Lipophilic Tracers—DiI, DiO, DiD, DiA, and DiR

Table 1. Contents and storage information.

Material	Storage*	Stability
DiO (D275, D1125, D7778, N22881)	• ≤25˚C • Protect from light	When stored as directed these products are stable for at least 1 year
DiA (D3883, D291)		
DiI (D282, D383, D384, , D3911, D7776, D7777, D12730, N22880)		
DiD (D307, D337, D7757)		
DiR (D12731)		
DiO (D3898); DiA (D7758); DiI (D3886, D3899, D7756)	• ≤–20˚C • Protect from light	
Lipophilic Tracer Sampler Kit	• ≤25˚C • Protect from light	
NeuroTrace® Multicolor Tissue-Labeling Kit *DiO, DiI, DiD pastes		

*Carbocyanine and aminostyryl dyes with saturated alkyl substituents (C_{18}, C_{16}, or C_{12}) may be stored at room temperature, protected from light. $DiIC_{12}(3)$ (D383) is intrinsically a sticky gum at room temperature. The perchlorate salt of DiD (D307) is intrinsically an oil at room temperature; the 4-chlorobenzenesulfonate salt (D7757) is a solid. DiI and DiO are also available mixed into ready-to-use tissue labeling pastes (N22880, N22881). These paste preparations may be stored at room temperature, protected from light. Dyes with unsaturated alkyl substituents ($\Delta^{9,12}$-C_{18} or Δ^{9}-C_{18}) should be stored frozen at ≤–20°C, protected from light under an argon or nitrogen atmosphere. These products are packaged under argon. *FAST* DiI™ (D3899) is intrinsically an oil at room temperature. *FAST* DiI™ and *FAST* DiA™ are also available as solid 4-chlorobenzenesulfonate salts (D7756 and D7758).

Approximate fluorescence excitation/emission maxima: See Table 2.

Introduction

Long-chain dialkylcarbocyanines, in particular DiI, and dialkylaminostyryl dyes (DiA and its analogs) are widely used as anterograde and retrograde neuronal tracers in living[1,2] and fixed[3,4] tissues and cells. DiI labeling does not appreciably affect cell viability, development, or basic physiological properties.[1,2] DiI-labeled motoneurons reportedly have remained viable for up to four weeks in culture and up to one year *in vivo*.[5] The dyes uniformly label neurons via lateral diffusion in the plasma membrane at a rate of about 0.2–0.6 mm per day in fixed specimens;[4,6] in living tissue labeling is more rapid (6 mm per day), due to active dye transport processes.[1,4] In aldehyde-fixed tissue, diffusion of DiI can be followed for up to two years in some cases.[7,8] In general, the dyes do not transfer from labeled to unlabeled cells, although some transfer may occur when the membrane is disrupted, as occurs when sectioning.[1,9]

Invitrogen offers a range of dialkylcarbocyanines with a variety of spectroscopic and cellular labeling properties:

- DiI (D282, D3911, N22880) and DiO (D275) have fluorescence excitation and emission maxima separated by about 65 nm, facilitating two-color labeling (Figure 1).[1,4,10] DiO

staining is usually less intense than that of DiI, and occasionally fails completely in fixed tissues.[6,11]

- DiD (D307, D7757) is an analog of DiI with markedly red-shifted fluorescence excitation and emission spectra (Figure 1). This characteristic is useful in avoiding autofluorescence and phototoxic effects,[12] as well as for two-color labeling.[13,14] DiR (D12731) has excitation and emission maxima in the near infrared region, where many tissues are optically transparent.
- $DiIC_{12}(3)$ (D383), $DiIC_{16}(3)$ (D384), and $DiOC_{16}(3)$ (D1125) have shorter alkyl substituents (C_{12} or C_{16}) than DiI and DiO (C_{18}). These slightly less lipophilic probes have been found by some to incorporate into membranes more easily than the DiI and DiO.[15]
- *FAST* DiI™ (D3899, D7756) and *FAST* DiO™ (D3898) have diunsaturated $\Delta^{9,12}$-C_{18} alkyl substituents in place of the saturated C_{18} tails of DiI and DiO, resulting in accelerated diffusion within membranes.[16] A monounsaturated Δ^9-C_{18} analog of DiI is aso available (Δ^9-DiI; D3886).
- Sulfonated derivatives of DiI and DiO, and CM-DiI (a thiol-reactive DiI derivative) produce staining that persists after fixation and permeabilization treatments. These tracers are described in instruction manual MP 06999.

