1991) and has been used in conjunction with BCECF (see below) to investigate the mechanism of intracellular pH control by plasma membrane anion exchangers (Sekler *et al*., 1996).

The major intracellular pH indicators in current use are 8-hydroxypyrene-1,3,8-trisulphonic acid (HPTS, also known as pyranine) (Wolfbeis *et al*., 1983), BCECF (Rink *et al*., 1982) and carboxy SNARF-1 (Whitaker *et al*., 1991). All three indicators exhibit pH-dependent spectral shifts in the normal cytosolic pH range (~6.8–7.4) and can, therefore, be used for dual-wavelength ratio measurements (Owen, 1992; Overly *et al*., 1995) Unlike carboxy SNARF-1 and BCECF, HPTS cannot be prepared as a cell-permeant acetoxymethyl or acetate ester precursor for bulk loading. Intracellular pH changes

detected by fluorescent indicators and microelectrodes in side-by-side measurements have been found to be very similar, although absolute values may differ by about 0.1 pH units (Nett & Deitmer, 1996). Simultaneous measurements of intracellular pH and Ca^{2+} can be made using appropriately chosen indicators pairs, for instance carboxy SNARF-1 and fura-2 (Martínez-Zaguilán *et al*., 1996).

MEMBRANE POTENTIAL PROBES

Optical probes used in conjunction with imaging techniques allow variations of membrane potential to be mapped along neurons, within tissues or among cell populations. Probes can be divided into two categories based on their response mechanism. Fast-response probes operate via a direct effect of the surrounding electric field on the electronic structure (and, consequently, the fluorescence) of the fluorophore and are, therefore, capable of detecting millisecond transient potential changes in excitable cells (Loew, 1996). Slow-response probes operate via potential-dependent changes in their transmembrane distribution (a molecular level response as opposed to electronic level for fast probes) and are suitable for detecting changes in average membrane potentials of non-excitable cells caused by respiratory activity, ion channel permeability, drugs and other factors.

The magnitude of the potential-dependent fluorescence change generated by fast potentiometric dyes is usually small; ~2–10% per 100 mV is considered to be a good response. A novel technique based on fluorescence resonance energy transfer between a potential-sensitive oxonol dye in the membrane interior and a surface-bound fluorescent lectin has produced millisecond fluorescence signal changes as high as 34% per 100 mV (González & Tsien, 1995). The ANEP (aminonaphthethenylpyridinium) dyes, developed by Leslie Loew and colleagues (Fluhler *et al*., 1985) exhibit consistently sensitive responses in a variety of tissue, cell and model membrane systems (Loew *et al*., 1992). ANEP dyes respond to alterations in membrane potential with opposite changes in fluorescence excited at approximately 440 and 505 nm (Montana *et al*., 1989; Loew *et al*., 1992), permitting the use of ratiometric methods to correlate the change in fluorescence signal with membrane potential. Using these methods, Loew and colleagues have been able to follow changes in membrane potential along the surface of a single mouse neuroblastoma cell (Bedlack *et al*., 1992) and to define differences between transmembrane potentials of neurites and somata (Bedlack *et al*., 1994). More recently, highly water-soluble ANEP dyes designed for microinjection have been developed to provide intensified voltage-sensitive signals from remote neuronal processes (Antic & Zecevic, 1995).

The methyl and ethyl esters of tetramethylrhodamine (TMRM and TMRE) are currently the preferred dyes for the determination of membrane potential by quantitative imaging (Gross & Loew, 1989; Loew, 1993). Determination of membrane potential by the application of the Nernst equilibrium requires that the transmembrane distribution of the dye depends only on the membrane potential and that other processes, such as dye aggregation and potential-independent interactions with intracellular components, contribute minimally. TMRM and TMRE fulfill these requirements in several respects (Ehrenberg *et al*., 1988; Farkas *et al*., 1989). They are readily membrane permeant, and their strong fluorescence means that they can be used at low concentrations, thus avoiding aggregation. As their fluorescence is relatively insensitive to environment, spatially resolved fluorescence of TMRM and TMRE presents an unbiased profile of their transmembrane distribution that can be related directly to the membrane potential via the Nernst equation (Loew, 1993, 1998).

The large internally negative membrane potential of mitochondria is the basis for the selective staining of this organelle by a variety of cationic probes, including rhodamine 123 (Chen, 1989) and CMXRos (Poot *et al*., 1996). Other cationic probes, including nonyl Acridine Orange and MitoFluor Green (Fig. 2), stain mitochondria via a potential-independent mechanism, possibly involving interaction with the anionic phospholipid cardiolipin, which is abundant in the inner mitochondrial membrane (Rashid & Horobin, 1990).

PROBES FOR PROTEINS AND ENZYMES

Green fluorescent protein

The green fluorescent protein (GFP) expression system is perhaps the most important recent development in fluorescent labelling technology. This 238 amino acid protein from the jellyfish *Aequorea victoria* incorporates an intrinsic fluorophore formed post-translationally by spontaneous cyclization of a Ser-Tyr-Gly sequence (residues 65–67) (Prasher *et al*., 1992; Heim *et al*., 1994). The crystal structure of the protein (Ormö *et al*., 1996) shows the fluorophore located in the centre of an 11-stranded β-barrel that completely shields it from the surrounding solvent. Heterologous expression of the cDNA for GFP provides the basis for its utilization as a powerful non-invasive labelling tool, whereby labelled proteins can be generated *in situ* within living cells (Chalfie *et al*., 1994). GFP expression has been demonstrated in a wide variety of cell types and organisms, including mammalian cells, plants, yeast, bacteria, nematodes, zebrafish embryos, mosquitoes and fruit flies. Primary applications are the

detection of gene expression (Galbraith *et al*., 1995; Anderson *et al*., 1996) and the imaging of intracellular protein localization and transport (Olson *et al*., 1995; Stauber *et al*., 1995; Htun *et al*., 1996; Moores *et al*., 1996). Co-localization of fluorescence from GFP fusion proteins and organelle-selective probes provides a powerful method for detecting the targeted translocation of proteins (Wang *et al*., 1996).

As implied by its name, the fluorescence of native GFP has an emission maximum at 508 nm; the excitation maximum is at 395 nm, although a weaker long-wavelength band (476 nm) allows excitation by argon-ion laser sources at 488 nm (Heim *et al*., 1994; Fig. 5). Site-directed mutagenesis of the protein has produced a number of GFP variants with modified spectral properties (Heim *et al*., 1994; Heim & Tsien, 1996). For example, replacement of serine-65 by threonine (S65T) shifts the excitation maximum to 489 nm, yielding improved detection sensitivity in flow cytometry using 488 nm excitation (Heim *et al*., 1995; Ropp *et al*., 1995; Fig. 5). Furthermore, some GFP mutants exhibit higher expression levels than the native protein, also resulting in greater detection sensitivity (Reichel *et al*., 1996). The combined use of spectrally distinct GFP mutants allows the simultaneous localization of two or more proteins in imaging applications (Rizzuto *et al*., 1996) and the detection of expression from two different genes within the same cell by flow cytometry (Anderson *et al*., 1996). Of course, the elements required to construct GFP fusion proteins are not always accessible, and the alternative approach of chemical derivatization of purified proteins with reactive fluorescent labels (Table 1) followed by microinjection of the resulting conjugate remains valuable (Wang, 1989). For example, fluorescent labelling of protein kinase C in conjunction with new quantitative imaging techniques based on fluorescence energy transfer has enabled observation of PKC translocation in response to phorbol ester stimulation to be observed in living fibroblasts (Bastiaens & Jovin, 1996).

