Handouts: 7A -- Sorting of Proteins (Overview) & Cross section of a capillary
7B -- Labeling

You will also need Handout 6B.

I. Capillary Structure (review) -- see handout 7A, bottom. (And PPT slides 1 & 2 for lecture #6.) Here are links for a diagram of a capillary, a diagram of transcytosis, and an electron micrograph of a capillary.

   A. Pores -- How small molecules get in and out by simple diffusion through liquid (in pores) between cells.

   B. Transcytosis -- How proteins (macromolecules) get in and out by going across (through) cells. 7A shows transcytosis from inside of capillary to outside. More on transcytosis below.

   C. Terminology -- epithelial cells around blood vessels are called endothelial cells

II. Ways that Big Molecules Enter (Eukaryotic) Cells -- Types of Endocytosis. In all cases net effect is that cell membrane folds in and pinches off, forming a vesicle in the cytoplasm that contains material from the outside, as diagrammed before.

   A. Pinocytosis = bulk phase endocytosis; no receptor. Cells take in random samples of surrounding fluid containing a random selection of extracellular substances.

   B. Phagocytosis -- -- extensions of cells (pseudopods) reach out and engulf solids. See Becker fig. 12-14. Vesicle that is formed is called a phagocytic vesicle (or vacuole) or phagosome.
      * Requires MF
      * Occurs in specialized cells only

   C. RME = receptor mediated endocytosis. Cells take in specific substances from surrounding fluid using a receptor. See Becker fig. 12-15 (diagram) & 12-16 (micrograph). Different cell types have different combinations of receptors.

III. RME -- Receptor Mediated Endocytosis (See Handout 6B)

   A. General and/or important Features.
      1. It's a cycle -- Exocytosis balances endocytosis so cell surface area stays the same. See Sadava fig. 6.16 (6.18) or Becker fig. 12-15. For LDL receptor, it takes about 10-20 minutes for one "round trip."
      2. Receptors -- Need specific receptor for each substance (or class of closely related substances) to be transported -- substance must bind to its receptor.
      3. Concentrates substances transported -- usually moves them up their gradient.
      4. Requires energy -- multiple stages in process use ATP or GTP. Energy must be required because substances move against their gradients. Energy is required to form the vesicles and to process and/or transport the vesicles inside the cell.
5. Topology -- material can enter and/or exit cell without being in contact with cytoplasm. Material can remain inside a vesicle or outside cell at all times. (See Transcytosis.)

6. Possible fates of endocytosed material -- Where does vesicle go? Where do receptor &/or ligand end up?

   a. Degradation -- vesicle fuses with lysosomes and contents are degraded.

   b. Return to surface -- material recycles -- vesicle fuses with plasma membrane.

   c. Sorting -- not everything in the vesicle may go to the same place. Vesicle may fuse with endosome (sorting vesicle), and different parts of the endocytosed material may be directed to different destinations. (More details on a-c below.)

   d. Transcytosis -- vesicle crosses cell and fuses with plasma membrane on opposite cell surface. See handout 7A, bottom.

      (1). Requires Receptor: Transcytosis requires a receptor for each substance transported.

      (2). Transcytosis = endocytosis + exocytosis = 2 steps

         (a). Material binds to receptor and is endocytosed on one surface of the cell.

         (b). Vesicle moves across cell and material is exocytosed on a different surface.

      (3). Function. Can be used to move proteins, across a layer of cells, in either direction. Examples:

         (a). How proteins (macromolecules as vs small molecules) enter or exit capillaries.

         (b). How antibodies cross epithelial layers in the body (to enter secretions, or exit lumens, etc.).

         For diagrams of transcytosis by antibodies, see figures from MBOC (Alberts) or from Lodish.

Question: In diagram on 7A, a receptor is drawn only once. Where else does it belong?

7. Role of coat protein -- A peripheral membrane protein is always needed to deform a membrane and allow vesicles to form -- it provides a coat. (See Becker figs. 12-15 to 12-18 and/or Sadava fig. 6.17 (6.19) Other proteins are required as well to complete cycle, but will not be discussed.

   a. Clathrin is the coat protein for vesicles forming from plasma membrane and trans-Golgi*.