In addition to neuronal tracing, lipophilic carbocyanines have many other applications including:

- Detection of cell-cell fusion[17-19] and adhesion[20]
- Tracing cell migration during development and after transplantation[21-23]
- Lipid diffusion in membranes by FRAP (**F**luorescence **R**ecovery **A**fter **P**hotobleaching)[24,25]
- Cytotoxicity assays[26,27]
- Labeling of lipoproteins[28,29]

The lipophilic aminostyryl dyes DiA (D3883), and 4-Di-10-ASP (D291) are also often used for neuronal tracing.[30,31] DiA has been used as a second-color neuronal tracer in conjunction with DiI.[6,32,33] Fixed tissue has been successfully labeled with DiA in some cases where DiO staining failed.[6] *FAST* DiA™ (D7758), a diunsaturated $\Delta^{9,12}$-C_{18} DiA analog designed for accelerated diffusion within membranes is also available from Invitrogen.

Spectral Characteristics

The spectral properties of the dialkylcarbocyanines are largely independent of the lengths of the alkyl chains, but are instead determined by the heteroatoms in the terminal ring systems and the length of the connecting bridge. They have extremely high extinction coefficients, moderate fluorescence quantum yields, and short excited state lifetimes in lipid environments (~1 ns).[34] They are insoluble in water, but their fluorescence is readily detected when incorporated into membranes. A summary of spectral properties is shown in Table 2, together with appropriate filter sets for fluorescence microscopy applications.

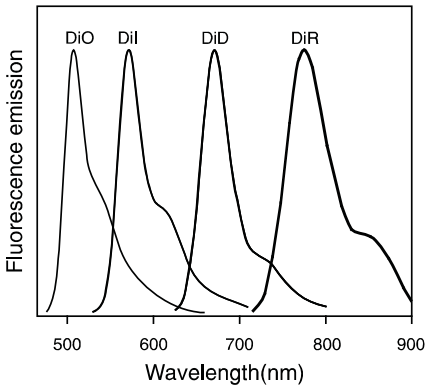

Figure 1. Normalized fluorescence emission spectra of DiO, DiI, DiD, and DiR bound to phospholipid bilayer membranes.

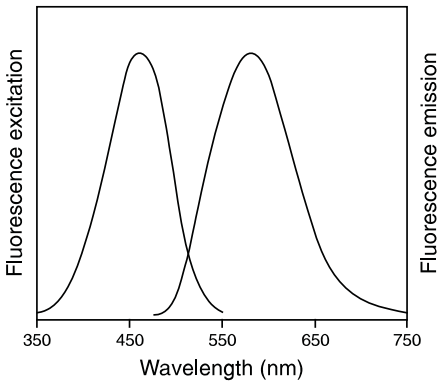

Figure 2. Fluorescence excitation and emission spectra of DiA bound to phospholipid bilayer membranes.

Aminostyryl dyes are insoluble in aqueous environments but their fluorescence is easily detected upon insertion into membranes or when diluted into organic solvents. DiA's excitation and emission maxima measured in DOPC phospholipid bilayers are about 460 nm and 580 nm, respectively (Figure 2). As is typical for aminostyryl dyes, there is a significant wavelength shift relative to spectra measured in solvents such as methanol (Table 2). The emission spectrum of DiA is very broad, allowing it to be detected as green, orange, or even red fluorescence depending on the optical filter used.

Experimental Applications

Preparing Stock Solutions

Prepare stock solutions of lipophilic tracers in dimethylformamide (DMF), dimethylsulfoxide (DMSO), or ethanol at 1 to 2.5 mg/mL. DMF is preferable to ethanol as a solvent for DiO. Stock solutions can be stored for at least six months without deterioration under the same conditions as the undissolved product.

Dissolving DiO in DMF may require sonication and heating to 50°C, and the dye may precipitate from this solution after several hours. To obtain a stable concentrated solution of DiO, perform the following procedure.[35]

1.1 Dissolve solid DiO in chloroform to prepare a 50 mg/mL DiO/chloroform stock solution. Mix one volume of the DiO/chloroform stock solution with an equal volume of octadecylamine. Ratios of DiO/chloroform stock solution and octadecylamine of up to 1:10 have also worked.

1.2 Heat the solution to 50°C, then allow it to precipitate by placing it on ice with two volumes of methanol.

1.3 Pellet the precipitate by centrifugation for 5 minutes at 13,000 rpm in a microcentrifuge. Carefully remove the supernatant and discard it.

1.4 Dry the pellet using a vacuum microcentrifuge or by allowing the solvent to evaporate overnight through a fine hole poked in the lid of the tube's cap.