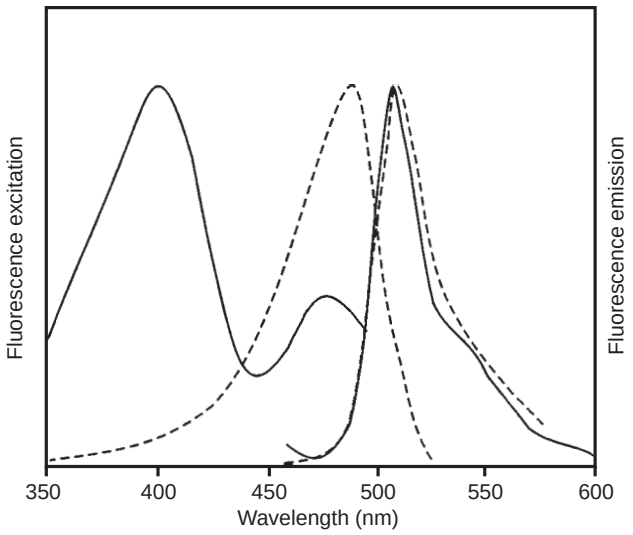

Fig. 5. Normalized fluorescence excitation and emission spectra of wild-type (——) and S65T mutant (– – –) green fluorescent proteins (adapted from published data with permission).

Fluorogenic enzyme substrates. Fluorogenic substrates, probes that are converted from a non-fluorescent to a fluorescent form by the action of an enzyme, have been developed primarily for hydrolytic enzymes, such as glycosidases, peptidases and phosphatases, and oxidative enzymes, such as peroxidases and microsomal dealkylases (Haugland & Johnson, 1993; Haugland, 1996c). The primary applications of these substrates are for the quantitative identification of enzyme activity on a cell-by-cell basis, usually by flow cytometry. Localization of activity within or on the surfaces of cells has been precluded by the fact that most substrates generate diffusible products. Substrates that generate non-diffusible fluorescent products have been developed more recently but have so far been used primarily for the analysis of fixed cells and tissue sections (Larison *et al*., 1995; Chao *et al*., 1996; Pecorino, *et al*., 1996). The following discussion presents three selected examples of enzymes that can be detected in living cells using fluorogenic substrates.

β-Galactosidase. Fluorescein di-β-D-galactopyranoside (FDG; Fig. 6) was originally described in 1963 (Rotman *et al*., 1963) and subsequently developed for detection of the *Escherichia coli lacZ* reporter gene in transformed cells by flow cytometry (Nolan *et al*., 1988; Roederer *et al*., 1991). The *gus* gene, in conjunction with FDGlcU, a β-glucuronidase substrate analogous to FDG, can be used as an alternative reporter in cell types that are unable to express *lacZ* (Lorincz *et al*., 1996). This method illustrates some practical problems with the application of fluorogenic substrates in living cells:

1. The substrate is not cell permeant and must be loaded by transient hypotonic shock permeabilization.
2. The amount of substrate loaded is finite and cannot readily be replenished. In cells with high enzyme activity levels, measurements must either be made very soon after loading or in the presence of a competitive inhibitor to prevent the fluorescence intensity from being attenuated because of substrate depletion.
3. Analysis must be carried out at 4°C to minimize efflux of the fluorescent product (fluorescein).

Structural modifications to FDG have been made by

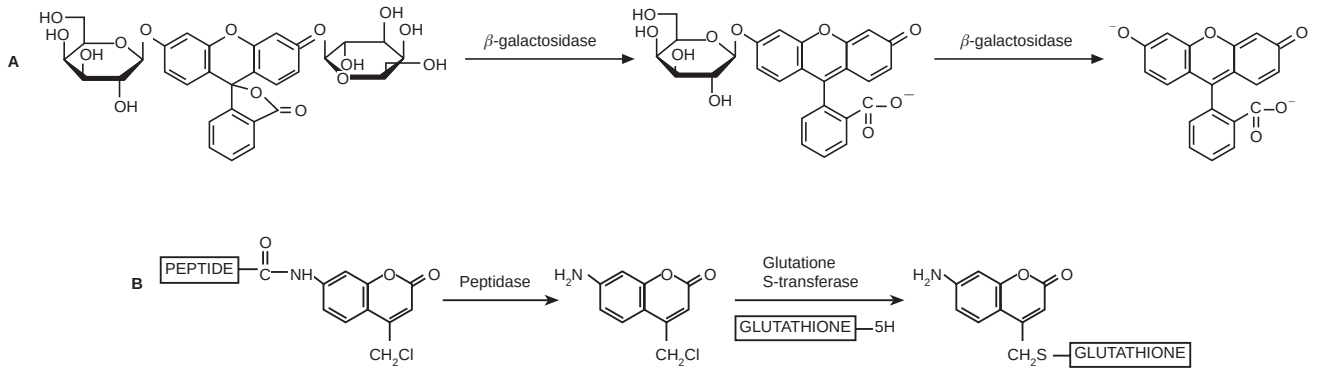

Fig. 6. (A) Conversion of non-fluorescent fluorescein di-β-D-galactopyranoside (FDG) to highly fluorescent fluorescein by sequential β-galactosidase cleavages. (B) Peptidase cleavage of a weakly fluorescent amide derivative of 7-amino-4-chloromethylcoumarin followed by glutathione *S*-transferase-mediated coupling of the highly fluorescent product to intracellular glutathione.

researchers at Molecular Probes, Inc., adding lipophilic or thiol-reactive chloromethyl substituents (see Fig. 6) to provide improved loading and product retention. (Zhang *et al*., 1991; Plovins *et al*., 1994; Brustugun *et al*., 1995).

Aminopeptidases. Fluorogenic substrates for aminopeptidases can be prepared by coupling the carboxy terminus of an enzyme-specific peptide sequence to the amine groups of 7-amino-4-methylcoumarin (AMC) or rhodamine 110 (R110). These substrates do not require invasive loading procedures and exhibit little toxicity in several mammalian cell types. R110-based substrates are analogous to fluorescein-based glycosidase substrates (Fig. 6), in that they require two successive cleavages to release the final fluorescent product, which can be excited at 488 nm for analysis by flow cytometry (Klingel *et al*., 1994; Ganesh *et al*., 1995), As with FDG, derivatization of the substrate with a thiol-reactive chloromethyl group improves the cellular retention of the fluorescent product (Fig. 6) (Rosser *et al*., 1993).

Probes for oxidative activity. Dichlorodihydrofluorescein diacetate (H_2DCFDA) is a freely membrane-permeant probe that is converted to dichlorodihydrofluorescein (H_2DCF) by cytoplasmic esterases. The fluorescence increase resulting from oxidation of H_2DCF to fluorescein can be used to detect the enzymatic generation of reactive oxygen species in processes such as the oxidative burst response to the stimulation of neutrophils (Bass *et al*., 1983; Robinson *et al*., 1994). However, the sensitivity and response time of this technique is impaired by the location of the probe in the cytoplasm rather than at the site of oxidative activity in the phagocytic vacuole. Coupling H_2DCF to an immune complex overcomes this problem, producing a probe targeted to the phagocytic vacuole via Fc receptor-mediated internalization (Ryan *et al*., 1990). Furthermore, cellular processing of the H_2DCF-labelled immune complex (FcOxyBURST Green; Molecular Probes, Inc.) does not involve the acetate cleavage step needed for the activation of H_2DCFDA, providing a further advantage in cells, such as monocytes, that have low levels of esterase activity.

PROBES FOR LIPIDS AND MEMBRANES

Fluorescent lipid probes include lipophilic tracers for long-term labelling of the plasma membrane, probes that are more peripherally membrane associated for intracellular labelling and detection of recycling processes, and analogues of endogenous lipids for investigating trafficking and metabolism (Fig. 7). The highly lipophilic carbocyanine dye DiI is the pre-eminent probe for plasma membrane labelling (Honig, 1993). The properties of DiI that give rise to its widespread utility include low cytotoxicity, lack of physiological perturbation, fidelity of labelling (minimal transfer from labelled to unlabelled cells) and strong, photostable fluorescence that is relatively insensitive to environment. Applications include neuroanatomical tracing (Honig & Hume, 1989; Honig, 1993), cell lineage tracing (Fraser, 1996), transplant labelling and detection of lipoprotein endocytosis, cell–cell fusion and adhesion. Microinjection of a concentrated solution of DiI in soybean oil produces selective labelling of the endoplasmic reticulum in living sea urchin eggs (Terasaki & Jaffe, 1991). Unlike DiI, the moderately lipophilic carbocyanine dye $DiOC_6(3)$ is readily internalized and can be used to label the endoplasmic reticulum by incubation in an extracellular staining solution (Terasaki, 1989; Terasaki & Reese, 1992).