*Note on terminology: Trans side of Golgi = "far end" = side away from nucleus and ER = last part that proteins travel through as they are processed in Golgi. Also called "TGN" for "trans Golgi Network." See Sadava fig. 5.10 (5.11) or Becker fig. 12-8 for labeled pictures. More details on structure and function of Golgi later.

   b. Budding of other membranes involve different "coat" proteins. Best known are COPI & COPII which are involved in ER-Golgi transport. (See Becker for details if interested.)
Types of coats are summarized in table 12-2.)

B. Stages of Cycle (Numbers match steps on Handout 6B.)

1. **Receptors bind material** (ligand) to be internalized

2. **Receptors are in (or migrate to) coated pits** = clathrin-coated parts of membrane

3. **Membrane starts to invaginate** to form coated vesicle. A single vesicle can contain more than one type of receptor plus ligand.

4. **Coated vesicle forms** (pinching off of vesicle is an energy requiring step)

5. **Uncoating occurs** relatively quickly (uncoating requires energy) -- coat protein (clathrin) is recycled.

6. **Vesicle is acidified** to become endosome (or fuses with pre-existing endosome), and sorting of receptor(s) and ligand(s) begins.
   - A single endosome may contain many different receptors and ligands, and different ones are sorted differently. (Some examples are given in detail below.)
   - Terminology: The uncoated, acidified vesicle can be called an endosome, early endosome, or a sorting vesicle.
   - Acidification requires energy to run proton pump -- to move $\text{H}^+$ into vesicle at expense of ATP. Pump is in membrane of vesicle.

   **Note:** Details of sorting and recycling -- the remaining steps -- vary with material endocytosed. More details below for individual cases.

7. **Endosome splits.** The substance we are following, and/or its receptor, can end up in either half.

   In example shown on handout, one half gets the receptor and one half gets the ligand, as is the case for LDL. Other examples will be discussed in class and are outlined in detail below.

   Note: endosome may not simply split in one step; process of sorting may be gradual. Pieces of different composition may gradually bud off as internal composition of remainder changes.

8. **What Happens to the Different Parts of the Endosome?**

   8A. Fate of vesicle with materials to be recycled (receptors and/or carriers) -- this vesicle fuses with plasma membrane in step 9. (In case of LDL, this vesicle would contain the receptor for LDL.)

   8B. Fate of vesicle with material that remains inside the cell -- Vesicle delivers contents to appropriate cell compartment. (For LDL, vesicle delivers LDL to lysosomes, so material is degraded.)

9. **Exocytosis occurs** -- returns receptors and/or other components to the plasma membrane or outside of cell.

   **Try Problem 2-6.**

C. Some Specific Examples — Any parts of this not covered will be done in #7.

1. **LDL (Low density lipoprotein)** -- receptor recycled, but ligand (including protein part) degraded. For a diagram of the cycle, see the diagram from *Lodish*, or See Becker, Box 12B or text of Sadava Ch. 51.4. Many of LDL
a. What is LDL? A lipoprotein particle containing cholesterol esters + some other lipids + a protein. Particle contains esterified cholesterol covered by monolayer of amphipathic lipid (phospholipid plus some unesterified cholesterol) + one molecule of protein (apoB). Click here for a picture of LDL or see slides 3 & 4.

b. Why LDL?

(1) Why a monolayer on outside? Solubility. Cholesterol is insoluble in blood. (Too hydrophobic.) Need a way to ferry cholesterol through blood and into cell -- Cholesterol transport requires formation of particle with hydrophilic surface.

(2) Why a protein (apoB)? For binding to cell surface receptor (LDL receptor). A protein is needed as ligand to bind to receptor.

