Reactive Oxygen Species (ROS) Detection Reagents

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Reactive Oxygen Species (ROS) Detection reagents	See label	• ≤–20°C • Desiccate • Protect from light • Protect from air * • Avoid freeze-thaw cycles	When stored as directed, this product is stable for at least 3 months.
* These products are air sensitive and should be stored under dry argon or nitrogen.			
Approximate Fluorescence Excitation and Emission, in nm : 492–495/517–527, in nm.			

Introduction

Molecular Probes offers derivatives of reduced fluorescein and calcein as cell-permeant indicators for reactive oxygen species. Chemically reduced and acetylated forms of 2′,7′-dichlorofluorescein (DCF) and calcein are nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell [1–4] (Figures 1 and 2). Esterase cleavage of the lipophilic blocking groups yields a charged form of the dye that is much better retained by cells than is the parent compound. Oxidation of these probes can be detected by monitoring the increase in fluorescence with a flow cytometer, fluorometer, microplate reader, or fluorescence microscope, using excitation sources and filters appropriate for fluorescein (FITC).[5–8] Because the dyes are susceptible to photo-oxidation, low light conditions should be used for fluorescence microscopy applications whenever possible.[9]

The carboxy derivative of fluorescein, carboxy-H_2DCFDA (C400), carries additional negative charges that improve its retention compared to noncarboxylated forms. The fluorinated derivative, H_2DFFDA (C13293), exhibits improved photostability compared to chlorinated fluorescein derivatives. Derivatives with a thiol-reactive chloromethyl group (C6827) or an amine-reactive succinimidyl ester group (D2935) allow for covalent binding to intracellular components, permitting even longer retention within the cell. H_2DCFDA, SE (D2935) may also provide extracellular labeling.

Dihydrocalcein, AM is freely permeant to cell membranes and is oxidized to green-fluorescent calcein, which has excellent retention properties. However, unlike the fluorescein derivatives, calcein fluorescence can be quenched by Fe^{3+}, Co^{2+}, Ni^{2+}, and Cu^{2+} at pH 7. Other reduced fluorescein ROS indicators available from Molecular Probes include aminophenyl fluorescein (A36003) and hydroxyphenyl fluorescein (H36004).

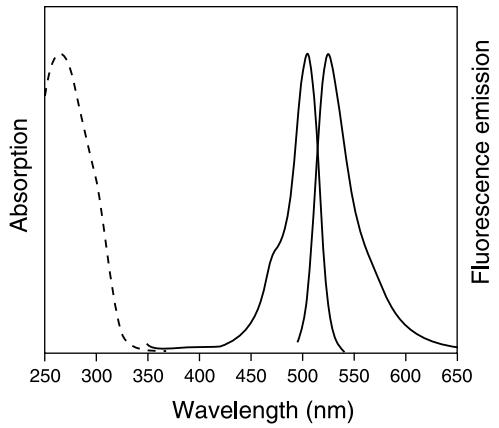

Figure 1. Absorption spectrum of reduced dye (---) and absorption/emission spectra of oxidized dye (—).

Guidelines for Use

Materials Required but Not Provided

- DMSO
- DMF
- Ethanol
- Buffer, such as PBS, HBSS, or HEPES

Preparing the Stock Solutions

Do not dissolve products until immediately prior to use. Acetylated dyes should be reconstituted only as required using high quality anhydrous dimethylsulfoxide (DMSO), dimethylform-amide (DMF), or 100% ethanol. Working solutions should be freshly prepared; discard excess diluted probe at the end of the work session. These probes oxidize more readily in solution, and the presence of moisture will facilitate the decomposition of the dye.

General Considerations

Protocols for loading a particular dye and/or cell type can be found in the literature. The AM or acetate ester concentration should be kept as low as possible to reduce potential artifacts from overloading, including incomplete hydrolysis, compartmentalization, and the toxic effects of hydrolysis by-products (e.g., formaldehyde, acetic acid). Loading may be done at the optimal temperature for cell growth; however, some investigators have reported a greater degree of compartmentalization at physiological temperatures than is observed at room temperature.

