Bill. Problem Set 1. Due Monday, March 5.

1a. The Balbiani ring particle is a massive mRNA-RiboNuclearParticle that unfolds as it exits the nucleus through the nuclear pore. Below are electron micrographs of Balbiani Rings (BR RNP) exiting nuclear pores. Smaller, electron dense particles are found associated with the Balbiani fiber as it emerges from the nuclear pore. The authors speculate that these particles are ribosomes. How could you prove that they are ribosomes?

![Image of electron micrographs](image.png)

The “easiest” way would be if you had an antibody that 1) recognized some component of ribosomes and 2) worked in immunogold EM. If so, you could then ask whether gold spots coincide with the particles near nuclear pores where the BR- RNPs are exiting.

(In practice, by the way, while it is fairly straightforward to obtain antibodies capable of Western blotting and such, it is more difficult to obtain antibodies that will work in EM with immunogold on fixed samples. Preparing samples for conventional, thin section EM frequently results in loss of the epitope binding site on the protein. The epitope is still physically present, but is somehow masked by the hard fixation and plastic embedding required for thin section EM. New techniques such as high pressure freezing as opposed to fixing are becoming more common and might help preserve antigenecity.)

1b. Turns out they were right! Your experiment proves that the smaller particles associated with the emerging Balbiani ring are in fact ribosomes. Given this information, which end of the mRNA do you think exits the pore first and why do you think this?

You would predict that the 5 prime end of the mRNA is coming out first because protein translation proceeds from 5’ to 3’ and the ribosome binds to the 5’ end first where the start codon is found.
2. You have attached a nuclear localization sequence (NLS) to Green Fluorescent Protein (GFP) and you have a microscope and camera capable of fast acquisition of single molecules. You use this setup to image movement of single NLS-GFP molecules through nuclear pores. The construct has a mean dwell time of ~10 msec at the pore and then it diffuses away. Half the unbinding events result in the NLS-GFP returning back to the cytosol. The other half of the unbinding events results in NLS-GFP going into the nucleus. How can NLS-GFP concentrate in the nucleus if each NLS-GFP molecule has a 50:50 chance of ending up in the nucleus versus in the cytosol?

Directionality of nuclear import is mediated by a Ran•GTP/Ran•GDP gradient on either side of the nuclear pore. In the case of nuclear import, nuclear import factors bind to NLS sequences in the cytosol, diffuse across the pore where cargo is released in the nucleoplasm by the high concentration of nuclear Ran•GTP which binds to the import factor. Import factors cannot bind cargo and Ran•GTP at the same time. In the case of export, export cargo binds specifically to an export factor-Ran•GTP complex in the nucleus. This complex diffuses across the pore and cargo is released into the cytosol due to high concentrations of Ran-GAP that catalyze GTP hydrolysis to break up the complex. The exported cargo therefore cannot diffuse back into the nucleus.

In this model, which is the current favorite, the pore acts simply like an exchange catalyst. The catalyst lowers the barrier for crossing the pore, but like a true catalyst, it does not itself contribute to the reaction. Enrichment of cargo on either side of the pore is determined by the Ran•GTP gradient. Le Chatelier Rules!

3. Some researchers used to think that the nuclear pore was lined with many binding sites for importins and that these binding sites would be distributed vectorially along the pore such that importin would encounter progressively stronger binding sites as it worked its way into the nucleus. Therefore, bunny hopping from binding site to a better binding site closer to the nucleoplasm would drive nuclear import. Is this model consistent or inconsistent with fast nuclear import? Why or why not?

The model is inconsistent with fast import/export because cargo would end up stuck in the pore due to slow off rates. (Of course it is still possible that cargo interacts with factors lining the pore, but these interactions are now thought to be of low affinity. In addition, while fewer researchers think cargo moves along an affinity gradient, many think that cargo might move through the pore along a specific path as opposed to moving through the pore along a random trajectory. A specific path could imply preferential cargo binding sites inside the pore, but increasing affinity gradients doesn’t seem right).