Phospholipid, sphingolipid and fatty acid analogues incorporating the green fluorescent dyes BODIPY FL and NBD are versatile tools for dissecting pathways of lipid transport and metabo-

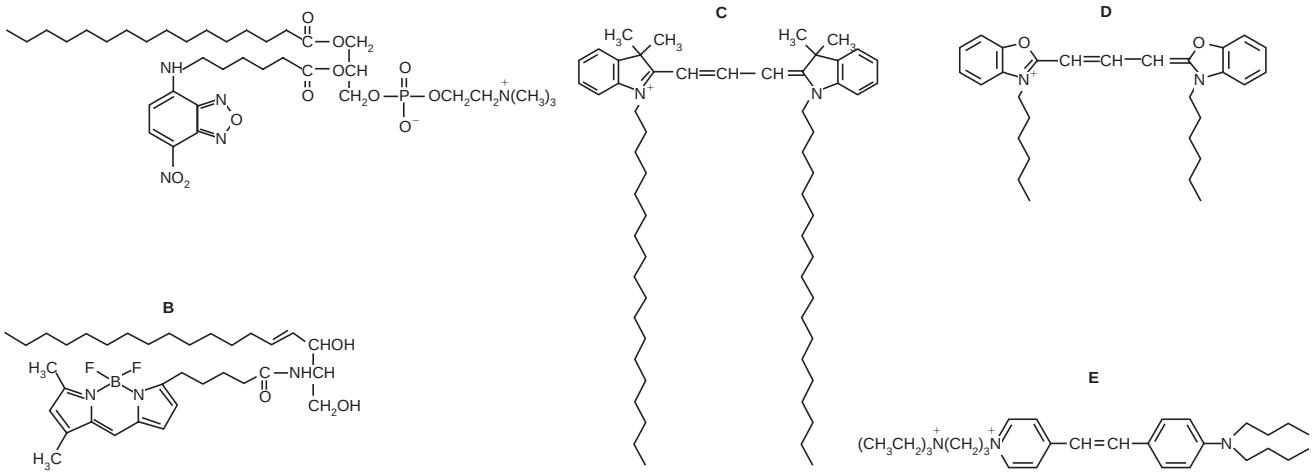

Fig. 7. Fluorescent lipid probes. (A) C_6-NBD-PC; (B) BODIPY FL C_5-ceramide; (C) DiI; (D) $DiOC_6(3)$; (E) FM 1-43.

lism using fluorescence imaging and biochemical analysis (Cornell-Bell *et al*., 1993; Kok & Hoekstra, 1993). The optical properties of the BODIPY FL fluorophore are generally superior to those of NBD (Table 1); in particular, it exhibits a fluorescence emission shift from ~520 nm to ~620 nm when it becomes highly concentrated. Consequently, cells labelled with BODIPY FL C_5-ceramide exhibit bright red staining of the Golgi apparatus, the primary site of metabolic accumulation (Pagano *et al*., 1991). The same technique has also been used recently to differentiate populations of endosomes based on the sorting of BODIPY FL C_5-sphingomyelin (Chen *et al*., 1997). Although NBD-labelled lipids exhibit some unusual biophysical characteristics (Chattopadhyay, 1990), in some cases, they are metabolized in a more native-like manner than their BODIPY FL-labelled counterparts (Paul *et al*., 1996).

The cationic styrylpyridinium dye FM 1-43 is highly soluble but non-fluorescent in water, becoming strongly fluorescent on binding to membrane surfaces. These characteristics have been very successfully exploited for activity-dependent staining of motor nerve terminals (Betz & Bewick, 1992; Betz *et al*., 1992). Trapping of the membrane-bound dye in synaptic vesicles during endocytosis produces staining; nerve stimulation after the removal of extracellular dye causes destaining as a result of exocytosis, releasing the water-soluble dye into the extracellular medium, in which it is non-fluorescent (Henkel *et al*., 1996). FM 1-43 has also proved to be highly effective for the detection of endocytosis and exocytosis in non-excitable cells (Bi *et al*., 1995; Whalley *et al*., 1995).

CYTOPLASMIC LABELS

The strategy of loading polar fluorescent indicators into cells in the form of membrane-permeant precursors that are subsequently activated by intracellular esterases (Fig. 4) can be used to produce stable cytoplasmic labelling, to the extent that the fluorescent product is retained by the cell (Fig. 8). Furthermore, enzymatic generation of a well-retained fluorescent product from a non-fluorescent precursor

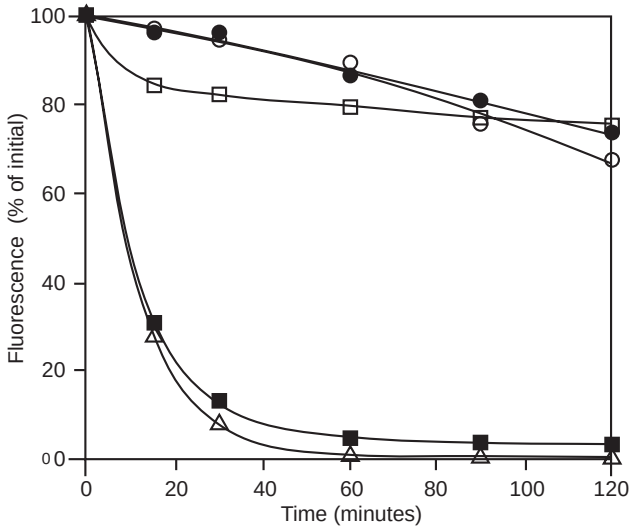

Fig. 8. Retention characteristics of cytoplasmic labelling probes. Cells of a human lymphoid line (GePa) were loaded with the following cell-permeant acetoxymethyl ester or acetate derivatives of fluorescein: calcein AM (●), BCECF AM (○), 5-chloromethylfluorescein diacetate (□), carboxyfluorescein diacetate (■) and fluorescein diacetate (△). After incubation in a 4 μM staining solution for 30 min at 37°C, cell samples were immediately analysed by flow cytometry to determine the initial average fluorescence per cell. Retained cell samples were subsequently washed twice by centrifugation, resuspended in medium, maintained at 37°C and then analysed by flow cytometry at various intervals during the next 2 h. The decrease in the average fluorescence intensity per cell in these samples relative to the initial samples indicates the extent of dye leakage.

is indicative of cell viability. Fluorescein diacetate is the prototype of a family of probes with these characteristics, the premier example of which is calcein AM (Fig. 9). As labelling is dependent on the membrane permeability of the acetoxymethyl ester or acetate precursor and on accessibility to intracellular esterases, it is not successful in all cell types (Kaneshiro *et al*., 1993). This technical problem does have advantageous aspects; certain cell types can be labelled selectively and differentiated from others by calcein AM labelling (Weston & Parish, 1992; Eddleman *et al*., 1995). Calcein fluorescence is not appreciably sensitive to cytoplasmic pH or other environmental factors, and labelling does not interfere with the functional properties of cells (De Clerck *et al*., 1994), leading to a wide assortment of applications:

- Cell viability. Intracellular calcein retention is sufficient to monitor cell-mediated cytotoxicity over periods of several hours (Fig. 8); labelling has no effect on killer cell–target cell conjugate formation (Callewaert *et al*., 1991; Lichtenfels *et al*., 1994).
- Chemotaxis. Unlike BCECF AM, labelling with calcein AM does not affect the migration of leukocytes in response to chemotactic stimuli (Denholm & Stankus, 1995).
- Cell–cell communication. Communication via gap junctions can be detected by calcein transfer from labelled to unlabelled cell populations (Tomasetto *et al*., 1993; Lampe, 1994).
- Cell volume. Because calcein is well-retained and homogeneously distributed, its concentration (and therefore fluorescence intensity) within the cytoplasm is inversely proportional to cell volume (Alvarez-Leefmans *et al*., 1995; Farinas *et al*., 1995).
- Multidrug resistance (MDR). The P-glycoprotein transporter responsible for the multidrug resistance of malignant cells extrudes non-fluorescent calcein AM but not its fluorescent hydrolysis product. Intracellular accumulation of calcein can, therefore, be used to measure P-glycoprotein expression and inhibition levels (Homolya *et al*., 1993; Holló *et al*., 1994).

