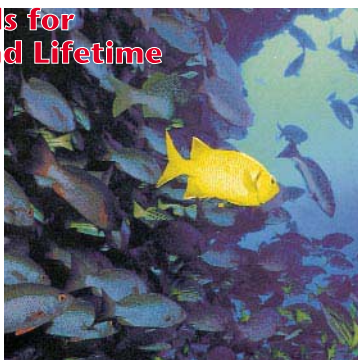


New Fluorescent Labels for Polarization Assays and Lifetime Imaging

Fluka is pleased to introduce a series of reactive dyes for use in fluorescence polarization assays and fluorescence lifetime imaging applications. Designed specifically to have long fluorescence lifetimes, these dyes were first synthesized in the labs of Dr. Joseph Lakowicz at the Center for Fluorescence Microscopy, University of Maryland Medical School. These laser-excitable metal ligand complexes, based on ruthenium, have fluorescence lifetimes between 400 and 800 nanoseconds [1].

Traditional fluorophores such as fluorescein and rhodamine have fluorescence lifetimes of less than 10 nanoseconds. Dyes with very short lifetimes can be used in fluorescence polarization assays if the molecular weight of labeled antigen is relatively low ($M_r < 1,000$). On binding to an antibody, the change in the effective molecular weight of the complex is great enough to be reflected as a change in anisotropy. If the molecular weight of the antigen is large ($M_r > 20,000$), changes in rotational correlation time on Ag:Ab complex formation are small. In this case, fluorophores with short (<10 ns) lifetimes are no longer useful. Dyes with lifetimes in the range of 400 to 500 ns range are better matched to the rotational



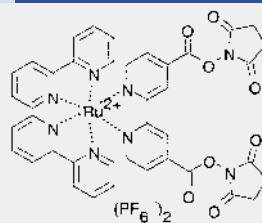
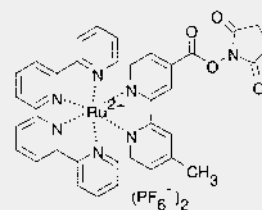
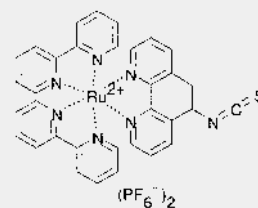
correlation times of very high molecular weight complexes.

These dyes can be used to label amines on biomolecules under mild conditions. Human serum albumin (HSA) has been labeled with $\text{Ru}(\text{bpy})_2\text{dcbpy}$, succinimidyl ester (figure 1) by adding a 100-fold excess of the reactive dye in *N,N*-dimethylformamide (DMF) to a solution of HSA in 0.2 mM carbonate buffer, pH between 8.0 and 9.0. The labeled protein is purified on Sephadex G-25 or G-50 eluting with 0.1 M PBS at pH 7.2. These are sufficient conditions for the labels mentioned below. The resulting conjugate had an emission greater than 540 nm when excited at 483 nm and had a fluorescence lifetime of 336 ns at pH 7.0 [2]. This labeled HSA was used in a model fluorescence polarization immunoassay for polyclonal anti-HSA [3].

Fluka 71603	Bis-(bipyridine)-5-(isothiocyanatophenanthroline)- $\text{Ru}(\text{PF}_6)_2$
Fluka 96631	Bis-(bipyridine)-4'-methyl-4-carboxy-bipyridine- Ru - <i>N</i> -succinimidyl ester (PF_6) ₂
Fluka 96632	Bis-(bipyridine)-4,4'-dicarboxy-bipyridine- Ru -di- <i>N</i> -succinimidyl ester (PF_6) ₂

Advantages of these dyes as protein labels include high photostability, good water solu-

Fig. 1:
A selection of reactive fluorescent ruthenium complexes



Contents:

- **New fluorescent labels for polarization assays and lifetime imaging**
- **powerful cell-permeable fluorophores for life cell assays**
- **improved ion probes**

bility, a lack of dye-dye interactions and large Stokes' shifts.

In addition, the fluorescence signal of long-lived fluorophores can be gated to eliminate the emission from short-lifetime fluorophores and autofluorescence from cells and biomolecules to further improve sensitivity.