1.5 Dissolve the resulting powder in DMF at 5% (w/v) with sonication, heating to 50°C. Clear the solution by centrifuging for 10 minutes at 13,000 rpm in a microcentrifuge.

Staining Methods

Methods for cellular labeling with dialkylcarbocyanine and dialkylaminostyryl tracers based on published procedures and recommendations from our customers are summarized below.

Direct application of dye crystals. Dye crystals can be applied directly to intact or cut neurons for retrograde or anterograde labeling.[4,6,31,36] We offer specially prepared large crystals of DiI (D3911) for this purpose. FAST DiI™ (D7756) is a crystal but has the appearance of tar. In some cases, direct application of crystals produces more consistent labeling than injection of a dye solution.[36] Crystals are typically applied to a micropipette tip for delivery to the desired labeling site. Water-setting glue [5] or pieces of gelatin sponge (Gelfoam, Pfizer, Kalamazoo, MI) [31] may be used to hold the crystals in place during the time required for transport of the dye along the neuronal pathway. For labeling relatively large areas, a piece of nylon filter or Gelfoam may be soaked with concentrated dye solution and inserted into a natural or dissected cavity.[3]

NeuroTrace® tissue labeling pastes. NeuroTrace® tissue labeling pastes consist of DiI, DiO, or DiD mixed into an inert, water-resistant gel. The pastes are 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. In similar situations, direct application of dye crystals or microinjection of concentrated solutions will only label neurons on the surface. This labeling method has also been found to increase the rate of dye transport by 50–80%.[37]

Loading of tracers supplied as oils. *FAST* DiI™ (D3899), Δ^9-DiI (D3886), and DiD (D307) are packaged as a sticky oil. The sticky oil residue may be warmed slightly and applied directly to tissue samples with forceps. The tissue should then be warmed to ~40°C to facilitate transport of the dye. *FAST* DiI™, *FAST* DiA™, and DiD are also available in solid form (D7756, D7758, D7757) for direct application to cells or tissue.

Loading by injection. Pressure microinjection of a small bolus of concentrated dye solution is an alternative to direct application of crystalline dye for retrograde and anterograde neuronal tracing.[1,13] A 2.5 mg/mL (0.25% w/v) solution of dye in DMF is typically used. Sonication, centrifugation, or filtration (5 µm pore size) of the concentrated dye solution prior to injection is recommended to remove undissolved dye crystals that might clog the pipette tip. Iontophoretic injection of DiI (5 mg/mL in ethanol) produces precise labeling of small groups of 2–30 cells for lineage tracing studies.[21]

Staining fixed and mounted tissue. Specimens for labeling with dialkylcarbocyanine and dialkylaminostyryl tracers (usually by direct application of dye crystals) are typically fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at ambient temperature.[38] Other fixatives, particularly glutaraldehyde, tend to produce unacceptably high levels of background fluorescence.[3,4] Storage during the time required for diffusive staining of neuronal pathways (typically several weeks) can be at 4°C or ambient temperature. Permeabilizing reagents, detergents, and high concentrations of organic solvents usually result in loss of staining.[3] Tissues stained with DiI and other lipophilic carbocyanines can be sectioned by cryostat or vibratome methods.[38] It is often reported that cryostat sectioning severely degrades the resolution of DiI labeling, but a recent report describes the use of polyethylene glycol (PEG) for this purpose.[39] Avoid mounting media containing glycerol, which can extract membrane-bound dyes.

Labeling cell suspensions or adherent cells. Vybrant® DiI, DiO, and DiD cell-labeling solutions can be added directly to normal culture media to uniformly label suspended or attached culture cells. Cell suspensions or adherent cells on coverslips are incubated with the loading solution for 5 minutes to 2 hours at 37°C. After loading, the cells are spun down, rinsed, and resuspended in fresh medium. For adherent cells, labeling in culture while attached results in improved viability compared to labeling after dissociation.[10] Methods for labeling cultured cells using the Vybrant® DiI, DiO, and DiD cell-labeling solutions are described in instruction manual MP 22885.