For long-term tracing applications, such as tracking cell migration during development or after transplantation, intracellular retention of calcein is insufficient. For these applications, probes modified by the addition of thiol-reactive chloromethyl substituents that result in conjugation with glutathione and other intracellular peptides (Fig. 6B) have been developed. An example of this approach is 5-chloromethylfluorescein diacetate (CellTracker Green; Molecular Probes Inc.; Fig. 8; Knecht & Sheldon, 1995); other examples include fluorinated derivatives of fluorescein diacetate (Gee *et al*., 1996) and the amine-reactive carboxyfluorescein diacetate succinimidyl ester (Paramore *et al*., 1992). Other probes providing the stable retention and low cytotoxicity necessary in these applications are the plasma membrane label DiI (see above) and microinjected fluorescent dextran conjugates (Fraser, 1996).

Summary and prospectus

The objective of this article has been to illustrate the scope and versatility of fluorescent probe applications in living cells. Many important applications, notably probes for nucleic acids, channels and receptors and photoactivated 'caged' probes, have not been covered. For more detailed presentations of these topics, readers are referred to comprehensive surveys published elsewhere (Mason, 1993; Haugland, 1996d). However, among the extensive array of fluorescent probes that is now available, none has yet been developed that works in every cell type, every time. In particular, differences in membrane permeability, enzyme activity and intracellular compartmentalization have made it difficult

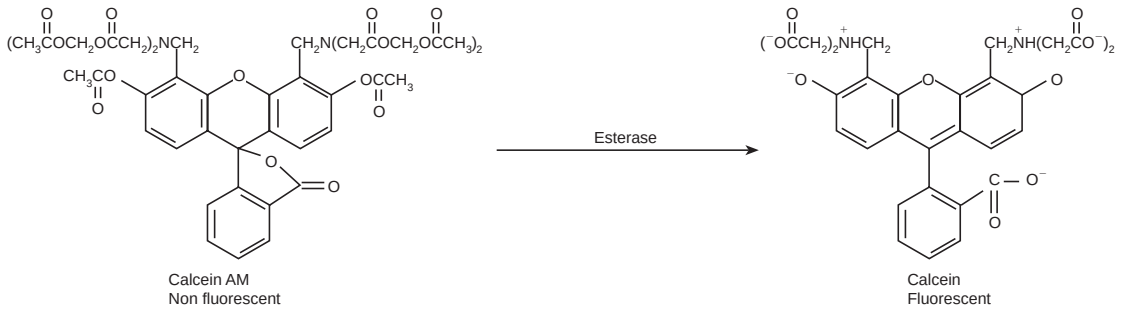

Fig. 9. Conversion of calcein AM to calcein by intracellular esterases. Note the non-polar structural characteristics of calcein AM, which result in membrane permeability, in contrast to the polar character of calcein.

to extend the applications of probes from mammalian cells to plant cells, bacteria and yeast. An appreciation as to how probes are designed and operate can help researchers to analyse and solve these technical problems, as well as stimulating the development of new probes and new applications.

New fluorescence imaging techniques currently under development, including two-photon excitation microscopy (Xu *et al*., 1996), fluorescence lifetime imaging microscopy (Wang *et al*., 1992) and optical near-field microscopy (Haydon *et al*., 1996), will certainly require adaptations to be made to existing probes or the design and synthesis of completely new ones. Probes for fluorescence detection of many key biological molecules, such as ATP, acetylcholine and glucose, and processes such as protein phosphorylation remain to be developed (Czarnik, 1995). Among other developments that can be anticipated are techniques for loading and detecting probes in thick tissue samples and for highly localized detection of ions and molecules within single cells, including the use of targeted probes and submicron-sized fibre optic sensors (Tan *et al*., 1992).

Acknowledgements

I would like to thank Dr Richard Haugland for creating the Molecular Probes bibliography database, the source of many of the papers cited in this article. Search access to this database is available via Molecular Probes' World Wide Web site (http://www.probes.com). Thanks also to the following for permission to reproduce data shown in figures: Dr Roger Tsien (Fig. 5), Dr Paul Millard and Janell Bishop-Stewart (Fig. 8) and Jennifer Krämer (Fig. 2).

References

ADAMS, S.R., HAROOTUNIAN, A.T., BUECHLER, Y.J., TAYLOR, S.S. & TSIEN, R.Y. (1991) Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* **349**, 694–7.

ALLBRITTON, N.L., OANCEA, E., KUHN, M.A. & MEYER, T. (1994) Source of nuclear calcium signals. *Proc. Natl. Acad. Sci. USA* **91**, 12458–62.

ALVAREZ-LEEFMANS, F.J., ALTAMIRANO, J. & CROWE, W.E. (1995) Use of ion-selective microelectrodes and fluorescent probes to measure cell volume. *Methods Neurosci*. **27**, 361–91.

AMORINO, G.P. & FOX, M.H. (1995) Intracellular Na^+ measurements using Sodium Green tetraacetate with flow cytometry. *Cytometry* **21**, 248–56.

ANDERSON, M.T., TJIOE, I.M., LORINCZ, M.C., PARKS, D.R., HERZENBERG, L.A., NOLAN, G.P. & HERZENBERG, L.A. (1996) Simultaneous fluorescence-activated cell sorter analysis of two distinct transcriptional elements within a single cell using engineered green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **93**, 8508–11.

ANTIC, S. & ZECEVIC, D. (1995) Optical signals from neurons with internally applied voltage sensitive dyes. *J. Neurosci*. **15**, 1392–405.

ATAR, D., BACKX, P.H., APPEL, M.M., GAO, W.D. & MARBAN, E. (1995) Excitation-transcription coupling mediated by zinc influx through voltage-dependent calcium channels. *J. Biol. Chem*. **270**, 2473–7.

BARBER, K., MALA, R.R. LAMBERT, M.P., QIU, R., MACDONALD, R.C. & KLEIN, W.L. (1996) Delivery of membrane-impermeant fluorescent probes into living neural cell populations by lipotransfer. *Neurosci. Lett*. **207**, 17–20.

BASS, D., PARCE, J.W., DECHATELET, L.R., SZEJDA, P., SEEDS, M.C. & THOMAS, M.J. (1983) Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol*. **130**, 1910–17.

BASTIAENS, P.I.H. & JOVIN, T.M. (1996) Microspectroscopic imaging tracks the intracellular processing of a signal transduction protein: fluorescent-labeled protein kinase C βI. *Proc. Natl. Acad. Sci. USA* **93**, 8407–12.

BEDLACK, R.S., WEI, M-D. & LOEW, L.M. (1992) Localized membrane depolarizations and localized calcium influx during electric field-guided neurite growth. *Neuron* **9**, 393–403.

BEDLACK, R.S., WEI, M-D., FOX, S.H., GROSS, E. & LOEW, L.M. (1994) Distinct electric potentials in soma and neurite membranes. *Neuron* **13**, 1187–93.