(3) Summary of Roles of Parts of LDL:

(a). Protein (apoB) = Ligand = what actually binds LDL receptor = protein part of LDL

(b). Cholesterol -- What cell actually needs is the cholesterol part (for building its membranes &/or hormone synthesis).

c. Receptor, but not protein part of LDL, is recycled. Note: there are 2 separate proteins here that are easily confused

(1) Receptor protein on the cell surface = LDL receptor = binds LDL and allows uptake of cholesterol

(2) Protein in LDL (apoB) = ligand for LDL receptor = part of LDL and helps carry cholesterol through the blood.

d. Receptor and apoB are separated inside sorting vesicles/endosomes. All of LDL (including protein) stays together; separates from receptor

e. Need lysosomes to degrade LDL protein and release cholesterol (cholesterol esters in LDL must be split for cholesterol to be used). How does LDL reach lysosomes? Through fusion of vesicles. Vesicles/endosomes holding sustrate fuse with either of the following:

(1). Pre-existing lysosomes, or

(2). Vesicles from Golgi carrying newly made hydrolases that will form new lysosomes. (More details on how hydrolases pass through the Golgi and are targeted to lysosomes to be discussed later.)

f. Function of LDL uptake -- to supply a nutrient (cholesterol), and to remove cholesterol from the blood. Defects in LDL uptake cause hypercholesterolemia -- high cholesterol in the blood, which causes premature heart attacks if not treated. (See Becker, Box 12B.)

g. Current terminology: relationship of early endosomes, late endosomes & lysosomes. Note: Most of this is FYI. In this course, the term "endosomes" will be used for both early and late endosomes.

(1). Early endosome = sorting vesicle. Term is used differently by different authors. Can be "early" on pathway into cell (by endocytosis) and/or "early" on
pathway from Golgi to lysosomes. Therefore, early endosomes can mean:

(a) Uncoated & acidified vesicles from invagination of plasma membrane carrying newly endocytosed material,

(b). Vesicles coming from Golgi carrying newly made proteins (more on this later).

2. Late endosome = vesicle containing hydrolytic enzymes destined for lysosomes (but not yet activated) plus potential substrate. More acidic than early endosome. Material not destined for lysosomes has been jettisoned. Formed by maturation of early endosome.

3. Lysosomes = vesicle containing active hydrolytic enzymes and substrate. More acidic than late endosome. Formed by maturation of late endosome and/or fusion with pre-existing lysosome.

4. Older terminology found in some texts (FYI only):

(a). Primary lysosome = vesicle with hydrolytic enzymes only.

(b). Secondary lysosome = enzymes + substrate = result of fusion of primary lyso. + another vesicle containing substrate.

2. EGF (Epidermal Growth Factor) -- all the protein involved (ligand + receptor) is degraded

a. No separate protein ligand required; EGF is a protein -- unlike cholesterol, or Fe (see case below). EGF itself binds to receptor = ligand for cell surface receptor & substance that will be transported into the cell.

b. Function of uptake -- to regulate signaling. EGF is a signaling molecule. Uptake turns off signal and down regulates receptors (reduces # of cell surface receptors).

c. Receptor not recycled -- Ligand (signal molecule) and receptor degraded together.

d. Need lysosomes (to degrade both receptor and ligand).

3. Fe/Transferrin -- none of the protein involved is degraded -- all recycled. For a diagram of the cycle see Lodish, or slide 3.

a. What is transferrin? Fe needs a protein (like cholesterol needs apoB) for transport and binding to receptor; protein (= ligand for cell receptor) is called (apo)transferrin.

b. Both apotransferrin & receptor are recycled.

c. No lysosomes needed -- iron is transported out of endosome (using transporter protein or channel in membrane); no protein is degraded.

d. Transferrin and receptor separate outside cell after recycled

(1). Outside cell/Entry: Fe/transferrin binds to receptor at neutral pH and enters cell by RME.

(2). Inside cell: Fe transported out of vesicle into cytoplasm, leaving apotransferrin stuck to receptor ("apo" means without ligand, cofactor, etc.). Apotransferrin does not separate from receptor at low pH (remains in endosome).
(3). Outside cell/Exit: Apo-transferrin (= transferrin without Fe) separates from receptor at neutral pH (outside cell).

(4). Overall Binding to Receptor: Binding in this case is contrary to usual behavior -- Most ligands stick to receptors at neutral pH but separate at low pH found in endosome. (Low pH breaks many weak bonds.) This case is the reverse.

(5). Apo-transferrin vs Fe/Transferrin: Note that apo-transferrin and Fe/transferrin have different affinities for the receptor at neutral pH. Under these conditions (neutral pH)

- Fe/transferrin binds to the receptor
- Apo-transferrin separates from the receptor.

e. Function of uptake -- to supply a nutrient (Fe).

