Chapter 17

Ligand Acidification by Nonadherent Cells

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Introduction

The goal of methods for ligand acidification by nonadherent cells is to obtain nearly continuous measurements of the pH to which a receptor-bound ligand is exposed after endocytosis. The protocols presented below are based on the method developed by Sipe and Murphy [1] for adherent (BALB/c3T3) cells and modified by Sipe et al. [2] for nonadherent (K562) cells. Reviews of acidification of endocytic compartments [3, 4] and flow cytometric methods for analysis of endocytosis [5, 6] may be consulted for additional background. The protocols below describe analysis of transferrin (Tf) acidification, but they may be easily modified for use with a different ligand.

Materials

Fluorescent transferrin conjugates

The ligand to be used must be conjugated with a pH sensitive fluorescent probe, normally fluorescein isothiocyanate (FITC), and, separately, with a pH-insensitive probe, such as lissamine rhodamine sulfonyl chloride (LRSC) or Cy5. Such conjugates may be purchased (Molecular Probes, Eugene, OR) or prepared following standard procedures (see [1] for preparation of Tf conjugates). Conjugates with stable, small molecular weight dyes are preferable since some larger probes (e.g. phycobiliproteins) may be unstable or undergo changes in properties after internalization and acidification in endosomes and lysosomes. Best results are usually obtained with a dual-laser flow cytometer equipped with an argon laser to excite FITC at 488 nm (530-nm emission filter with 30-nm bandwidth) and a krypton laser or

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dye laser to excite LRSC at 568 nm (625-nm emission filter with 35-nm bandwidth) or a krypton laser or He-Ne laser to excite Cy5 at 647 nm or 633 nm. Stock solutions of Tf conjugates at 1 mg/ml are normally prepared. Each conjugate should be tested for specificity of binding to competition with unlabeled ligand and an optimal labeling concentration chosen. For Tf, this should be 5-10 µg/ml. For the protocols below, a 10 mg/ml stock solution of unconjugated diferric human Tf in phosphate-buffered saline (PBS) is also needed to confirm the specificity of the fluorescence conjugates.

Standard pH buffers are used for calibrating the pH dependence of the fluorescence of ligand conjugates. All contain 50 mM hydroxyethyl-piperazine ethanesulfonic acid 50 mM 2-[N-morpholino] ethane sulfonic acid (MES), 50 mM NaCl, 30 mM NH₄Ac, and 40 mM NaN₃. The pH calibration curve requires (at a minimum) buffers adjusted to pH 4.00, 4.25, 4.75, 5.00, 6.00, 6.50, 7.00, 7.25, 7.50, and 8.00 (all when measured at 0 °C).

- PBS containing 8 mM NaH₂PO₄, 2.7 mM KCl, 140 mM NaCl, and 1.5 mM KH₂PO₄ (adjusted to pH 7.4) is used for washing cells. The appropriate base salt solution for a given cell type may be substituted (e.g., α-minimum essential medium salts).
- RPMI (or other growth medium; without serum) is used for cell labeling.

### Procedure

Measurement of acidification kinetics for internal ligand requires completion of all four protocols below. The common labeling procedure, which is the starting point for each of these protocols, is described first.

#### Cell labeling

1. Collect cells and wash twice with PBS at room temperature.
2. Resuspend cells in a small volume of RPMI (without serum).
3. Count cells.
4. Dilute cells to 10⁷/ml with RPMI and place on ice.
5. The cells to be used for one of the protocols should be labeled together. Place the required number of cells in an Eppendorf or other conical tube on ice. Determine the volume(s) of labeled Tf(s) and the additional volume of RPMI needed for a final concentration of 5 x 10⁶ cells/ml and a