Cell-permeable Fluorophores for Life Cell Assays

We would like to highlight three important fluorescent indicators that have proven to be powerful tools for the direct, real-time imaging of biological events – Calcein-AM, BCECF-AM and Fluo-3 AM. These three dyes form the basis for a wide variety of reported live-cell assays. They are available in a nonpolar form that passively crosses the plasma membrane and accumulates in the cytoplasm of living cells. After cleavage of the protecting groups by intracellular esterases, the polar fluorophore (4–6 negative charges/molecule) remains trapped within the cell. The indicators can be excited at 488 nm. Since the dye is nonfluorescent prior to cleavage, only the dye that has accumulated in living cells gives a fluorescent signal.

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These indicators:

- are excited by the 488 nm argon laser line and used with standard fluorescein filters
- stain only live cells with intact membranes
- accumulate in the cytoplasm to give a large fluorescence signal
- are nonfluorescent in the cell-permeable form, so that there is little or no background fluorescence from the extracellular dye
- can be used with orange and red fluorescent fluorophores for multi-color imaging applications
- can be used to perform cell viability assays using a variety of instruments such as fluorescence well-plate readers, flow cytometers, microscopes and fluorometers.

Loading live cells with AM esters:

Use of these dyes in cell viability assays is straightforward:

1. The protected form of the dye (AM) is dissolved in dry DMSO to give a concentration of 1 millimolar.
2. An aliquot is then diluted into buffer and added to cultured cells to give a final dye concentration of 1 to 50 μ M. The cells are bathed in this solution for 30 minutes to 2 hours.
3. The extracellular dye can then be washed away, or if a small background signal is tolerable, the cells can be imaged directly.

Calcein-AM ester has proven to be one of the most important and versatile fluorescent dyes for cell viability research. The dye passively crosses the plasma membrane of living cell where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm.

Calcein can be excited by the argon laser line at 488 nm and, unlike most fluorescein derivatives, its emission intensity is not sensitive to changes in pH over the physiological range.

Assays using Calcein-AM include determining cell viability [4,5] proliferation [6] and adhesion [7]. In NIH 3T3 cells, Calcein-AM has been shown to be more sensitive than Annexin V for detection of the early phases of programmed cell death (apoptosis) [8]. Cytotoxicity assays using Calcein AM have been developed as an alternative to radioactive ^{51}Cr -release assays [9].

Cobalt chloride quenches cytoplasmic calcein, and this has been used to monitor the permeability of mitochondrial pores. Fluo-3 AM is a laser-excitable (488 nm) fluorescent calcium indicator. Like Calcein-AM, Fluo-3 AM is nonfluorescent and is converted to the polar Fluo-3, which accu-

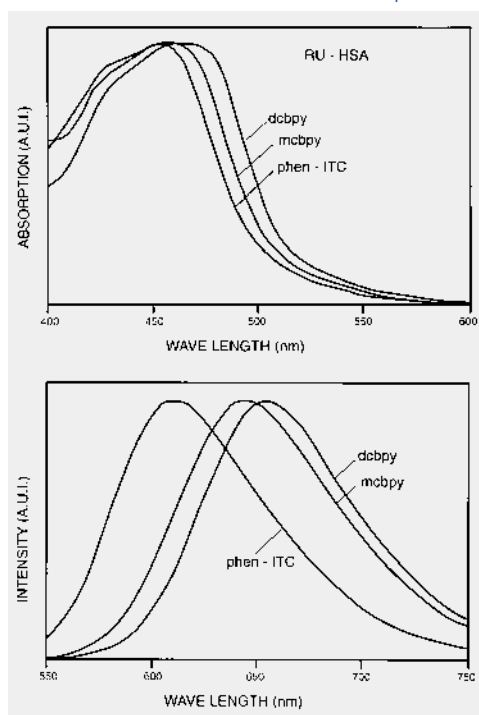


Fig. 2
Absorption and emission spectra of fluorescent ruthenium complexes bound to human serum albumin, 20°C, 10 mM MOPS pH 7.3. Excitation wave length 460 nm (from [1])

