Labeling Pinocytotic Vesicles and Cytoplasm with Fluorescently Labeled Ficoll or Dextran

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot5572

Protocol

Labeling Pinocytotic Vesicles and Cytoplasm with Fluorescently Labeled Ficoll or Dextran

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INTRODUCTION

Dextran, a high molecular weight poly-D-glucose, and ficoll, a synthetic polymer of epichlorhydrin and sucrose, are electroneutral hydrophilic polysaccharides whose size can be varied. This variability in size coupled with their membrane impermeability can make them useful for studying fluid phase pinocytosis, the size of membrane pores such as nuclear membrane pores, or the environmental conditions and the size of a cell compartment. In this protocol, two fluorophores based on fluorescein and rhodamine are used to label either dextran or ficoll. The resulting probes can then be used to label cells.

RELATED INFORMATION

For more detailed information, see Luby-Phelps (1989) and Berlin and Oliver (1980) on probe use and preparation. A protocol for **Mounting Live Cells onto Microscope Slides** (Chazotte 2011) is also available.

This protocol assumes that cells were grown on glass microscope coverslips and immersed in small Petri dishes containing culture medium. At no time in the following protocol should the cells be allowed to dry out. Generally, labeling conditions vary by cell type, and readers may find it necessary to alter the protocol for their own particular use.

Note that autofluorescence, from endogenous cellular molecules, such as NADH (nicotinamide adenine dinucleotide, reduced form) or $FADH_2$ (flavin adenine dinucleotide, reduced form), can interfere with imaging by reducing the signal-to-noise ratio. This occurs when the excitation and/or emission wavelengths of the probe and the autofluorescing molecules are similar. This occurs frequently with excitation wavelengths <500 nm, particularly at ultraviolet (UV) wavelengths. Autofluorescence can be reduced by careful selection of the excitation and the emission wavelengths used, by treatment of fixed cells with reducing agents such as $NaBH_4$ (1% solution for 20 min), and by comparison between the experimental images and the unlabeled control slides. Avoid fixation with glutaraldehyde because it can increase interference from cellular autofluorescence, most frequently at wavelengths <500 nm.

MATERIALS

CAUTIONS AND RECIPES: Please see the end of this protocol for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Aminoethylcarboxymethyl (AECM)-ficoll or AECM-dextran
<**R**>Carbonate-bicarbonate buffer stock (pH 9.2)

Adapted from *Imaging: A Laboratory Manual* (ed. Yuste). CSHL Press, Cold Spring Harbor, NY, USA, 2010.
Cite as: Cold Spring Harb Protoc; 2011; doi:10.1101/pdb.prot5572

Cells of interest, grown on coverslips
Complete medium (e.g., Dulbecco's modified Eagle's medium [DMEM] containing 10% fetal bovine serum [FBS])
<!>Dimethylformamide (DMF, for labeling with TRITC; see Step 2.i)
<!>Fluorescein isothiocyanate (FITC), MW = 389.38 (Invitrogen/Molecular Probes F-143)

<!>Tetramethylrhodamine-5-[and-6]-isothiocyanate (TRITC), MW = 443.52 (Invitrogen/Molecular Probes T-490 may be used instead of FITC.

Alternatively, commercial versions of fluorescently labeled dextrans and ficolls are available.

<!>NaOH (0.1 N)
<!>Paraformaldehyde (4%)
<R>Phosphate-buffered saline (PBS)

Prepare PBS with added $CaCl_2$ and $MgCl_2$ (PBS^+). This solution allows cells to adhere to each other and to the substrate. If cells are in medium containing no Ca^{2+} or Mg^{2+}, they will round up and detach from the substrate.

Sephadex G-25

Equipment

Beaker (1-L)
Cell culture dishes, sterile
Chromatography column (1 × 30-cm)
Dialysis tubing
Incubator preset to 37°C
Lyophilizer
Magnetic stirrer
Microscope, fluorescence, equipped with a fluorescein (for FITC) or rhodamine (for TRITC) filter set

<!>For fluorescence recovery after photobleaching (FRAP) and confocal microscopy laser excitation, use the following lasers:

For FITC: 488-nm line argon-ion laser

For TRITC: 514-nm line argon-ion laser or 543-nm line of a green He:Ne laser

Stir bar
Water bath preset to 40°C

METHOD

FITC or TRITC Labeling of AECM-Ficoll or AECM-Dextran

This portion of the protocol is based on the Inman method as per Luby-Phelps (1989) and can be used if commercially labeled material is unavailable.