4. Electron microscopy clearly gives the most informative images of nuclear pore structure. Why don’t cell biologists simply acquire a timelapse movie of a single import or export event using electron microscopy?
All EM techniques with biological samples requires some degree of chemical fixation with, for example, glutaraldehyde, (Conventional EM) or it requires rapid freezing to incredibly low temperatures (cryo-EM). In either case, things are no longer moving. Shooting movies of processes in cells can be done using light microscopy such as fluorescence imaging. Light microscopy is clearly superior for dynamics but EM gives much higher spatial resolution.

5. Below is an experiment by Brown and Goldstein on LDL processing by fibroblasts isolated from a healthy individual (Panel A) and a patient named J. D. with familial hypercholesterolemia (Panel B). LDL was labeled with radioactive iodine (I125) and added to both normal and mutant cells. The cells are incubated at 37C for various lengths of time thereafter and then quickly chilled to 4C to block any further reactions and then analyzed. Circles show the amount of I125 LDL associated with cells that can be easily released from cells with a very gentle chemical treatment (wash the cells with heparin). Triangles show the amount of I125 LDL associated with cells but cannot be released with the gentle heparin treatment. Squares show the amount of I125 associated with cells but is no longer attached to LDL. Squares therefore imply that the LDL has been degraded.
5a. What is the defect in the cells from the patient J.D.?

The defect is in internalization of LDL. J.D.’s cells can bind LDL just like a normal subject’s. This experiment does not reveal whether J.D.’s cells also have a defect in degradation/processing of LDL.

5b. Which organelle do you think is responsible for the degradation of LDL and why?

LDL is normally degraded/processed in the lysosomes. We already learned that LDL is internalized by clathrin-dependent, receptor-mediated endocytosis. Clathrin coated endosomes can eventually fuse with lysosomes to degrade internalized factors.
5c. Some genetic diseases compromise formation or function of lysosomes. You repeat the Brown and Goldstein I125 LDL experiment shown above using cells from a patient suffering from a lysosomal disorder. What would that data look like?

![Graph showing internalized, surface bound, and released LDL]

6. Sba1 is a small ATPase protein that is essential for viability in yeast. Brain Freeman’s group here at UIUC is interested in understanding the function of Sba1 in cells and recently found that Sba1 has defects in the secretory pathway but precise molecular mechanisms are not known. A temperature sensitive mutation of Sba1 is active at 25C but inactive at 37C. They also have antibodies to Sba1. To test if Sba1 is necessary for secretion, you assay the extracellular media from yeast grown at the permissive (25C) and restrictive (37C) temperatures for invertase activity. You find that indeed, invertase activity is low in the growth media of SbaI cells grown at the restrictive temperature. You now want to prove that SbaI is inhibiting secretion of invertase as opposed to its synthesis. Design a simple experiment with the proper controls to prove that Sba1 is required for secretion not synthesis of invertase.

You could compare the amount of invertase enzymatic activity in lysed yeast cells that had been grown at the permissive temperature to the activity present in lysed yeast that had been grown at the restrictive temperature. Enzyme activity would have to normalized in some way, such as amount of invertase activity per milligram of yeast extract protein.

If you had an antibody to invertase (commercially available for $), you could simply run a Western blot on equal amounts of yeast grown at the permissive versus restrictive temperature.
7. Electron microscopy (EM) of mutant yeast at the restrictive temperature for Sba1 reveals an accumulation of coated vesicles near the endoplasmic reticulum and cis golgi. A combination of immunogold EM imaging and fluorescence light microscopy reveals that the cells have accumulated an excessive number of COPI coated vesicles. State two alternative hypotheses as to why Sba1 might be required for secretion in yeast.

Sba1 might be required for uncoating of COPI coated vesicles, or excessive retrograde flow to the ER. Excessive flow could be caused by any number of problems and everybody’s answer was good. For example, loss of Sba1 results in more ER residents leaking to the golgi that therefore must be returned via COPI vesicles.

8. How would you start to address whether Sba1 is required for secretion in mammalian cells? Assume you have antibodies against mammalian Sba1, a tissue culture facility and standard fluorescence microscopes.

The most common and easiest approach would be to knock the expression of Sba1 down using RNAi methods. Use the Sba1 antibody to confirm the extent of knockdown (control) and measure secretion of a factor that is normally secreted from cells (such as extracellular matrix or a secreted protease...