Transferrin Uptake Assays

Transferrin Recycling using Fluorescence Microscopy

1. Culture cells on coverslips to 60-70% confluence.
2. Starve cells for 30 mins at 37˚C in pre-warmed serum-free medium supplemented with 25 mM HEPES pH7.4 and 0.5% (w/v) BSA medium. This can be done on coverslips by placing them face up on parafilm in a dish and dropping 65 µl of medium on top.
3. Pulse cells with labeled Tfn (biotinylated, FITC, Alexa dye, etc.) in the same serum-free medium/ 0.5% BSA/ HEPES at 37˚C. Be sure to use the appropriate species Tfn – mouse cells bind poorly to human transferrin and vice versa. The range of concentration is usually 20-50 µg/ml. Incubation time depends on the purpose:

   - to label sorting endosomes only, incubate 5-10 min at 37°C.

   - to label recycling endosomes only, wash and chase as in step 4

   - to label the entire early endosomal compartment, incubate 30-45 min
4. If desired, chase cells in regular pre-warmed medium **WITH SERUM** for various time points at 37˚C (a 30 min chase will visualize recycling endosomes; a time course of chase will allow you to determine recycling kinetics).
5. Wash cells by dipping in warm medium or PBS 10X rapidly, and then put coverslip in fixative (formaldehyde, paraformaldehyde, or glutaraldehyde as needed). Then proceed for fluorescence microscopy or permeabilize and proceed with immunofluorescence with antibodies against proteins of interest.

The protocol below was set up by Anand, modified by Mickey.

2. Tansferrin recycling trafficking using FACS analysis (quantitative)

(How many cells shall be used? What centrifugation speeds?—just the normal speed?) *generally for FACS we like to have 5 x 10^5 cells per sample for analysis. Remember that for FACS you will need to have a sample that is unlabeled to set the parameters for the instrument. Centrifugation is just as you would for any assay in which you do not want to kill the cells. Typically around 200-300g for HeLa, NRK, or most other cultured cells. Also be aware that the cells at the time of analysis need to be in 12x75mm plastic tubes to use in the flow cytometer. You can either do all of your incubations/ washes in these tubes or transfer them to the tubes at the end, whichever is easier.*

----- *Endocytosis assay*

1) Wash Hela cells with PBS and suspend the cells with ~~Trypsin~~ (?)/EDTA. *NO, definitely no trypsin – this will likely chew off your transferrin receptor from the cell surface. Incubate cells in PBS containing 5-10mM EDTA. They will round up and you can then wash them off the plate. For HeLa cells, it takes about 5 min at 37˚C; I think COS cells are more sticky and may take longer. Some cells are very quick (our melanocytes round up in seconds).* Then transfer the cells to test tubes.
2) Starve cells for 30 mins in serum-free, 25mM HEPES,

0.5% BSA medium at 37°C.

3) Pulse cells at 4°C with ~~FITC-Tfn or PE-Tfn~~ **biotinylated-transferrin** *for internalization assay, you need something that will disappear from the cell surface. fluorochrome-conjugated stuff won't work – it may indeed be quenched in endosomes, but not completely and not synchronously. Thus, use biotinylated stuff, which you can then follow its disappearance using PE- or FITC-conjugated streptavidin* in DMEM, 0.5% BSA for 30 min (FITC *fluorescence* is ~~unstable~~ *quenched* in acid pH of the endosomes *– it's the quenching and unquenching upon deacidification that complicates this assay using fluorochrome-conjugated stuff*).

4) Change the media to regular medium (serum supplemented) ~~with holo-Tfn~~ and shift the temperature to 37°C. Chase the cells in various time points (0, 1', 2', 5') and place the tubes in ice to stop trafficking.

5) Stain cells with FITC- or PE-conjugated streptavidin. Wash 2X with PBS. Proceed for FACS.

----- *Recycling assay*

1) Wash Hela cells with PBS and then suspended with ~~Trypsin (?)~~PBS/EDTA. And transfer the cells to test tubes.

2) Starve cells for 30 mins in serum-free, 25mM HEPES, 0.5% BSA medium at 37°C.

3) Pulse cells at 37 °C with Alexas-Tfn for 30 mins (Alexa dyes are insensitive to pH in the endosomal compartments). *just beware here – the flow cytometer, at least the inexpensive one here, is not able to detect Alexa594, which we use a lot for IFM.*

4) Wash cells with PBS on ice.

5) Warm up to 37°C in regular media containing ~~holo-Tfn~~ serum. Chase at different time points (0', 5', 10', 15', 20', 30') and place the cells on ice to terminate trafficking. Wash 2X with PBS (just to ensure that you get rid of released Tf).

6) Proceed for FACS. If FACS facility is not ready, fix the cells for future FACS analyses.