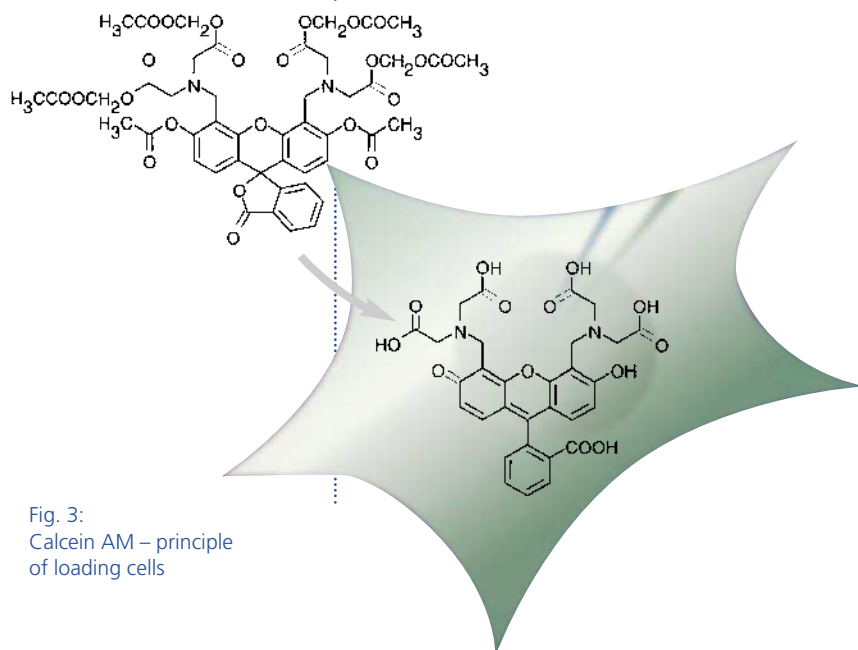


Fig. 3:
Calcein AM – principle of loading cells

mulates in the cytosol. Intracellular Fluo-3 is sensitive to changes in intracellular free calcium [10]. Because of the importance of Ca^{2+} as a messenger, Fluo-3 AM has found widespread utility in high throughput screening assays. Some assays utilizing Fluo-3 AM in live cells include flow cytometric assays for multi-drug resistance [11,12] and for detecting the expression of $\text{Na}^+/\text{Ca}^{2+}$ -exchanger clones [13].

BCECF-AM is a pH indicator ($\text{pK}_a = 6.98$) with an excitation wave length of 488 nm that is useful for imaging changes in cytosolic pH. Like the two dyes above, BCECF-AM itself is nonfluorescent and passively crosses the plasma membrane of cells. BCECF has a pH-insensitive isobestic point at about 527 nm, allowing for ratio imaging of pH changes to correct for differences in path length, dye concentration and bleaching.

Assays have been described that use BCECF-AM in drug screening, cytotoxicity/proliferation [14] and cell viability [15]. The pH response has been used to indirectly determine intracellular potassium levels and monitor nitric oxide (NO)-induced apoptosis. High throughput assays using BCECF include screens for cancer cell attachment [16] and for drugs that alter proton flow through the cell membrane [17].

Related products offered by Fluka:

Ethidium homodimer, unlike Calcein-AM, stains only dead cells. This dye enters cells that have compromised (leaky) membranes and is essentially nonfluorescent until binding to DNA, which elicits a bright red fluorescence. This dye has a much higher affinity for DNA than ethidium bromide. The dye:DNA complexes are extremely stable. In a sensitive cell viability assay, it has been used to detect tissue necrosis factor (TNF) activity[18]. Because ethidium homodimer binds only double-stranded DNA, it has proven useful

as an indicator of nucleotide hybridization[19]. Dihydroethidium is a cell-permeable derivative of the DNA stain ethidium bromide. In parts of the cell where there is oxidative activity or stress, dihydroethidium is oxidized to ethidium bromide. The ethidium bromide then binds to intracellular DNA producing a bright red fluorescence. Dihydroethidium has been shown to exhibit increased fluorescence in six models of apoptosis[20]. It has also been used to detect superoxide generation in the mitochondria of living cells[21].

Fluka 17783	Calcein-AM
Fluka 46394	Fluo 3-AM
Fluka 14562	BCECF-AM
Fluka 46043	Ethidium homodimer
Fluka 37291	Dihydroethidium
Fluka 11696	BAPTA-AM
Fluka 16569	4-Br-A23187
Fluka 21879	5(6)-Carboxyfluorescein diacetate

BAPTA-AM ester and Br-A23187 are used to calibrate the signal from Fluo-3 in cells. BAPTA-AM chelates intracellular Ca^{2+} resulting in a minimum signal of Fluo-3, whereas Br-A23187 is an ionophore that allows extracellular Ca^{2+} to enter the cytoplasm of live cells, resulting in a maximum Fluo-3 signal.

