SYLLABUS
Freshman Research Initiative: Sarah Simmons Cell Signaling Stream

Instructor: Stanley Roux
Research Educator: Greg Clark

Our Discovery Laboratory this semester will be a unique opportunity for you to carry out original experiments on a topic of significant current interest in biology. The course will emphasize learning methods of experimental design, data gathering, data interpretation, and data presentation. To achieve these educational goals you will carry out four rounds of experiments that have never been done before, and you will design most of these experiments. This year's experiments will address the question of what signaling steps mediate the effects of extracellular nucleotides on the polarized growth of single-celled root hairs, structures that are crucial for plants' absorption of water and nutrients from the soil. The experiments will constitute a novel test of a relatively new hypothesis, first published in 2003, which predicts that extracellular ATP (eATP) can influence plant growth and development by functioning like hormones, as they do in animal cells, and that they initiate growth-affecting changes in cells through the mediation of a cell-surface protein called a receptor that can bind either ATP or ADP. In animals these receptors are called **purinoceptors,** because they bind best to purine nucleotides**.**

Extracellular nucleotides have been confirmed to act as hormones in animals cells, where they rapidly induce an increase in $[Ca^{2+}]_{cyt}$, a change that commonly leads to the activation of signaling pathways that greatly influence cell activities in both plants and animals. Recent publications show that extracellular nucleotides can induce an increase in $[Ca^{2+}]_{cyt}$ in plant cells, too. The reason why an increase in $[Ca^{2+}]_{cyt}$ induces a signaling cascade is because it can activate calcium-binding proteins that play major regulatory roles in plant and animal cells. Among the best known calcium-binding proteins is calmodulin, which becomes activated when the $[Ca^{2+}]_{cyt}$ rises above a low level. Once activated, calmodulin can then bind to cellular enzymes and regulate their activity. Among the better known enzymes activated either directly by calcium or indirectly by calcium-activated calmodulin are nitrate reductase and nitric oxide synthase, both of which can catalyze the synthesis of nitric oxide (NO), a powerful signaling molecule, and NADPH oxidase, which catalyzes the synthesis of superoxide (O_2^-), another major signaling molecule. Previous results indicate that changes in [NO] and $[O_2^-]$ may be key downstream signaling steps of eATP and that NO and O_2^- may react with each other to form peroxynitrite ($ONOO^-$) a potent oxidant and nitrating species. Another way in which NO could mediate eATP-mediated changes in root hair growth is through its role as an activator of the enzyme guanylate cyclase which produces the messenger cGMP.

When testing the effects of nucleotides on growth, scientists often use poorly hydrolysable analogues of ATP and ADP, ATPγS and ADPβS, so as to avoid confusion about whether the results are due to the nucleotides or to the phosphates released when the nucleotides are hydrolyzed. An example of a dose-response curve for ADPβS is shown on the next page. In this experiment, students in a previous FRI stream laboratory found that application of ADPβS at concentrations of 150 and 300 μM could statistically significantly inhibit root hair growth while treatment with 50 and 100 μM ADPβS had no effect on root hair growth.

Enzymes called ectoapyrases can destroy extracellular ATP and ADP (eATP, eADP), and thus help maintain a low [eATP] and [eADP]. In Arabidopsis there are two apyrases that appear to act as ectoapyrases, named APY1 and APY2. We have previously documented a role for these two apyrases in regulating auxin transport in roots. Specifically, we found that a loss-of-function apyrase mutant disrupted in its ability to express these two apyrases showed inhibited auxin transport, and gain-of-function apyrase mutants with higher levels of expression of these two apyrases showed increased auxin transport. In order to learn more about the growth changes induced by eATP and the role of auxin in regulating these growth changes, we have obtained loss-of-function mutants that are disrupted in their ability to express certain auxin transporters. This year we plan to use all of these mutants in our novel experiments.

In past years, FRI classes made significant advances that revealed some of the signaling steps involved in converting the extracellular nucleotide stimulus to growth changes. These discoveries resulted in a major 2010 publication co-authored by 12 former FRI students (*Plant Molecular Biology* **74**: 423-435). However, there are still many questions unanswered, and our goal will be to address and begin to solve some of these questions, using the

transgenic plants described above.

Root hairs are relatively small cells, and measuring their growth will require some practice. To achieve this we will start a **preliminary** experiment on January 28 by planting seeds after lecture. For this **preliminary** experiment you will need to learn how to take clear digital images of the hairs on primary roots of *Arabidopsis* seedlings. In class on February 4 you will take digital images of the growing root hairs at two different time points, and will be instructed on how to analyze these images to evaluate the rate of root hair growth. The results of this preliminary experiment will be reported and briefly discussed at the beginning of class on February 5. Your first set of experiments ("Round 1") will begin with planting seeds on February 4. This set of experiments will be the only one designed by Drs. Roux and Clark. As noted in the next paragraph, there will be three other Rounds of experiments this semester, and these will be designed by you.