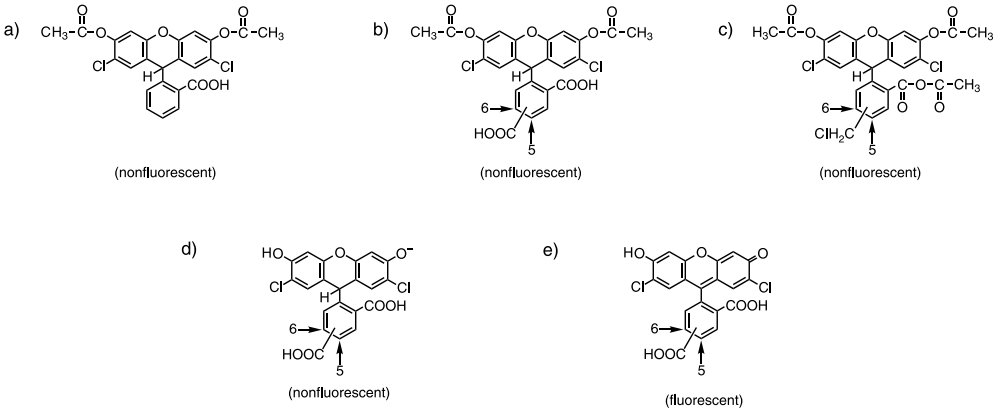

Figure 2. The structures of (a) H_2DCFDA, (b) carboxy-H_2DCFDA, (c) chloromethyl-H_2DCFDA, (d) deacetylated H_2DCF, and (e) the deacetylated, oxidized product, DCF.

To determine the optimal dye-loading concentration, temperature, incubation, recovery, and retention time for your specific application, we recommend using a cell-permeant oxidized form of the ROS indicator such as carboxy-DCFDA (C369), fluorescein diacetate (F1303), or calcein, AM (C1430).

Extracellular hydrolysis of the AM and acetate esters can be minimized by using a loading buffer that is free of primary and secondary amines (such as PBS). Cells should be maintained in medium that is free of phenol red and other colorimetric dyes prior to and throughout the assay and washed with dye-free buffer or media after loading.

Note: The time the dye is retained within the cells must cover the duration of the experiment. Cells vary in their response to dyes; some dyes are lost by passive diffusion over time, or more quickly by active efflux. If the assay is to be carried over an extended period of time, dye-retention times need to be determined beforehand. This should also be done for derivatives that allow for covalent attachment, since cells vary in the rates at which they turn over various cellular components, and in their viability subsequent to covalent modification.

Protocols

The following protocols provide general guidelines derived from various publications, and should be modified for the particular application and sensitivity required.

1.1 Shortly before performing the experiments, reconstitute the ROS indicator to make a concentrated stock solution. Keep tightly sealed until ready to use.

1.2 Remove cells from growth media via centrifugation or pipetting. Resuspend cells in prewarmed buffer (PBS, HBSS, HEPES, or other simple physiological buffer) containing the probe to provide a final working concentration of ~1–10 µM dye. The optimal working concentration for your application must be empirically determined.

1.3 Incubate at the optimal temperature for the cells. Generally, a loading time of 5–60 minutes is sufficient.

1.4 Remove the loading buffer; return the cells to prewarmed growth medium and incubate at the optimal temperature. For derivatives with acetoxymethyl ester (AM) and/or diacetate groups, allow a short recovery time for cellular esterases to hydrolyze the AM or acetate groups and render the dye responsive to oxidation. The optimal recovery time can vary widely, as some cell types normally exhibit low levels of esterase activity.[10]

1.5 Determine the baseline fluorescence intensity of a sample of the loaded cells prior to exposing the cells to experimental inducements. If using the succinimidyl ester derivative (C2935), any extracellularly bound dye can be quenched using Trypan Blue (~0.0025% w/v) in order to better distinguish the signal from the intracellular ROS response.

