

### Receptor Endocytosis

1. Prior to biotinylation, cells were treated with 100ug/ml of the lysosomal protease inhibitor leupeptin for 1hr.
2. Cool cells on ice.
3. Wash cells three times with ice-cold PBS containing 1 mM MgCl<sub>2</sub> and 0.1 mM CaCl<sub>2</sub> (PBS/CM) to remove any contaminating proteins.
4. Add 1 mg/ml of Sulfo-NHS-SS-Biotin (Pierce) per ml of reaction volume(3ml for 100mm dish; 1ml for 60mm dish) , incubated at 4C for 30 min.
5. Cells were then incubated at either 4C to block membrane trafficking ( as background)or37C for various times to allow endocytosis to occur.
6. Wash cells once with ice-cold PBS/CM and quench by incubation with 0.1 M glycine, followed by three washes in ice-cold PBS.
7. Cleave the remaining surface biotin by reducing its disulfide linkage with glutathione cleavage buffer (50mM glutathione in 75 mM NaCl , 10 mM EDTA , 1% BSA, and 0.075 N NaOH) (two times for 15 min each at 4C).
8. Wash cells three times with ice-cold PBS.
9. Cells were harvested in modified RIPA buffer.
10. Centrifuge at 14,000 rpm for 15 min at 4c.
11. The resulting supernatant was incubated with 100 ul of 50% streptavidin agarose (Molecular Probes, Eugene, Oregon) with rotating overnight at 4c.
12. Beads were washed five times with RIPA buffer, bound proteins were eluted with SDS sample buffer by boiling for 5 min.
13. Total protein and isolated biotinylated proteins were analyzed by immunoblotting.