Like Calcein-AM, the diacetates of 5(6)-carboxyfluorescein can be used as indicators of cell viability. The diacetates are nonfluorescent and are converted intracellularly to the fluorescent carboxyfluorescein on cleavage by esterases. Although these dyes are more sensitive to changes in intracellular pH and are not retained in cells as well as Calcein-AM, they provide a less expensive alternative for the analysis of cell viability over short periods.

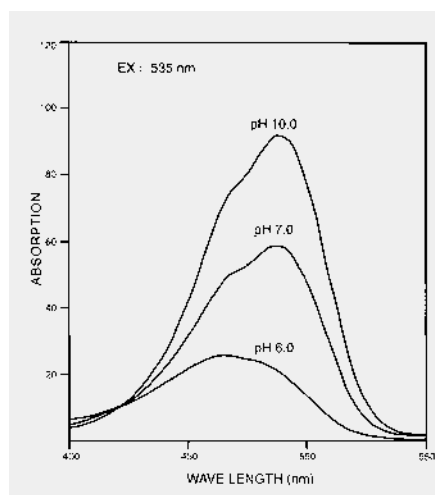


Fig. 4:
BCECF excitation
spectra – response to pH

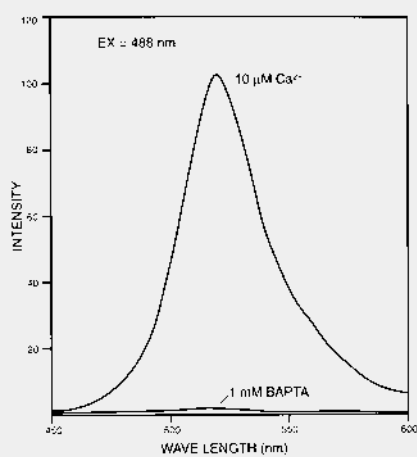


Fig. 5:
Fluo 3 emission
spectra – response to calcium

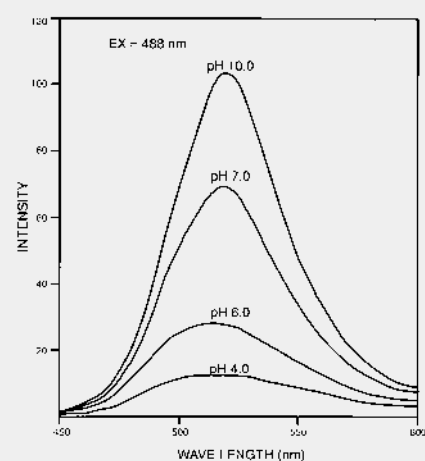


Fig. 6:
BCECF emission
spectra – response to pH

Superior Calcium Indicators

Fluorescent indicators have enabled the investigation of changes in intracellular free Ca^{2+} concentrations. Although they are undisputedly the most sensitive tools for this purpose, some problems are associated with the most popular Ca^{2+} indicators, Fura 2, Fluo 3 and Indo 1, in some applications.

Furthermore, the use of these indicators is limited, with respect to the range of Ca^{2+} -concentration as well as to the location of Ca^{2+} .

Fluka now offers new calcium indicators that overcome these traditional problems or limitations.

High calcium indicators

Indicators specially modified for measuring high calcium have:

- reduced sequestration of intracellular calcium
- reduced perturbation of calcium transients
- measurement of short-lived transients
- absence of Mg^{2+} -effects

Excitation and emission wave lengths, photostability and quantum yields are comparable to the related low Ca^{2+} -indicators Fura-2, Indo-1 and Fluo-3.

Near membrane calcium indicators

Near membrane indicators associate primarily with the plasma membrane. FFP18 has been described as the best near membrane indicator in that it shows much larger and faster transients compared to Fura-2.

Leakage resistant calcium indicators

Fura-2, Indo-1 and Fluo-3 tend to leak out of cells or accumulate in organelles. For some types of cells this leakage can be so great that it becomes difficult to obtain reliable measurements. This problem can be solved by using modified Ca^{2+} -indicators, like Fura-PE3 developed by Drs. Poenie and Minta (US patent 5,576,433). Leakage resistant modified indicators show the same spectral properties as the parent compounds.