1.6 Negative controls should be assessed as follows:

a) Examine unstained cells for autofluorescence in the green emission range.

b) For flow cytometry, ascertain that the forward and side scatter of cells is unchanged after dye-loading and treatment. Changes in cell dimensions may be related to blebbing or shrinkage resulting from handling or a toxic response.

c) Examine the fluorescence of cell-free mixtures of dye and buffer/media with and without the inducer/stimulant. In the absence of extracellular esterases and other oxidative enzymes, the ROS indicator should exhibit a gradual increase in fluorescence over time, which may be related to spontaneous hydrolysis, atmospheric oxidation, and/or light-induced oxidation.[11–13]

d) Examine the fluorescence of untreated loaded cells that have been maintained in growth medium or simple buffer. In healthy cells, oxygen radicals are eliminated by cellular enzymes and/or natural antioxidants. Following the dye-loading recovery period, healthy cells should exhibit a low level of fluorescence that is relatively stable for the duration of the experiment;

however, a gradual increase (due to auto-oxidation) or decrease (due to loss of dye from cells or photobleaching) in fluorescence may be observed. In the absence of any stimulus or inducement, a burst of fluorescence in healthy, untreated cells could indicate progress to cell death or some other oxidative event.

1.7 To create positive controls, oxidative activity may be stimulated with:

a) the tumor promoter 4β-phorbol 12-myristate 13-acetate (PMA; stock solution 1 mM in DMF; working concentration 100 pM to 10 µM)

b) the bacterial chemotatic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; stock solution 1 mM in DMF; working concentration 1–10 µM)

c) H_2O_2 or *tert*-butyl hydroperoxide (TBHP) to a final concentration of ~100 µM (increase or decrease based on the sensitivity and response of the cells).

1.8 To assure that an added drug or compound will not quench the dye, examine the absorbance spectrum of the compound and determine that the absorbance peak does not overlap with either the excitation or emission peak of the oxidized dye. Alternatively, you can mix a solution of the drug/compound with carboxy-DCF (C368), fluorescein (F1300), or calcein (C481), then compare the fluorescence intensity to a control solution of the dye, or subject a culture loaded with the cell-permeant, oxidized form of the dye to the drug/compound and compare to dye-loaded cells untreated.

References

1. Anal Biochem 11, 6 (1965); **2.** Anal Biochem 11, 1 (1965); **3.** Anal Biochem 134, 111 (1983); **4.** Cell Biol Int 24, 757 (2000); **5.** J Immunol Methods 156, 39 (1992); **6.** J Immunol Methods 178, 89 (1995); **7.** Toxicol in Vitro 11, 531 (1997); **8.** J Histochem Cytochem 50, 289 (2002); **9.** Biochem Biophys Res Commun 304, 619 (2003); **10.** J Leukoc Biol 43, 304 (1988); **11.** FEBS Lett 511, 21 (2002); **12.** Free Radic Biol Med 33, 938 (2002); **13.** Free Radic Biol Med 30, 1418 (2001).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
C13293	5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) *mixed isomers*	5 mg
C400	5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) *mixed isomers*	25 mg
C6827	5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) *mixed isomers* *special packaging*	20 x 50 µg
D23805	dihydrocalcein, AM *special packaging*	20 x 50 µg
D2935	2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester (OxyBURST® Green H_2DCFDA, SE)	5 mg
D399	2′,7′-dichlorodihydrofluorescein diacetate (2′,7′-dichlorofluorescin diacetate; H_2DCFDA)	100 mg
C2938	6-carboxy-2′,7′-dichlorodihydrofluoresce in diacetate, di(acetoxymethyl ester)	5 mg

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