Each "Round" of experiments will consist of 3 lab sessions: two sessions of planting and then measuring the effects on root hair growth of genetically altering the expression of apyrase (overexpression or suppression) or using chemical agonists or antagonists to alter the signaling pathways induced by extracellular nucleotides, and one session of data presentation and analysis, including statistical treatment to determine the significance of any differences noted. At the end of each round, students will react to the results obtained by refining the experimental design to get more detailed and/or more accurate data.

Note: There is a Canvas website for the course where this syllabus and class information will be posted.

The lab schedule will be:

Jan. 19	Introduction to the course and the experimental system.
26	Begin preliminary experiment by learning techniques needed and planting Arabidopsis seeds.
Feb. 2	Measure effects of treatment with different concentrations of ATP. Begin **Round 1** of experiments by planting seeds.
9	Report results from Feb 5 measurements. Measure effects of different treatments for **Round 1** and tabulate results from **Round 1** of experiments.
16	Meet to present and discuss results. Propose second round of experiments to test alternative hypotheses that may account for results of first round of experiments.
23	Begin **Round 2** of experiments.
Mar. 2	Measure effects of different treatments; tabulate results from **Round 2** of experiments.
9	Meet to discuss results. Propose third round of experiments to test alternative hypotheses that may account for results of second round.
3/23-4/6	**Round 3** of Experiments [3 weeks, same procedure as first two rounds]
4/13-4/27	**Round 4** of experiments [3 weeks, same procedure as first 3 rounds]
May 4	**Final Discussion**
May 12	**9-12 Open Book Final Exam; Final Reports due**

Dose-Response Assay Showing Effects of ADPβS on Root Hair Growth
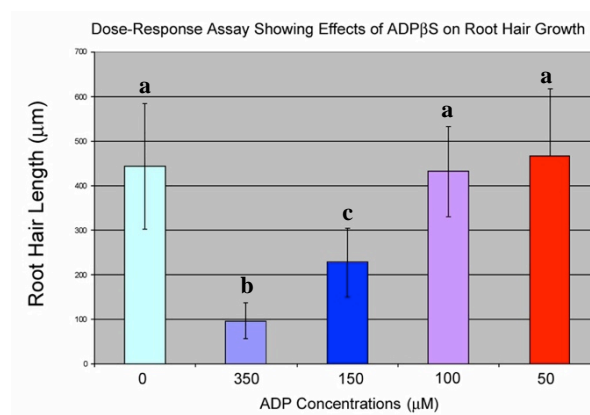
INSTRUCTIONS FOR WRITTEN & ORAL REPORTS FOR NSC 109/BIO 206L

Each team will present one paper and one oral report for each round of experiments. One member will write the written report and the other member will present a short oral report (Powerpoint), so each student will do two written and two oral reports during the semester. Teams should work together when preparing reports.

Each written Lab Report should include:

1. A Cover page with date and names of team members (place an asterisk next to the author's name), a title for the report, and a VERY brief (less than 250 words) description of the experiment done. The description should focus on the hypothesis tested and results.

2. A graphical rendition of the results in the form of one or more bar graphs. Each bar in the graph should give the standard deviation around the mean value.

3. A table (Table I) with the raw data in columns including:
> The treatment being measured
> The range of values measured
> The mean of the values measured
> The standard deviation of the values measured
> The number of samples measured

4. A table (Table II) which describes the t-comparison being measured, the t-value measured for that comparison and a column stating the level of significance. Note that Level of Significance values above 0.05 are considered insignificant. Each Table and Graph should have a descriptive title.

An example of such a report is attached.

Each Oral Report should:
1. Be 10 min or less.
2. Include an introduction that gives some background information & specifies the hypothesis being tested.
3. Show and discuss the summarized Table & Graph results.
4. Interpret the results and present what might be the next experiment.

COURSE GRADING (same grade assigned for 109/206L)
2 ORAL REPORTS:	**25**
2 WRITTEN REPORTS:	**25**
Final exam	**15**
Final Report	**20**
Overall performance*	**15**

*The success of this laboratory experience, as is true for the success of all original research, will depend strongly on how much thought and consultation time you put into your project. Although Dr. Roux and Dr. Clark cannot predict the outcome of your experiments, we can surely help you think about your results, solve technical problems that you may encounter, and evaluate options for the new experiments you will plan. The research you will do this semester is original and could generate potentially exciting results. We ask you to take on both the joy and responsibility of discovering new insights about how plants grow and develop, and we hope you will enjoy this collaboration with us and with your student colleagues. On-time attendance is a key factor considered in determining the Overall Performance grade. Active participation in the design and execution of your experiments and in the discussion sessions at the end of each round will enhance your Overall Performance grade; and, of course, surfing the web, texting, or other signs of inattention during the labs or discussion sessions will significantly hurt your performance grade.