DiI Derivatives for Long-Term Cellular Labeling

Introduction

The strong, photostable fluorescence, excellent cellular retention, and minimal cytotoxicity of DiI and other long-chain carbocyanine membrane probes make them particularly suitable for long-term labeling and tracking of cells,[1-4] intracellular membranes,[5,6] liposomes,[7] viruses,[8] and lipoproteins.[9,10] Unfortunately, currently available lipophilic carbocyanines, including DiI and DiO and the PKH2 and PKH26 dyes,[11,12] sometimes require the presence of osmolarity regulating agents or the absence of salts to avoid dye precipitation from the staining medium during labeling of cell suspensions. Furthermore, DiI labeling is not compatible with long-term preservation using standard tissue fixation procedures. To circumvent these limitations, Molecular Probes has developed several lipophilic carbocyanine derivatives with improved water solubility and staining persistence after fixation:

- Chloromethylbenzamido (CellTracker™ CM-DiI; C7000, C7001, N22883, V22888) and methylbenzamido (FM®-DiI, F6999) derivatives of DiI
- Sulfonated DiI derivatives SP-DiIC$_{18}$(3) (D7777) and DiIC$_{18}$(3)-DS (D7776)
- Sulfonated derivatives of DiO (SP-DiOC$_{18}$(3); D7778) and the red-fluorescent carbocyanine tracer DiD (DiIC$_{18}$(5)-DS; D12730)

CM-DiI incorporates a mildly thiol-reactive chloromethyl substituent that confers aldehyde fixability via conjugation to thiol-containing peptides and proteins. Unlike DiI and PKH26, CM-DiI is retained in cells throughout fixation, permeabilization, and paraffin embedding procedures.[13,14]

Labeling with sulfonated carbocyanine tracers also appears to be compatible with aldehyde fixation and acetone permeabilization treatments, although the mechanism responsible for the persistence of labeling is not currently known. We have observed excellent retention of SP-DiIC$_{18}$(3) and SP-DiOC$_{18}$(3) staining in human B cells under conditions in which virtually all of the PKH26 dye is lost during the lipid extraction step (Figure 1).

Furthermore, we have observed that in some cases acetone treatment actually enhances the fluorescence of cells stained with SP-DiIC$_{18}$(3) and SP-DiOC$_{18}$(3) (particularly the latter), a phenomenon that has not been seen with other carbocyanine membrane stains. Although these properties have not yet been extensively tested, they could make SP-DiIC$_{18}$(3) and SP-DiOC$_{18}$(3) particularly useful for immunohistochemical studies requiring tissue dehydration, clearing, and lipid extraction.

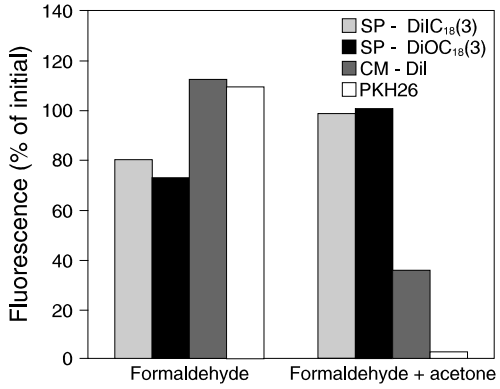

Figure 1. Persistence of lipophilic tracer fluorescence following fixation. Cultured human B cells were stained with 20 µM SP-DiOC$_{18}$(3), SP-DiIC$_{18}$(3), CM-DiI or PKH26 and then fixed with 3.7% formaldehyde or 3.7% formaldehyde + acetone. The fixed cells were analyzed by flow cytometry (Becton Dickinson FACS Vantage) to generate a comparison of their fluorescence with that of the original live-cell population.

Materials

CellTracker™ CM-DiI (C7000) and FM DiI (F6999) are supplied in specially packaged sets of 20 vials, each containing 50 µg solid for reconstitution as required. CellTracker™ CM-DiI is also supplied in single vials containing 1 mg (C7001), and as a ready-made cell-labeling solution (V22888) or tissue-labeling paste (N22883). Sulfonated carbocyanine tracers are supplied in 5 mg units. Upon receipt, CM-DiI should be stored frozen at ≤–20°C, desiccated, and protected from light (desiccation not required for N22883 or V22888). FM DiI and the sulfonated carbocyanine tracers may be stored at room temperature, protected from light. Stock solutions (see *Preparing Stock Solutions*) can be stored under the same conditions as the undissolved product. When stored properly, both the solid materials and the stock solutions are stable for at least six months.

Spectral Characteristics

Lipophilic carbocyanine tracers have extremely high extinction coefficients, moderate fluorescence quantum yields, and short excited state lifetimes (~1 ns) in lipid environments. SP-DiIC$_{18}$(3) and SP-DiOC$_{18}$(3) have higher fluorescence quantum yields than DiI and DiO.[15] Lipophilic carbocyanines are weakly fluorescent in water, but highly fluorescent and photostable when

Table 1. Spectral characteristics of lipophilic carbocyanine tracers arranged in order of increasing excitation maximum.