BETZ, W.J. & BEWICK, G.S. (1992) Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science* **255**, 200–3.

BETZ, W.J., MAO, F. & BEWICK, G.S. (1992) Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J. Neurosci*. **12**, 363–75.

BEUMER, T.L., VEENSTRA, G.J.C., HAGE, W.J. & DESTRÉE, O.H.J. (1995) Whole mount immunohistochemistry on *Xenopus* embryos using far-red fluorescent dyes. *Trends Genet* **11**, 9.

BI, G.-Q., ALDERTON, J.M. & STEINHARDT, R.A. (1995) Calcium-regulated exocytosis is required for cell membrane resealing. *J. Cell Biol*. **131**, 1747–58.

BIWERSI, J. & VERKMAN, A.S. (1991) Cell-permeable fluorescent indicator for cytosolic chloride. *Biochemistry* **30**, 7879–7883.

BLOOM, J.A. & WEBB, W.W. (1984) Photodamage to intact erythrocyte membranes at high laser intensities. *J. Histochem. Cytochem*. **32**, 608–16.

BRANDES, R. & BERS, D.M. (1996) Increased work in cardiac trabeculae causes decreased mitochondrial NADH fluorescence followed by slow recovery. *Biophys. J*. **71**, 1024–35.

BRIGHT, G.R., KUO, N.-T., CHOW, D., BURDEN, S., DOWE, C. & PRZYBYLSKI, R.J. (1996) Delivery of macromolecules into adherent cells via electroporation for use in fluorescence spectroscopic imaging and metabolic studies. *Cytometry* **24**, 226–33.

BRUNE, M., HUNTER, J.L., CORRIE, J.E.T. & WEBB, M.R. (1994) Direct, real-time measurement of rapid inor-

ganic phosphate release using a novel fluorescent probe and its application to actinomyosin subfragment 1 ATPase. *Biochemistry* **33,** 8262–71.

BRUSTUGUN, O.T., MELLGREN, G., GJERTSEN, B.T., BJERKVIG, R. & DØSKELAND, S.O. (1995) Sensitive and rapid detection of β-galactosidase expression in intact cells by microinjection of fluorescent substrate. *Exp. Cell. Res*. **219,** 372–8.

BUSH, D.S. & JONES, R.L. (1990) Measuring intracellular Ca^{2+} levels in plant cells using the fluorescent probes indo-1 and fura-2. *Plant Physiol*. **93,** 841–5.

CALLEWAERT, D.M., RADCLIFF, G., WAITE, R., LEFEVRE, J. & POULIK, M.D. (1991) Characterization of effector–target conjugates for cloned human natural killer and human lymphokine activated killer cells by flow cytometry. *Cytometry* **12,** 666–76.

CHACON, E., REECE, J.M., NIEMINEN, A.-L., ZAHREBELSKI, G., HERMAN, B. & LEMASTERS, J.J. (1994) Distribution of electrical potential, pH, free Ca^{2+} and volume inside cultured adult rabbit cardiac myocytes during chemical hypoxia: a multiparameter digitized confocal microscopic study. *Biophys. J*. **66,** 942–52.

CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W.W. & PRASHER, D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–5.

CHAO, J., DEBASIO, R., ZHU, Z., GIULIANO, K.A. & SCHMIDT, B.F. (1996) Immunofluorescence signal amplification by the enzyme-catalyzed deposition of a fluorescent reporter substrate (CARD) *Cytometry* **23,** 48–53.

CHATTOPADHYAY, A. (1990) Chemistry and biology of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled lipids: fluorescent probes of biological and model membranes. *Chem. Phys. Lipids* **53,** 1–15.

CHEN, C.-S., MARTIN, O.C. & PAGANO, R.E. (1997) Changes in the spectral properties of a plasma membrane lipid analog in the first seconds of endocytosis in living cells. *Biophys J*. **72,** 37–50.

CHEN, L.B. (1989) Fluorescent labeling of mitochondria. *Methods Cell Biol*. **29,** 103–23.

CORNELL-BELL, A.H., OTAKE, L.R., SADLER, K., THOMAS, P.G., LAWRENCE, S., OLSEN, K., GUMKOWSKI, F., PETERSON, J.R. & JAMIESON, J.D. (1993) Membrane glycolipid trafficking in living, polarized pancreatic acinar cells: assessment by confocal microscopy. *Methods Cell Biol*. **38,** 221–40.

CZARNIK, A.W. (1995) Desperately seeking sensors. *Chem. Biol*. **2,** 423–8.

DE CLERCK, L.S., BRIDTS, C.H., MERTENS, A.M., MOENS, M.M. & STEVENS, W.J. (1994) Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. *J. Immunol. Methods* **172,** 115–24.

DENHOLM, E.M. & STANKUS, G.P. (1995) Differential effects of two fluorescent probes on macrophage migration as assessed by manual and automated methods. *Cytometry* **19,** 366–9.

DIVE, C., WATSON, J.V. & WORKMAN, P. (1990) Multiparametric analysis of cell membrane permeability by two colour flow cytometry with complementary fluorescent probes. *Cytometry* **11,** 244–52.

EDDLEMAN, C.S., GODELL, C.M., FISHMAN, H.M., TYTELL, M. & BITTNER, G.D. (1995) Fluorescent labeling of the glial sheath of giant nerve fibers. *Biol. Bull*. **189,** 218–19.

EHRENBERG, B., MONTANA, V., WEI, M.-D., WUSKELL, J.P. & LOEW, L.M. (1988) Membrane potential can be determined in individual cells from the Nernstian distribution of cationic dyes. *Biophys. J*. **53,** 785–94.

ETTER, E.F., MINTA, A., POENIE, M. & FAY, F.S. (1996) Near-membrane [Ca^{2+}] transients resolved using the Ca^{2+} indicator FFP18. *Proc. Natl. Acad. Sci. USA* **93,** 5368–73.

FARINAS, J., SIMANEK, V. & VERKMAN, A.S. (1995) Cell volume measured by total internal reflection microfluorimetry: application to water and solute transport in cells transfected with water channel homologs. *Biophys. J*. **68,** 1613–20.

FARKAS, D.L., WEI, M.-D., FEBBRORIELLO, P., CARSON, J.H. & LOEW, L.M. (1989) Simultaneous imaging of cell and mitochondrial membrane potentials. *Biophys. J*. **56,** 1053–69.

FLUHLER, E., BURNHAM, V.G. & LOEW, L.M. (1985) Spectra, membrane binding and potentiometric responses of new charge shift probes. *Biochemistry* **24,** 5749–55.

FRASER, S.E. (1996) Iontophoretic dye labeling of embryonic cells. *Methods Cell Biol* **51,** 147–60.

GALBRAITH, D.W., LAMBERT, G.M, GREBENOK, R.J. & SHEEN, J. (1995) Flow cytometric analysis of transgene expression in higher plants: green fluorescent protein. *Methods Cell Biol*. **50,** 3–13.

GANESH, S., KLINGEL, S., KAHLE, H. & VALET, G. (1995) Flow cytometric determination of aminopeptidase activities in viable cells using fluorogenic rhodamine 110 substrates. *Cytometry* **20,** 334–40.

GEE, K.R., SUN, W.-C., KLAUBERT, D.H., HAUGLAND, R.P., UPSON, R.H., STEINBERG, T.H. & POOT, M. (1996) Novel derivatization of protein thiols with fluorinated fluoresceins. *Tetrahedron Lett*. **37,** 7905–7908.

GIULIANO, K.A., POST, P.L., HAHN, K.M. & TAYLOR, D.L. (1995) Fluorescent protein biosensors: Measurement of molecular dynamics in living cells. *Annu. Rev. Biophys. Biomol. Struct*. **24,** 405–34.

GODWIN, H.A. & BERG, J.M. (1996) A fluorescent zinc probe based on metal-induced peptide folding. *J. Am. Chem. Soc*. **118,** 6514–515.