D. For Reference: Compare & Contrast for the examples described above for transport of X

<table>
<thead>
<tr>
<th></th>
<th>Transferrin</th>
<th>LDL</th>
<th>EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>What's carried in (what is X)?</td>
<td>Fe</td>
<td>Cholesterol</td>
<td>Growth Factor</td>
</tr>
<tr>
<td>Function of X</td>
<td>Metabolism (Fe is cofactor for many proteins)</td>
<td>Metabolism (cholesterol is a component of cell membranes; used for hormone synthesis)</td>
<td>Signal</td>
</tr>
<tr>
<td>Ligand (What binds to receptor?)</td>
<td>Transferrin = apotranferrin + Fe</td>
<td>LDL</td>
<td>EGF</td>
</tr>
<tr>
<td>Does ligand include protein in addition to X?</td>
<td>Yes (apotransferrin)</td>
<td>Yes (ApoB)</td>
<td>No</td>
</tr>
<tr>
<td>Ligand Fate (protein part)</td>
<td>Recycled</td>
<td>Digested</td>
<td>Digested</td>
</tr>
<tr>
<td>Receptor Fate</td>
<td>Recycled</td>
<td>Recycled</td>
<td>Digested</td>
</tr>
<tr>
<td>Do protein part of ligand &amp; receptor separate inside cell?</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lysosomes Involved?</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Where do ligand &amp; receptor separate?</td>
<td>Outside the cell</td>
<td>In endosomes</td>
<td>Not separated -- both degraded</td>
</tr>
</tbody>
</table>

IV. Labeling -- How do you follow material coming into the cell? See handout 7B.

A. Types of Labeling (using added tracers)

1. Continuous Labeling -- switch from regular, ordinary material to labeled material (material containing radioactivity, fluorescence, etc.) and follow what gets labeled first (with radioactivity, fluorescence, etc.), what gets labeled next, and so on. We will discuss radioactive labeling, but the principle is the same whether the label is radioactivity, fluorescence, etc.
2. Pulse-Chase Experiments -- supply radioactive material for a brief time (pulse) and then switch back to ordinary, non-radioactive material (chase). Follow where the radioactivity goes. The "pulse" passes through the cell like a mouse through a boa constrictor. Just as different parts of the boa constrictor bulge out temporarily as the mouse passes down the snake, so different parts of the cell become radioactive temporarily, one at a time, as the radioactive material passes through. Then as the "pulse" or the "mouse" passes on, each part will return to normal -- non radioactive or normal size, depending on whether we are referring to the cell or to the snake.

For a nice video (& explanation) of a pulse chase experiment, try this link:
This site has many interesting animations. To select a topic, go to
http://sumanasinc.com/webcontent/animation.html

B. Detection -- How do you find where the radioactivity (or whatever tracer/label you used) is?

1. Autoradiography -- Cover a layer of labeled cells with photographic emulsion and count radioactive grains over each organelle or part of the cell. This method is similar to doing in situ assays, in that you examine intact cells to pin down the location of what you are looking for. See Becker, Appendix, A-18 (A-17) or Guide to Microscopy.

Note: Becker's Appendix (or Guide to Microscopy in 5th ed.) has a lot of useful background info on microscopic methods, including immunofluorescence, freeze fracture, etc.

For a picture of typical results, see fig. 12-10 of Becker or click here. These pictures were obtained by following newly made material out of the cell (as described below), not following it in. However the principle is the same -- it shows label in different cell parts at different times.

2. Fractionate First -- Break up labeled samples, fractionate into various organelles, and measure radioactivity in each fraction. This is similar to the "grind and find" procedure, in that you break up the cells, separate them into their parts, and test a solution or suspension of each part for what you are looking for.

To review labeling and RME, try (or retry) problems 2-8 & 2-11.

V. Sorting of Proteins to their Proper Place: Overview See Handout 7A, or Becker fig. 22-13 (22-14) or Sadava 14.18 (14.19) -- terminology in Sadava is slightly different.

A. What determines the fate (final location) of each individual protein? The amino acid sequence of the protein itself. The ability of each protein to reach its proper destination is built into the protein. The presence (or absence) of localization signals in the amino acid sequence of the protein is the determining factor.

