Lumiprobe
Life science solutions

Staining Cell Membranes with Lipophilic Carbocyanine Dyes

Lipophilic carbocyanine (DiI, DiO, DiD, DiR, etc.) and dialkylaminostyryl (DiA) dyes are fluorogenic membrane probes that exhibit weak fluorescence in aqueous media and intense fluorescence upon incorporation into the lipid bilayer of cell membranes due to their long hydrocarbon "tails". Once incorporated into the membrane, the dyes can diffuse laterally within it, providing uniform, complete staining.

Carbocyanine dyes can be used to stain both live and fixed cells. The label is retained by live cells in culture for weeks. Immediately after staining, the label is predominantly localized on the plasma membrane; however, after a few hours of culturing, labeled membranes are internalized into intracellular vesicles, causing the staining to become predominantly intracellular.

Due to the different cyanine fluorophores in the cores of the DiI family dyes, they emit fluorescence across a range of wavelengths (from green to near-infrared), enabling multicolor cell analysis.

Minimal toxicity and the absence of label transfer between cells allow the use of these dyes in studies investigating cell fusion, membrane internalization, cell adhesion, and migration.

1. Preparation of Stock Solutions

Most lipophilic cyanine dyes are soluble in DMSO, DMF, or ethanol. For cell membrane staining, it is recommended to use stock solutions in ethanol or DMSO at a concentration of 1–2 mM (~1–2 mg/mL).

- Dissolution of the dye usually takes at least an hour with periodic vortexing. To accelerate this process, heating to 55 °C can be used.
- For DiI and Neuro-DiO, vegetable oil can be used as a solvent. A stock concentration of 1–2 mM is typically prepared by heating to 55 °C and ultrasonic treatment for 30 minutes or more.
- DiO is highly hydrophobic, and DMF is preferable for preparing its stock solution; however, if experimental conditions do not allow it, DMSO and ethanol can be used:
  - 2 mM (1.76 mg/mL) in DMF with heating to 55 °C and vortexing;
  - 1 mM (0.88 mg/mL) in DMSO with heating to 55 °C and vortexing;
  - 2 mM in DMSO:EtOH 1:1 mixture (first add DMSO to the dye, mix, then add an equal volume of ethanol, heat to 55 °C with periodic vortexing).
- Storage of stock solutions: at 4 °C in a dark place for up to 12 months. Crystals may appear in the stock solution during storage. If they appear, heat the solution to 55 °C or sonicate until the crystals are completely dissolved.
- Dye solutions in vegetable oil should be stored at room temperature, protected from light. Freezing is not recommended.

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2. Preparation of Working Solutions

- The final concentration of the working solution should be determined empirically for different cell types and/or experimental conditions.
- Dilute the stock solution in a suitable buffer, for example, serum-free culture medium, HBSS, or PBS, to obtain a working concentration of 1–10 μM. Mix the resulting solution well.

Important! Serum proteins and lipids in the medium can bind the dye, thereby reducing its effective concentration. Divalent cations (Ca^{2+}, Mg^{2+}) can promote dye precipitation; therefore, it is recommended to use buffers that do not contain them.

3. Staining Live Cells in Suspension

1. Suspend cells at a density of 1×10^6/mL in the dye working solution.

   (Optional) The dye stock solution can be added directly to the cell suspension, followed by thorough mixing by gentle pipetting.
2. Incubate for 20 minutes at 37 °C. The optimal incubation time for uniform labeling varies by cell type and should be determined empirically.
3. Centrifuge at 1000–1500 rpm for 5 minutes.
4. Remove the supernatant and gently resuspend the cells in pre-warmed (37 °C) culture medium.
5. Repeat centrifugation and washing twice.
6. Cells can be visualized directly in the culture medium.

4. Staining Live Adherent Cells

1. Grow cells on sterile coverslips.
2. Remove the culture medium.
3. Add the dye working solution in a volume sufficient to completely cover the cells.
4. Incubate the cells for 20 minutes at 37 °C. The optimal incubation time for uniform labeling varies by cell type and is determined empirically.
5. Remove the dye solution.
6. Wash the cells by adding fresh warm culture medium and incubating at 37 °C for 5 minutes. Repeat this wash twice.
7. Cells can be visualized directly in the culture medium.

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5. Post-staining Cell Fixation

- Cells stained with carbocyanine dyes should be fixed with 2–4% paraformaldehyde (PFA).
- Fixation with methanol, acetone, or other organic solvents extracts lipids, resulting in poor cell staining.
- Glutaraldehyde is not recommended for cell fixation, as even its low concentrations (0.5%) can enhance autofluorescence and affect the interpretation of subsequent immunocytochemical analysis.

6. Staining Fixed Cells

1. Fix cells with PFA.
2. Wash cells several times with PBS.
3. Replace the buffer with the dye working solution prepared in PBS.
4. Incubate for 10 minutes or longer at room temperature in the dark. The optimal incubation time for uniform labeling varies with cell type and fixation quality and should be determined empirically.
5. Wash cells 3 times with PBS.
6. Visualize cells in PBS. Do not use mounting media, as their components can wash the dye out of the membranes.

7. Combination with Immunocytochemical Staining

- Because lipophilic dye staining is sensitive to detergent treatments, it is recommended to perform fixation, permeabilization, and immunocytochemical procedures first, followed by lipophilic dye staining.
- It is recommended to permeabilize cells with 0.1% Triton® X-100 in PBS for 10 minutes at room temperature. This method better preserves plasma membrane staining than permeabilization with digitonin or saponin.
- It is not recommended to use detergents in blocking buffers, antibody dilution buffers, or wash buffers.
- If the use of detergents for immunocytochemistry is unavoidable, it is better to use the chloromethyl derivative CM-DiI instead of regular lipophilic dyes. Upon aldehyde fixation, CM-DiI covalently binds to thiol-containing peptides and proteins, providing labeling that is resistant to detergent and organic solvent treatments.

8. Mounting Samples for Visualization

- Do not use a mounting medium for mounting cells stained with carbocyanine dyes. Glycerol or organic solvents in mounting media wash them out of membranes, leading to high background and increased intracellular staining.
- For mounting cells, use PBS or another aqueous buffer. Coverslips should be sealed using sealant or nail polish. Stained samples can be stored in PBS at 4 °C for several weeks.
- If mounting cells in specialized media or optical clearing agents is required, use CM-DiI followed by fixation instead of regular carbocyanine dyes.

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9. Detection

Microscopy:

The choice of filter sets for DiD, DiO, DiI, and DiR (and their derivatives) is based on the spectral properties of the dyes:

Dye	Max. excitation, nm	Max. emission, nm	Channel
DiO	487	501	Green
Neuro-DiO	497	514	Green
RAPID DiO	487	501	Green
DiA	492	607	Yellow-green
DiI	551	566	Orange-red
RAPID DiI	551	565	Orange-red
CM-DiI	556	571	Orange-red
DiD	647	666	Far-red
DiR	750	775	Near-infrared

For simultaneous detection of multiple dyes, multi-band filter sets are available:

- DiI and DiO: Omega XF52, Chroma 51004
- DiI and DiD: Omega XF92, Chroma 51007
- DiI, DiO, and DiD: Omega XF93, Chroma 61005

Flow Cytometry:

Cells labeled with DiO, DiI, and DiD (and their derivatives) can be analyzed using standard flow cytometer detection channels FL1, FL2, and FL3, respectively.

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