New Mounting and Embedding Media for Microscopy

Fluka is pleased to offer an extended range of embedding media kits and ready-made mounting media for both light and electron microscopy. New non-fluorescent aqueous mounting media that delay fluorescence fading are now available. PVA-NPD is the medium of choice for AMCA, Cascade blue-labeled tissue sections and cell cultures. Either PVA-NPD or PVA-DABCO are recommended for FITC, TRITC, DTAF, Cy-3 labeled sections or cell cultures. Glycerol-Gelatin is another, well-known mounting medium for histochemistry that is compatible with fluorescent microscopy.

Fluka 49927 Glycerol-Gelatin

Fluka 10981 PVA-DABCO

Fluka 10979 PVA-NPD

High calcium indicators

Name and Product No.	Solubility	Excitation/Emission (bound to Ca^{2+})	Kd
Fluo-3FF* 17022	H ₂ O	500, 515 / 526	41 μM
Fluo-3FF AM 17079	DMSO	500, 515 / 526 on hydrolysis	
Indo-1FF* 17091	H ₂ O	350 / 435	33 μM
Indo-1FF AM 17088	DMSO	350 / 435 on hydrolysis	
Fura-2FF* 17085	H ₂ O	340 / 505	35 μM
Fura-2FF AM 17090	DMSO	340 / 505 on hydrolysis	

*potassium salt

Near membrane calcium indicators

Name and Product No.	Excitation	Emission maximum	Kd
FFP 18* 17089	Free: 335 Bound: 335	Free: 502 Bound: 492	400 nM
FFP 18 AM 17083	As above on hydrolysis	As above on hydrolysis	
FIP 18* 17087	Free: 346 Bound: 346	Free: 475 Bound: 408	450 nM

*potassium salt

Leakage resistant calcium indicators

Name and Product No.	Excitation	Emission maximum	Kd
Fura PE3* 17082	Free: 364 Bound: 335	Free: 502 Bound: 495	250 nM
Fura PE3-AM 17081	As above on hydrolysis	As above on hydrolysis	
Indo PE3* 17084	Free: 350 Bound to Ca^{2+} : 350	Free: 475 Bound: 408	260 nM
Indo PE3-AM 17086	As above on hydrolysis	As above on hydrolysis	

*potassium salt

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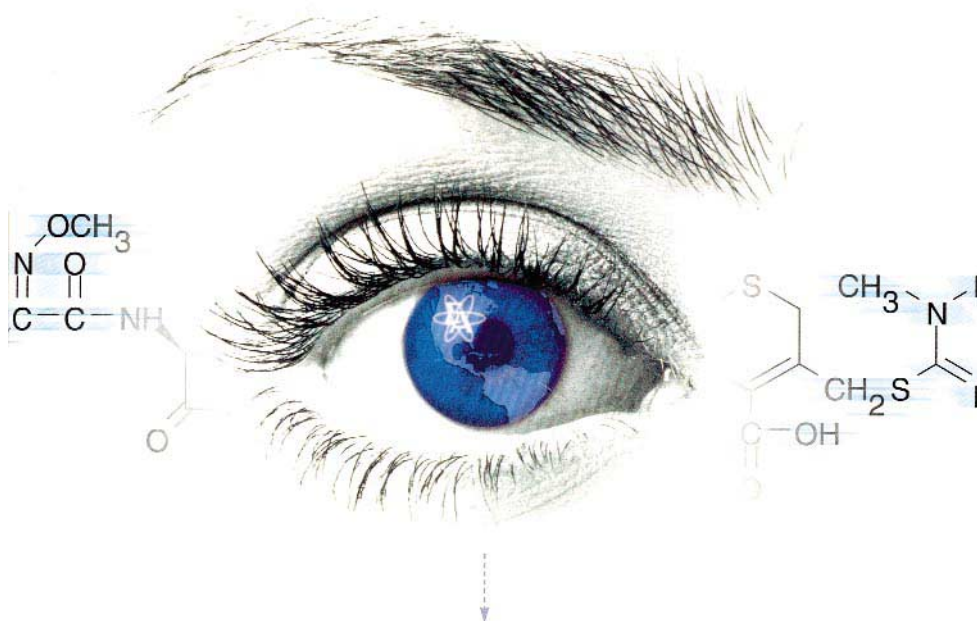
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