1. What's a localization signal: a group of amino acids acting as an "address" or "tag" directing the protein to a particular destination.

2. Terminology: The localization signal or "tag" is often called a localization sequence (LS) or patch.

   a. LS = localization signal. Signal or tag that consists of a continuous section in the peptide chain.

   b. Localization Patch. If signal or tag consists of a contiguous section in the folded protein. (But AA are not next to each other in the unfolded chain.)

   c. SP = Signal Peptide. SP is used two different ways.

      (1). General sense -- SP can be used as a synonym for LS (as in Sadava) -- it can
refer to any LS.

(2). **Restricted sense** -- SP can be used to refer only to the particular LS that is the signal to enter the ER. (This is the way it is used in the notes and problem sets.)

3. **Use of tags:** The localization sequence/patch directs the protein to the ER, nucleus, etc. Several different localization sequences, which are read sequentially, may be needed to direct a protein to its proper destination. If no "tag" or special sequence/patch is present at all, the protein remains in the (soluble) cytoplasm = cytosol = cytoplasm minus its organelles.

**B. The Big Divide -- Attach to the ER or not? Importance of signal sequence in peptide**

1. **Ribosomes start to make protein first.** Translation starts first; then ribosome location (attachment to ER or not) is determined by the sequence of the protein being made.

2. **Which ribosomes go to ER:** If protein has the right "tag" (a localization signal called a signal peptide (SP) or signal sequence) -- the ribosomes attach to the ER, and protein enters ER as it is made*.

3. **Which ribosomes stay in cytoplasm:** If there is no SP, ribosomes remain "free" in the cytoplasm -- they do not attach to ER or any other membrane.

*Co-translational import into the ER is the usual method in humans. In yeast (a unicellular eukaryote, and common model organism), most import into the ER is post-translational. We will stick with the human mechanism.

**C. Fate of proteins made on attached ribosomes** -- these become part of the endomembrane system and/or leave the cell. How they enter the ER & other parts of the EMS will be discussed in more detail as we go.

1. **Proteins enter the ER** as they are made (co-translational import). Protein can
   - Remain inserted in membrane -- become a transmembrane single or multipass protein (details later)
   - Pass all the way across the membrane and end up in lumen of ER (as soluble protein or peripheral, membrane associated protein on lumen side)

2. **Most proteins travel from ER to Golgi** (a few may remain in ER) -- either as part of the membrane of a vesicle or as 'cargo' in the lumen of a vesicle.

3. **What happens in ER & Golgi?** Modifications (such as glycosylation) occur in ER & Golgi, and sorting at the end of the Golgi (trans side). Details later.

4. **Where do proteins go when they leave the Golgi?**
   a. **To other parts of the EMS** -- back to ER, to endosomes, lysosomes, etc.

   b. **To the plasma membrane** -- Traffic to the plasma membrane (in a vesicle) can lead to one or both of the following:

   1. **Secretion -- Release of proteins inside vesicle to outside of cell (not necessarily to outside of body)**. Examples:
      - (a). Proteins of ECM (like laminin, fibronectin, collagen).
      - (b). Proteins that act as signal molecules (like EGF, insulin). More on signaling later.
(2). Addition of integral protein to cell membrane.

5. How do proteins travel from one part of EMS to another or to the plasma membrane? Vesicles carrying protein bud off ER, Golgi, etc., travel to another compartment (or plasma membrane) and fuse with target membrane.

a. Transmembrane proteins remain in the membrane. TM proteins move from membrane to membrane but always remain in the same orientation -- cytoplasmic domain remains in cytoplasm.

b. Soluble proteins stay soluble. Contents of lumen of compartment #1 → lumen of vesicle → lumen of next compartment or outside the cell.

D. Fate of proteins made on free ribosomes

1. Soluble Cytoplasm -- the default location for a soluble protein. If there are no "tags" at all, proteins stay in the cytoplasm.

2. Organelles. If proteins have the right "tags" they can be imported post-translationally (after synthesis) into organelles (nuclei, mito, chloro or peroxisomes) that are NOT part of the endomembrane system.