Catalog #	Name	Ex * (nm)	$\epsilon \times 10^{-3}$ †	Em * (nm)	Optical Filters ‡	
					Omega	Chroma
D7778	SP-DiOC$_{18}$(3)	497	175	513	XF23	31001
F6999	FM-DiI	553	133	570	XF32	31002
C7000, C7001	CM-DiI	553	134	570	XF32	31002
D7776	DiIC$_{18}$(3)-DS	555	144	570	XF32	31002
D7777	SP-DiIC$_{18}$(3)	556	164	573	XF32	31002
D12730	DiIC$_{18}$(5)-DS	650	247	670	XF47	31023

***** Fluorescence excitation (Ex) and emission (Em) maxima determined in methanol. Ex and Em of carbocyanine dyes in methanol and bound to membranes are very similar. **†** Molar extinction coefficient determined in methanol. Multiply the listed value by 1,000 to convert to units of $cm^{-1}M^{-1}$. **‡** Catalog numbers of optical filter sets recommended for fluorescence imaging. Omega filters are supplied by Omega Optical, Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

incorporated into membranes. A summary of spectral properties is shown in Table 1, together with recommended filter sets for fluorescence microscopy.

Experimental Application

Preparing Stock Solutions

Stock solutions of lipophilic tracers may be prepared in dimethylformamide (DMF), dimethylsulfoxide (DMSO), or ethanol at 1–2 mg/mL. DMF is preferable to ethanol or DMSO as a solvent for SP-DiOC$_{18}$(3).

Cell Labeling

The following protocol should be considered a starting point for researchers developing their own cell labeling techniques. Immediately before labeling, dilute the 1–2 mg/mL solution stock (see *Preparing Stock Solutions*) into a suitable medium such as Hanks' balanced salt solution (HBSS) or Dulbecco's phosphate-buffered saline (D-PBS). Suitable working concentrations generally range from 1–2 µM but may vary considerably depending on the application. Molecular weights for CM-DiI, FM-DiI, and the sulfonated carbocyanines are generally close to 1,000 (exact values are printed on the container label); therefore 1 µM ≈ 1 µg/mL. Dilute Vybrant® CM-DiI cell-labeling solution directly into HBSS or D-PBS (1–5 µL of labeling solution per mL of medium). CM-DiI and FM-DiI rapidly aggregate in more concentrated solutions (~5 µM). The sulfonated carbocyanines are soluble at concentrations up to 20 µM, although only for a limited time (significant precipitation is detectable after 4 hours). We suggest incubating cells in the working solution for 5 minutes or less at 37°C, and then for an additional 15 minutes at 4°C. Incubation at this lower temperature appears to allow the dye to label the plasma membrane but slows down endocytosis, thus reducing dye localization into cytoplasmic vesicles. After labeling, wash cells with phosphate-buffered saline (PBS) and resuspend in fresh medium. For adherent cells, labeling in culture while attached results in improved viability compared to labeling after dissociation.[3]

Tissue Labeling

NeuroTrace® CM-DiI tissue-labeling paste consists of CM-DiI mixed into an inert, water-resistant gel. The paste is ready to use as supplied and can be applied directly to live or fixed tissue specimens using the tip of a needle. This method of application improves the penetration of the dye into bundled neurons, labeling axons both on and below the surface.

Post-Labeling Processes

Cells stained with CM-DiI or the sulfonated carbocyanines may be fixed by suspension in 3.7% (w/v) formaldehyde in PBS at 37°C for 10 minutes and permeabilized in acetone for 10 minutes at –20°C (Figure 1). Plasma membrane staining of PC12 rat pheochromocytoma cells by CM-DiI withstands fixation with 2% (w/v) paraformaldehyde and permeabilization with 0.2% (w/v) Triton X-100.[13] CM-DiI labeled lymphocytes recovered from the lymphatic system of sheep have been fixed with formaldehyde, embedded in paraffin wax, sectioned, and then cleared through xylene, graded ethanol, and water without adverse effects on staining.[14] As with DiI,[16,17] sensitized photoconversion of diaminobenzidine (DAB) to an electron-dense precipitate allows long-term preservation of labeling, as well as correlation of fluorescence imaging with transmitted light and electron microscopy.

References

1. Methods Cell Biol 51, 147 (1996); **2.** Neuron 13, 813 (1994); **3.** Histochemistry 97, 329 (1992); **4.** J Cell Biol 103, 171 (1986); **5.** J Cell Biol 114, 929 (1991); **6.** Dev Biol 170, 607 (1995); **7.** Biochim Biophys Acta 1190, 99 (1994); **8.** Cytometry 14, 16 (1993); **9.** J Cell Biol 90, 595 (1981); **10.** Biochim Biophys Acta 1303, 193 (1996); **11.** Methods Cell Biol 33, 469 (1990); **12.** Cytometry 21, 160 (1995); **13.** J Cell Biol 131, 1327 (1995); **14.** J Immunol Methods 194, 181 (1996); **15.** Bioorg Med Chem Lett 6, 1479 (1996); **16.** J Histochem Cytochem 38, 725 (1990); **17.** J Neurosci Methods 64, 47 (1996).

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

Cat #	Product Name	Unit Size
C7000	CellTracker™ CM-DiI *special packaging*	20 x 50 µg
C7001	CellTracker™ CM-DiI	1 mg
D12730	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine-5,5′-disulfonic acid (DiIC$_{18}$(5)-DS)	5 mg
D7776	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid (DiIC$_{18}$(3)-DS)	5 mg
D7777	1,1′-dioctadecyl-6,6′-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine (SP-DiIC$_{18}$(3))	5 mg
D7778	3,3′-dioctadecyl-5,5′-di(4-sulfophenyl)oxacarbocyanine, sodium salt (SP-DiOC$_{18}$(3))	5 mg
F6999	FM® DiI *special packaging*	20 x 50 µg
L7781	Lipophilic Tracer Sampler Kit	1 kit
N22883	NeuroTrace® CM-DiI tissue-labeling paste	100 mg
V22888	Vybrant® CM-DiI cell-labeling solution	1 mL

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