1. Prepare 100 mg of AECM-dextran or AECM-ficoll in 2 mL of 10 mM carbonate-bicarbonate buffer (pH 9.2).
2. Prepare 15 mg of dye in 1 mL of 10 mM carbonate-bicarbonate buffer, as follows:

For TRITC

i. Add 15 mg of TRITC to 400 mL of DMF.
ii. Titrate the solution into 1 mL of 10 mM carbonate-bicarbonate buffer with constant stirring.
iii. Adjust the pH to 9.0 with 0.1 N NaOH.

For FITC

iv. Add 15 mg of FITC to 1 mL of 10 mM carbonate-bicarbonate buffer with stirring.

v. Continue stirring as the pH is adjusted to 9.0 with 0.1 N NaOH.

It is likely that the FITC will not completely dissolve until the proper pH has been reached.

3. Add the desired dye solution in a dropwise manner with constant stirring to either the ficoll or the dextran solution.
4. Incubate the resultant solution for 30 min at 40°C.
5. Use a 1 × 30-cm column of Sephadex G-25 to remove unreacted dye by desalting.
6. Dialyze the fluorescently labeled material twice versus 1 L of H_2O.
7. Lyophilize the material for storage, and protect it from light at –20°C until used.

Pinocytotic Studies

8. Prepare 5 mg/mL of labeled dextran or ficoll in a complete cell medium (e.g., DMEM with 10% FBS).
9. Immerse the cells grown on glass coverslips with labeling medium in a small Petri dish. Incubate for 30 min at 37°C.

   Pinocytotic uptake is concentration, time, and cell cycle dependent. There is greater uptake during mitosis than during interphase.
10. Prepare cells for imaging:

   For fixing cells

   i. Aspirate off the labeling medium, and wash the cells three times with PBS^+.

   ii. Immediately incubate the cells with 4% paraformaldehyde for 10 min.

   For live cells

   iii. Wash the cells three times with DMEM containing 10% FBS.
11. Mount fixed or live cells as described in **Mounting Live Cells onto Microscope Slides** (Chazotte 2011).
12. Image the cells.

   For FITC-labeled polysaccharides, $\lambda_{excitation}$ maximum ~460 nm and $\lambda_{emission}$ maximum ~534 nm (using methanol as the solvent). The emission spectrum of fluorescein depends on its local pH and ionic strength.

   For TRITC-labeled dextran, $\lambda_{excitation}$ maximum ~555 nm and $\lambda_{emission}$ maximum ~580 nm.

REFERENCES

Berlin RD, Oliver JM. 1980. Surface functions during mitosis. II. Quantitation of pinocytosis and kinetic characterization of the mitotic cycle with a new fluorescence technique. *J Cell Biol* **85:** 660–670.

Chazotte B. 2011. Mounting live cells onto microscope slides. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot5554.

Luby-Phelps K. 1989. Preparation of fluorescently labeled dextrans and ficolls. *Methods Cell Biol* **29:** 59–74.

RECIPES

[**NOTE:** Recipes for reagents marked with the <**R**> symbol not listed below can be found online at http://www.cshprotocols.org/recipes.]

Carbonate-bicarbonate buffer stock (pH 9.2)

MATERIALS

Reagents

<**!**>Sodium carbonate, anhydrous
Sodium bicarbonate

METHOD

1. Prepare a 0.2-M solution of anhydrous sodium carbonate (2.2 g/100 mL).
2. Prepare a 0.2-M solution of sodium bicarbonate (1.68 g/100 mL).
3. Combine 4 mL of carbonate solution from Step 1 and 46 mL of bicarbonate solution from Step 2.
4. Bring to 200 mL with H_2O.