3. Terminology: "free" means not attached to a membrane. All ribosomes making protein, "free" or not, are attached to mRNA.

Questions (to check or review your understanding of the topology):

1. Suppose domain X of a multipass integral membrane protein is on the inside of the ER, sticking into the lumen of the ER. When a vesicle forms off the ER, where will domain X be? Inside vesicle in lumen? On outside of vesicle in cytoplasm?

2. When vesicle fuses with Golgi, where will domain X be? In lumen of Golgi? Sticking out into cytoplasm?

VI. How do you follow newly made protein molecules? How did we find out that newly made proteins go from RER to Golgi etc.? In examples of detection discussed previously, emphasis was on following molecules going in to the cell. This section is about following newly made molecules on their way out.

A. Use of Labeling -- How do you follow newly made proteins out?

1. General idea -- Add labeled precursors (small molecules) and measure incorporation into macromolecules.

   a. Add labeled precursors, and take cell samples after increasing time intervals.

   b. For each sample, wash out unused ('unincorporated') small molecules -- removes labeled molecules not used for synthesis so not incorporated into macromolecules. Radioactivity remaining in dif. parts of the cell is in macromolecules.

   c. Measure amount of incorporation. Use autoradiography for measurement of radioactivity in each cell part, or measure amounts in each isolated fraction.

2. A specific example -- following secreted proteins out (in cells specialized for secretion*). See graphs on handout 7B. Re-label the curves, left to right, with Rough ER, Golgi, Vesicles, Outside the cell.

   a. Continuous label vs pulse-chase results (See graphs on handout 7B & fig. 12-10 of Becker.)

   b. Implications: newly made proteins to be secreted go → RER → Golgi → secretory
vesicles → outside (See Becker fig. 12-8.)

*Note: Secretion (the release of selected material, often material made in the cell, from a cell into the extracellular space or a lumen) is not the same as excretion (the elimination of material, often waste, from the body).

To review labeling of newly made material, try 3-4D.

B. Another Type of Labeling -- Cell makes its own labeled (fluorescent) protein containing GFP

GFP has been mentioned before. Here are the details.

1. What is it? GFP = green fluorescent protein = small fluorescent protein discovered in jelly fish. Click here for page with pictures of GFP and related fluorescent proteins.

2. What is it good for? GFP is used as tag to follow proteins inside the cell or organism.

3. How is GFP added to proteins? GFP is not added from outside. Instead, genetic engineering is used to splice the gene for GFP to the gene for the protein of interest. The recombinant gene makes a fusion protein = normal sequence of amino acid + sequence of amino acids in GFP. Fusion protein (including GFP) is made internally by the cell; the functioning protein part and the GFP part fold up separately (each forms a separate domain).

4. How does fusion protein work?
   a. GFP part fluoresces. In other words, cell makes its own fluorescently tagged version of the protein.
   b. Functioning protein part usually works normally, but location of protein can be easily followed in cell, because protein has GFP attached. GFP labeled protein is used for many purposes, including following newly made protein through the cell.
   c. Variants. Original protein was green, but variants of the protein are now available -- each variant fluoresces in a different color. All variants are used to make fusion proteins in the same way.

5. Examples. For examples of use of GFP, see Becker fig. A-14, or Sadava fig. 18.4 (18.5).

GFP is often used to identify cells that express (turn on) a particular gene. For an example see fig. 5 in the article on GFP at http://www.chm.bris.ac.uk/motm/GFP/GFP.htm. The cells that "light up" in fig. 5 are the only ones that express (turn on) the fusion gene. Only these cells produce a fusion protein containing GFP. (This example also illustrates why people use small, transparent organisms as "model organisms.") For a really startling picture and article, try this link. For an example of how to follow a protein (EGF) into the cells, go to http://spie.org/x8736.xml?ArticleID=x8736.

See also: http://nobelprize.org/nobel_prizes/chemistry/laureates/2008/presentation-speech.html.

See problem 2R-4 for an example of the use of GFP labeling.

Next Time: Wrap up of anything we don't finish above, and then: How do proteins get to ER or to other parts of the cell -- to mitochondria, peroxisomes, lysosomes, Golgi etc.? What happens in these organelles?