Labeling intracellular membranes. DiI and $DiIC_{16}(3)$ can be used to selectively label the endoplasmic reticulum (ER) in living sea urchin and ascidian eggs. A saturated solution of dye in soybean oil is pressure microinjected into the egg and diffusively labels the entire ER network within about 30 minutes.[39]

Combination with Other Labeling Techniques

DiI labeling can be used in combination with immunofluorescent or immunoperoxidase labeling for detailed neuroanatomical studies.[15,38,40-42] The primary limitation is that conventional permeabilization reagents such as Triton® X-100 cannot be used to enhance antibody penetration.[41] An optimized protocol developed by Lukas and co-workers[38] uses 0.3% Tween 20. DiI-sensitized photoconversion of diaminobenzidine (DAB) to an electron-dense precipitate allows long-term preservation of DiI labeling, as well as correlation of fluorescence imaging with transmitted light and electron microscopy.[3,7] Staining may be enhanced by Giesma counterstaining.[43] Typically, tissue sections are pre-incubated in a 2 mg/mL solution of DAB in 0.1 M Tris buffer, pH 8.2 for approximately 60 minutes at 4°C in the dark. Sections are then irradiated for 1–2 hours using the same excitation filter used for fluorescence microscopy, changing the DAB solution every 10–20 minutes.[3,43] Use of higher-magnification objectives (e.g., 20X) decreases the photoconverted area of the specimen but produces better image resolution and sensitivity and also reduces the irradiation time.[43]

References

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Table 2. Spectral characteristics of lipophilic carbocyanine and aminostyryl tracers.

Tracer	Catalog Numbers	Ex * (nm)	Em * (nm)	Optical Filters †	
				Omega	Chroma
DiO	D275, D1125, D3898, D7778, N22881	484	501	XF100, XF23	41001, 31001
DiA	D3883, D291, D7758	456	590	XF21	31024
DiI	D282, D383, D384, D3886, D3899, D7756, D7776, D7777, D12730, D3911, N22880	549	565	XF108, XF32	41002, 31002
DiD	D307, D337, D7757	644	665	XF110, XF47	41008, 31023
DiR	D12731	750	780 ‡	XF112	41009

* Fluorescence excitation (Ex) and emission (Em) maxima for membrane-bound dye. † Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).
‡ Fluorescence emission of this dye is invisible to the human eye and must be detected using a CCD camera or other infrared-sensitive detector.

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

Cat. no.	Product Name	Unit Size
D291	4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-ASP)	25 mg
D383	1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC$_{12}$(3))	100 mg
D3883	4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA; 4-Di-16-ASP)	25 mg
D1125	3,3′-dihexadecyloxacarbocyanine perchlorate (DiOC$_{16}$(3))	25 mg
D7778	3,3′-dioctadecyl-5,5′- di(4-sulfophenyl)oxacarbocyanine, sodium salt (SP-DiOC$_{18}$(3))	5 mg
D384	1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC$_{16}$(3))	100 mg
D7758	4-(4-(dilinoleylamino)styryl)-N -methylpyridinium 4-chlorobenzenesulfonate (FAST DiA™ solid; DiΔ^{9,12}-C$_{18}$ASP, CBS)	5 mg
D3898	3,3′-dilinoleyloxacarbocyanine perchlorate (FAST DiO™ solid; DiOΔ^{9,12}-C$_{18}$(3), ClO$_4$)	5 mg
D7756	1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate (FAST DiI™ solid; DiIΔ^{9,12}-C$_{18}$(3), CBS)	5 mg
D3899	1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (FAST DiI™ oil; DiIΔ^{9,12}-C$_{18}$(3), ClO$_4$)	5 mg
D275	3,3′-dioctadecyloxacarbocyanine perchlorate ('DiO'; DiOC$_{18}$(3))	100 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
D12730	1,1′-dioctadecyl-3,3, 3′,3′-tetramethylindodicarbocyanine- 5,5′-disulfonic acid (DiIC$_{18}$(5)-DS ()	5 mg
D282	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ('DiI'; DiIC$_{18}$(3))	100 mg
D3911	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ('DiI'; DiIC$_{18}$(3)) *crystalline*	25 mg
D7757	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt ('DiD' solid; DiIC$_{18}$(5) solid)	10 mg
D307	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate ('DiD' oil; DiIC$_{18}$(5) oil)	25 mg
D337	4,4′-diisothiocyanatostilbene- 2,2′-disulfonic acid, disodium salt (DIDS)	100 mg
D12731	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide ('DiR'; DiIC$_{18}$(7))	10 mg
D3886	1,1′-dioleyl-3,3,3′,3′-tetramethylindocarbocyanine methanesulfonate (Δ^9-DiI)	25 mg
L7781	Lipophilic Tracer Sampler Kit	1 kit
N22880	NeuroTrace® DiI tissue-labeling paste	500 mg
N22881	NeuroTrace® DiO tissue-labeling paste	500 mg
N22884	NeuroTrace® Multicolor Tissue-Labeling Kit *DiO, DiI, DiD pastes, 500 mg each*	1 kit

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