GONZÁLEZ, J.E. & TSIEN, R.Y. (1995) Voltage sensing by fluorescence resonance energy transfer in single cells. *Biophys. J*. **69,** 1272–80.

GROSS, D. & LOEW, L.M. (1989) Fluorescent indicators of membrane potential: microspectrofluorometry and imaging. *Methods Cell. Biol*. **30,** 193–218.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem*. **260,** 3440–50.

HAJNÓCZKY, G., ROBB-GASPERS, L.D., SEITZ, M.B. & THOMAS, A.P. (1995) Decoding of cytosolic calcium oscillations in the mitochondria *Cell* **82,** 415–24.

HAUGLAND, R.P. (1990) Fluorescein substitutes for microscopy and imaging. In *Optical Microscopy for Biology* (edited by HERMAN, B. & JACOBSON, K.), pp. 143–57. New York: Wiley-Liss.

HAUGLAND, R.P. (1996a) Fluorophores and their amine-

reactive derivatives. In *Handbook of Fluorescent Probes and Research Chemicals*, 6th edn (edited by SPENCE, M.T.Z.), pp. 7–46. Eugene, OR: Molecular Probes.

HAUGLAND, R.P. (1996b) Thiol-reactive probes. In *Handbook of Fluorescent Probes and Research Chemicals*, 6th edn (edited by SPENCE, M.T.Z.), pp. 47–62. Eugene, OR: Molecular Probes.

HAUGLAND. R.P. (1996c) Enzymes, enzyme substrates and enzyme inhibitors. In *Handbook of Fluorescent Probes and Research Chemicals*, 6th edn (edited by SPENCE, M.T.Z.), pp. 201–50. Eugene, OR: Molecular Probes.

HAUGLAND. R.P. (1996d) *Handbook of Fluorescent Probes and Research Chemicals*, 6th edn Eugene, OR: Molecular Probes.

HAUGLAND, R.P. & JOHNSON, I.D. (1993) Detecting enzymes in living cells using fluorogenic substrates. *J. Fluorescence* **3**, 119–27.

HAYDON, P.G., MARCHESE-RAGONA, S., BASARSKY, T.A., SZULCZEWSKI, M. & MCCLOSKEY, M. (1996) Near-field confocal optical spectroscopy (NCOS): subdiffraction optical resolution for biological systems. *J. Microscopy* **182**, 208–16.

HEIM R. & TSIEN, R.Y. (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol*. **6**, 178–82.

HEIM, R., PRASHER, D.C. & TSIEN, R.Y. (1994) Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **91**, 12501–4.

HEIM, R., CUBITT, A.B. & TSIEN, R.Y. (1995) Improved green fluorescence. *Nature* **373**, 663–4.

HENKEL, A.W., LÜBKE, J. & BETZ, W.J. (1996) FM 1-43 dye ultrastructural localization in and release from frog motor nerve terminals. *Proc. Natl. Acad. Sci. USA* **93**, 1918–23.

HOFER, A.M. & MACHEN, T.E. (1993) Technique for *in situ* measurement of calcium in intracellular inositol 1,4,5-triphosphate-sensitive stores using the fluorescent indicator mag-fura-2. *Proc. Natl. Acad. Sci. USA* **90**, 2598–602.

HOLLÓ, Z., HOMOLYA, L., DAVIS, W.C. & SARKADI, B. (1994) Calcein accumulation as a fluorometric functional assay of the multidrug transporter. *Biochim. Biophys. Acta* **1191**, 384–8.

HOMOLYA, L., HOLLÓ, Z., GERMANN, U.A., PASTAN, I., GOTTESMAN, M.M. & SARKADI, B. (1993) Fluorescent cellular indicators are extruded by the multidrug resistance protein. *J. Biol. Chem*. **268**, 21493–6.

HONIG, M.G. (1993) DiI labeling. In *Neuroscience Protocols* (edited by WOUTERLOOD, F.G.), pp. 93-050-16-01–93-050-16-20. Amsterdam: Elsevier Science Publishers.

HONIG, M.G. & HUME, R.I. (1989) DiI and DiO: versatile fluorescent dyes for neuronal labeling and pathway tracing. *Trends Neurosci*. **12**, 333–41.

HTUN, H., BARSONY, J., RENYI, I., GOULD, D.L. & HAGER, G.L. (1996) Visualization of glucocorticoid receptor translation and intranuclear organization in living cells with a green fluorescent protein chimera. *Proc. Natl. Acad. Sci. USA* **93**, 4845–0.

KANESHIRO, E.S., WYDER, M.A., WU, Y-P. & CUSHION, M.T. (1993) Reliability of calcein acetoxymethyl ester and ethidium homodimer or propidium iodide for viability assessment of microbes. *J. Microbiol. Methods* **17**, 1–16.

KLINGEL, S., ROTHE, G., KELLERMAN, W. & VALET, G. (1994) Flow cytometric determination of cysteine and serine proteinase activities in living cells with rhodamine 110 substrates. *Methods Cell. Biol*. **41**, 449–59.

KNECHT, D.A. & SHELDON, E. (1995) Three-dimensional localization of wild-type and myosin II mutant cells during morphogenesis of *Dictyostelium. Dev. Biol*. **170**, 434–44.

KNOWLES, R.B., SABRY, J.H., MARTONE, M.E., DEERINCK, T.J., ELLISMAN, M.H., BASSELL, G.B. & KOSIK, K.S. (1996) Translocation of RNA granules in living neurons. *J. Neurosci*. **16**, 7812–20.

KOK, J.W. & HOEKSTRA, D. (1993) Fluorescent lipid analogues: applications in cell and membrane biology. In *Fluorescent and Luminescent Probes for Biological Activity* (edited by MASON, W.T.), pp. 100–19. London: Academic Press.

LAMPE, P.D. (1994) Analyzing phorbol ester effects on gap junctional communication: a dramatic inhibition of assembly. *J. Cell Biol*. **127**, 1895–905.

LARISON, K.D., BREMILLER, R., WELLS, K.S., CLEMENTS, I. & HAUGLAND, R.P. (1995) Use of a new fluorogenic phosphatase substrate in immunohistochemical applications. *J. Histochem. Cytochem* **43**, 77–83.

LEE, G., DELOHERY, T.M., RONAI, Z., BRANDT-RAUF, P.W., PINCUS, M.R., MURPHY, R.B. & WEINSTEIN, I.B. (1993) A comparison of techniques for introducing macromolecules into living cells. *Cytometry* **14**, 265–70.

LICHTENFELS, R., BIDDISON, W.E., SCHULZ, H., VOGT, A.B. & MARTIN, R. (1994) CARE-LASS (calcein release assay), an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity. *J. Immunol. Methods* **172**, 227–39.

LIPP, P. & NIGGLI, E. (1993) Ratiometric confocal Ca^{2+} measurements with visible wavelength indicators in isolated cardiac myocytes. *Cell Calcium* **14**, 359–72.

LLOYD, Q.P., KUHN, M.A. & GAY, C.V. (1995) Characterization of calcium translocation across the plasma membrane of primary osteoblasts using a lipophilic calcium sensitive fluorescent dye, Calcium Green C_{18}. *J. Biol. Chem*. **270**, 22445–51.

LOEW, L.M. (1993) Confocal microscopy of potentiometric fluorescent dyes. *Methods Cell Biol*. **38**, 195–209.

LOEW, L.M. (1998) Measuring membrane potential in single cells with confocal microscopy. In *Cell Biology: A Laboratory Handbook*, 2nd edn (edited by CELIS, J.E.), pp. 375–379. San Diego, CA: Academic Press.

LOEW, L.M. (1996) Potentiometric dyes: imaging electrical activity of cell membranes. *Pure Appl. Chem.* **68**, 1405–9.