Final pH will be 9.2.

Phosphate-buffered saline (PBS)

Reagent	Amount to add (for 1X solution)	Final concentration (1X)	Amount to add (for 10X stock)	Final concentration (10X)
NaCl	8 g	137 mM	80 g	1.37 M
<!>KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM
If necessary, PBS may be supplemented with the following:				
<!>$CaCl_2 \cdot 2H_2O$	0.133 g	1 mM	1.33 g	10 mM
<!>$MgCl_2 \cdot 6H_2O$	0.10 g	0.5 mM	1.0 g	5 mM

<!>PBS can be made as a 1X solution or as a 10X stock. To prepare 1 L of either 1X or 10X PBS, dissolve the reagents listed above in 800 mL of H_2O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization. Store PBS at room temperature.

CAUTIONS

[**NOTE:** For reagents marked with the <!> symbol not listed below, please consult the manufacturer's Material Safety Data Sheet for further information.]

$CaCl_2$ (Calcium chloride)

$CaCl_2$ (Calcium chloride) is hygroscopic and may cause cardiac disturbances. It may be harmful by inhalation, ingestion, or skin absorption. Do not breathe the dust. Wear appropriate gloves and safety goggles.

Dimethylformamide (DMF, *N,N*-Dimethylformamide, $HCON[CH_3]_2$)

Dimethylformamide (DMF, *N,N*-dimethylformamide, $HCON[CH_3]_2$) is a possible carcinogen and is irritating to the eyes, skin, and mucous membranes. It can exert its toxic effects through inhalation, ingestion, or skin absorption. Chronic inhalation can cause liver and kidney damage. Wear appropriate gloves and safety glasses. Use in a chemical fume hood.

Fluorescein isothiocyanate (FITC)

Fluorescein isothiocyanate (FITC) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

HCl (Hydrochloric acid, Hydrochloride)

HCl (hydrochloric acid, hydrochloride) is volatile and may be fatal if inhaled, ingested, or absorbed through the skin. It is extremely destructive to mucous membranes, upper respiratory tract, eyes, and skin. Wear appropriate gloves and safety glasses. Use with great care in a chemical fume hood. Wear goggles when handling large quantities.

KCl (Potassium chloride)

KCl (Potassium chloride) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Laser radiation

Laser radiation, both visible and invisible, can be seriously harmful to the eyes and skin and may generate airborne contaminants, depending on the class of laser used. High-power lasers produce permanent eye damage, can burn exposed skin, ignite flammable materials, and activate toxic chemicals that release hazardous by-products. Avoid eye or skin exposure to direct or scattered radiation. Do not stare at the laser and do not point the laser at someone else. Wear appropriate eye protection and use suitable shields that are designed to offer protection for the specific type of wavelength, mode of operation (continuous wave or pulsed), and power output (watts) of the laser being used. Avoid wearing jewelry or other objects that may reflect or scatter the beam. Some non-beam hazards include electrocution, fire, and asphyxiation. Entry to the area in which the laser is being used must be controlled and posted with warning signs that indicate when the laser is in use. Always follow suggested safety guidelines that accompany the equipment and contact your local safety office for further information.

$MgCl_2$ (Magnesium chloride)

$MgCl_2$ (magnesium chloride) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

NaOH (Sodium hydroxide)

NaOH (Sodium hydroxide) and solutions containing NaOH are highly toxic and caustic and should be handled with great care. Wear appropriate gloves and a face mask. All concentrated bases should be handled in a similar manner.

Paraformaldehyde

Paraformaldehyde is highly toxic and may be fatal. It may be a carcinogen. It is readily absorbed through the skin and is extremely destructive to the skin, eyes, mucous membranes, and upper respiratory tract. Avoid breathing the dust or vapor. Wear appropriate gloves and safety glasses and use in a chemical fume hood. Keep away from heat, sparks, and open flame.

Sodium carbonate (Na_2CO_3)

Sodium carbonate (Na_2CO_3) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

Tetramethylrhodamine isothiocyanate (TRITC)

Tetramethylrhodamine isothiocyanate (TRITC) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.