LOEW, L.M., COHEN, L.B., DIX, J., FLUHLER, E.N., MONTANA, V., SALAMA, G. & JIAN-YOUNG, W. (1992) A naphthyl analog of the aminostyryl pyridinium class of potentiometric membrane dyes shows consistent sensitivity in a variety of tissue, cell and model membrane preparations. *J. Membr. Biol*. **130**, 1–10.

LORINCZ, M., ROEDERER, M., DIWU, Z., HERZENBERG, L.A.

& NOLAN, G.P. (1996) Enzyme-generated intracellular fluorescence for single-cell reporter gene analysis utilizing *Escherichia coli* β-glucuronidase. *Cytometry* **24**, 321–9.

MCNEIL, P.L. (1989) Incorporation of macromolecules into living cells. *Methods Cell. Biol*. **29**, 153–73.

MARTÍNEZ-ZAGUILÁN, R., PARNAMI, G. & LYNCH, R.M. (1996) Selection of fluorescent ion indicators for simultaneous measurements of pH and Ca^{2+} *Cell Calcium* **19**, 337–49.

MASON, W.T. (ed.) (1993) *Fluorescent and Luminescent Probes for Biological Activity*. London: Academic Press.

MASTERS, B.R. & CHANCE, B. (1993) Redox confocal imaging: intrinsic fluorescent probes of cellular metabolism. In *Fluorescent and Luminescent Probes for Biological Activity* (edited by MASON, W.T.), pp. 44–57. London: Academic Press.

MINTA, A. & TSIEN, R.Y. (1989) Fluorescent indicators for cytosolic sodium *J. Biol. Chem*. **264**, 19449–57.

MIYAWAKI, A., LLOPIS, J., HEIM, R., MCCAFFERY, J.M., ADAMS, J.A., IKURA, M. & TSIEN, R.Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882–7.

MONTANA, V., FARKAS, D.L. & LOEW, L.M. (1989) Dual-wavelength ratiometric fluorescence measurements of membrane potential. *Biochemistry* **28**, 4536–9.

MOORES, S.L., SABRY, J.H. & SPUDICH, J.A. (1996) Myosin dynamics in live *Dictyostelium* cells. *Proc. Natl. Acad. Sci. USA* **93**, 443–6.

NETT, W. & DEITMER, J.W. (1996) Simultaneous measurements of intracellular pH in the leech giant glial cell using 2′,7′-bis-(2-carboxyethyl)-5-,6-carboxyfluorescein and ion-sensitive microelectrodes. *Biophys. J*. **71**, 394–402.

NOLAN, G.P., FIERING, S., NICOLAS, J.-F. & HERZENBERG, L.A. (1988) Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*. *Proc. Natl. Acad. Sci. USA* **85**, 2603–7.

NOVAK, E.J. & RABINOVITCH, P.S. (1994) Improved sensitivity in flow cytometric intracellular ionized calcium measurements using fluo-3/Fura Red fluorescence ratios. *Cytometry* **17**, 135–41.

OLSON, K.R., MCINTOSH, J.R. & OLMSTED, J.B. (1995) Analysis of MAP4 function in living cells using green fluorescent protein chimeras. *J Cell Biol* **130**, 639–50.

ORMÖ, M., CUBITT, A.B., KALLIO, K., GROSS, L.A., TSIEN, R.Y. & REMINGTON, S.J. (1996) Crystal structure of the *Aequoria victoria* green fluorescent protein. *Science* **273**, 1392–5.

OVERLY, C.C., LEE, K.-D., BERTHIAUME, E. & HOLLENBECK, P.J. (1995) Quantitative measurement of intraorganelle pH in the endosomal–lysosomal pathway in neurons by using ratiometric imaging with pyranine. *Proc. Natl. Acad. Sci. USA* **92**, 3156–60.

OWEN, C.S. (1992) Comparison of the spectrum-shifting intracellular pH probes 5′(and 6′)-carboxy-10-dimethylamino-3-hydroxyspiro[7H-benzo[c]xanthene-7,1′(3′H)-isobenzofuran]-3′-one and 2′,7′-biscarboxyethyl-5 and 6)-carboxyfluorescein. *Anal. Biochem*. **204**, 65–71.

PAGANO, R.E., MARTIN, O.C., KANG, H.C. & HAUGLAND, R.P. (1991) A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J. Cell Biol*. **113**, 1267–79.

PARAMORE, C.G., TURNER, D.A. & MADISON, R.D. (1992) Fluorescent labeling of dissociated fetal cells for tissue culture. *J. Neurosci. Methods* **44**, 7–17.

PAUL, P., KAMISAKA, Y., MARKS, D.L. & PAGANO, R.E. (1996) Purification and characterization of UDP-glucose:ceramide glucosyltransferase from rat liver Golgi membranes. *J. Biol. Chem*. **271**, 2287–93.

PECORINO, L.T., BROCKES, J.P. & ENTWISTLE, A. (1996) Semi-automated positional analysis using laser scanning microscopy of cells transfected in a regenerating newt limb. *J. Histochem. Cytochem*. **44**, 559–69.

PERSECHINI, A., LYNCH, J.A. & ROMOSER, V.A. (1997) Novel fluorescent indicator proteins for monitoring free intracellular Ca^{2+}. *Cell Calcium* **22**, 209–16.

PLOVINS, A., ALVAREZ, A.M., IBANEZ, M., MOLINA, M. & NOMBELA, C. (1994) Use of fluorescein-di-β-D-galactopyranoside (FDG) and C_{12}-FDG as substrates for β-galactosidase detection by flow cytometry in animal, bacterial, and yeast cells. *Appl. Environ. Microbiol*. **60**, 4638–41.

POOT, M., ZHANG, Y.-Z., KRÄMER, J.A., WELLS, K.S., JONES, L.J., HANZEL, D.K., LUGADE, A.G., SINGER, V.L. & HAUGLAND, R.P. (1996) Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J. Histochem. Cytochem*. **44**, 1363–72.

PRASHER, D.C., ECKENRODE, V.K., WARD, W.W., PRENDERGAST, F.G. & CORMIER, M.J. (1992) Primary structure of the *Aequoria victoria* green fluorescent protein. *Gene* **111**, 229–33.

RAJU, B., MURPHY, E., LEVY, L.A., HALL, R.D. & LONDON, R.E. (1989) A fluorescent indicator for measuring cytosolic free magnesium. *Am. J. Physiol*. **256**, C540–8.

RASHID, F. & HOROBIN, R.W. (1990) A structure–activity analysis of mitochondrial staining by cationic probes and a discussion of the synergistic nature of image-based and biochemical approaches. *Histochemistry* **94**, 303–8.

READ, N.D., ALLAN, W.T.G., KNIGHT, H., KNIGHT, M.R., MALHÓ, R., RUSSELL, A., SHACKLOCK, P.S. & TREWAVAS, A.J. (1992) Imaging and measurement of cytosolic free calcium in plant and fungal cells. *J. Microscopy* **166**, 57–86.

REICHEL, C., MATHUR, J., ECKES, P., LANGENKEMPER, K., KONCZ, C., SCHELL, J., REISS, B. & MAAS, C. (1996) Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proc. Natl. Acad. Sci. USA* **93**, 5888–93.

RICHIERI, G.V., OGATA, R.T. & KLEINFELD, A.M. (1992) A fluorescently labeled intestinal fatty acid binding protein. *J. Biol. Chem*. **267**, 23495–501.

RINK, T.J., TSIEN, R.Y. & POZZAN, T. (1982) Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell Biol*. **95**, 189–96.

RIZZUTO, R., BRINI, M., DE GIORGI, F., ROSSI, R., HEIM, R., TSIEN, R.Y. & POZZAN, T. (1996) Double labeling of

subcellular structures with organelle-targeted GFP mutants *in vivo*. *Curr. Biol*. **6,** 183–8.

ROBINSON. J.P., CARTER, W.O. & NARAYANAN, P.K. (1994) Oxidative product formation analysis by flow cytometry. *Methods Cell Biol*. **41,** 437–47.

ROEDERER, M., FIERING, S. & HERZENBERG, L.A. (1991) FACS-Gal: flow cytometric analysis and sorting of cells expressing reporter gene constructs. In *METHODS: A Companion to Methods Enzymol*. **2,** 248–60.

ROPP, J.D., DONAHUE, C.J., WOLFGANG-KIMBALL, D., HOOLEY, J.J., CHIN, J.Y.W., HOFFMAN, R.A., CUTHBERTSON, R.A. & BAUER, K.D. (1995) *Aequorea* green fluorescent protein analysis by flow cytometry. *Cytometry* **21,** 309–17.

ROSSER, B.G., POWERS, S.P. & GORES, G.J. (1993) Calpain activity increases in hepatocytes following addition of ATP. *J. Biol. Chem*. **268,** 23593–600.

ROTMAN, B., ZDERIC, J.A. & EDELSTEIN, M. (1963) Fluorogenic substrates for β-D-galactosidases and phosphatases derived from fluorescein (3,6-dihydroxyfluoran) and its monomethyl ether *Proc. Natl. Acad. Sci. USA* **50,** 1–6.

RYAN, T.C., WEIL, G.J., NEWBURGER, P.E., HAUGLAND, R. & SIMONS, E.R. (1990) Measurement of superoxide release in the phagovacuoles of immune complex-stimulated human neutrophils. *J. Immunol. Methods* **130,** 223–33.

SCHEENEN, W.J.J.M., MAKINGS, L.R., GROSS, L.R., POZZAN, T. & TSIEN, R.Y. (1996) Photodegradation of indo-1 and its effect on apparent Ca^{2+} concentrations. *Chem. Biol*. **3,** 765–74.

SEKLER, I., KOBAYASHI, S. & KOPITO, R.R. (1996) A cluster of cytoplasmic histidine residues specifies pH dependence of the AE2 plasma membrane anion exchanger. *Cell* **86,** 929–35.

SHIBUYA, I. & DOUGLAS, W.W. (1992) Calcium channels in rat melanotrophs are permeable to manganese, cobalt, cadmium and lanthanum, but not to nickel: evidence provided by fluorescence changes in fura-2 loaded cells. *Endocrinology* **131,** 1936–41.

SIMONS, T.J.B. (1993) Measurement of free Zn^{2+} ion concentration with the fluorescent probe mag-fura-2 (furaptra) *J. Biochem. Biophys. Methods* **27,** 25–37.

SIXOU, S., SZOKA, F.C., GREEN, G.A., GIUSTI, B., ZON, G. & CHIN, D.J. (1994) Intracellular oligonucleotide hybridization detected by fluorescence resonance energy transfer (FRET). *Nucleic Acids Res.* **22,** 662–8.

SONG, L., HENNINK, E.J., YOUNG, I.T. & TANKE, H.J. (1995) Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy. *Biophys J* **68,** 2588–600.

SONG, L., VARMA, C.A.G.O., VERHOEVEN, J.W. & TANKE, H.J. (1996) Influence of the triplet excited state on the photobleaching kinetics of fluorescein in microscopy. *Biophys J* **70,** 2959–68.

STAUBER, R., GAITANARIS, G.A. & PAVLAKIS, G.N. (1995) Analysis of trafficking of Rev and transdominant Rev proteins in living cells using green fluorescent protein fusions: transdominant Rev blocks the export of Rev from the nucleus to the cytoplasm. *Virology* **213,** 439–49.

STEINBERG, T.H., NEWMAN, A.S., SWANSON, J.A. & SILVERSTEIN, S.C. (1987) ATP^4 permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biol. Chem*. **262,** 8884–8.

TAN, W., SHI, Z.-Y., SMITH, S., BIRNBAUM, D. & KOPELMAN, R. (1992) Submicrometer intracellular chemical optical fiber sensors. *Science* **258,** 778–81.

TERASAKI, M. (1989) Fluorescent labeling of the endoplasmic reticulum. *Methods Cell Biol*. **29,** 125–35.

TERASAKI, M. & JAFFE, L.A. (1991) Organization of the sea urchin endoplasmic reticulum and its reorganization during fertilization. *J. Cell Biol*. **114,** 929–40.

TERASAKI, M. & REESE, T.S. (1992) Characterization of endoplasmic reticulum by colocalization of BiP and dicarbocyanine dyes *J. Cell Sci*. **101,** 315–22.

TOMASETTO, C. NEVEU, M.J., DALEY, J. HORAN, P.K. & SAGER, R. (1993) Specificity of gap junction communication among mammary cells and connexin transfectants in culture. *J. Cell Biol*. **122,** 157–67.

TSE, F.W., TSE, A. & HILLE, B. (1994) Cyclic Ca^{2+} changes in intracellular stores of gonadotropes during gonadotropin-releasing hormone-stimulated Ca^{2+} oscillations. *Proc. Natl. Acad. Sci. USA* **91,** 9750–4.

TSIEN, R.Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19,** 2396–404.

TSIEN, R.Y. (1981) A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* **290,** 527–8.

USTER, P.S. (1993) *In situ* resonance energy transfer in microscopy: monitoring membrane fusion in living cells *Methods Enzymol*. **221,** 239–46.

VAN DER WOLK, J.P.W., KLOSE, M., DE WIT, J.G., DEN BLAAUWEN, T., FREUDL, R. & DRIESSEN, A.J.M. (1995) Identification of the magnesium-binding domain of the high-affinity ATP-binding site of the *Bacillus subtilis* and *Escherichia coli* SecA protein *J. Biol. Chem*. **279,** 18975–82.

VERKMAN, A.S. (1990) Development and biological applications of chloride-sensitive fluorescent indicators. *Am. J. Physiol*. **257,** C375–88.

WAGGONER, A. (1995) Covalent labeling of proteins and nucleic acids. *Methods Enzymol*. **246,** 362–73.

WANG, H.-G., RAPP, U.R. & REED, J.C. (1996) Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell* **87,** 629–38.

WANG, X.F., PERIASAMY, A. & HERMAN, B. (1992) Fluorescence lifetime imaging microscopy (FLIM): instrumentation and applications. *Crit. Rev. Anal. Chem*. **23,** 369–95.

WANG, Y-L. (1989) Fluorescent analog cytochemistry: tracing functional protein components in living cells. *Methods Cell Biol*. **29,** 1–12.

WESTON, S.A. & PARISH, C.R. (1992) Calcein: a novel marker for lymphocytes which enter lymph nodes. *Cytometry* **13,** 739–49.

WHALLEY, T., TERASAKI, M., CHO, M.-S. & VOGEL, S.S. (1995) Direct membrane retrieval into large vesicles after exocytosis in sea urchin eggs. *J. Cell Biol*. **131,** 1183–92.

WHITAKER, J.E., HAUGLAND, R.P. & PRENDERGAST, F.G. (1991) Spectral and photophysical studies of Benzo[c]-xanthene dyes: dual emission pH sensors. *Anal.*

Biochem. **194**, 330–44.

WOLFBEIS, O.S., FÜRLINGER, E., KRONEIS, H. & MARSONER, H. (1983) Fluorometric analysis. 1. A study on fluorescent indicators for measuring near-neutral ('physiological') pH values. *Fresnius Z. Anal. Chem*. **314**, 119–24.

XU, C., ZIPFEL, W. SHEAR, J.B., WILLIAMS, R.M. & WEBB, W.W. (1996) Multiphoton fluorescence excitation: New spectral windows for biological nonlinear microscopy. *Proc. Natl. Acad. Sci. USA* **93**, 10763–8.

ZHANG, Y.-Z., NALEWAY, J.J., LARISON, K.D., HUANG, Z. & HAUGLAND, R.P. (1991) Detecting *lac*Z expression in living cells with new lipophilic, fluorogenic β-galactosidase substrates. *FASEB J*. **5**, 3108–13.