Week 1

Background: Microscopic observation of anemones

Coral reefs depend on symbiosis with certain species of photosynthetic algae, which are nutritional endosymbionts (i.e., they live inside the cells of their hosts and provide their hosts with nutrients). In this class, we use the anemone *Exaptaisa pallida* as a model system for coral-algal symbiosis because it associates with the same types of algae, can be bleached like coral, and grows quickly in laboratory conditions. In class we will observe anemones in symbiosis with algae as well as aposymbiotic anemones (i.e., anemones that have been bleached and do not contain algae within their cells).

Background: Pipetting exercise

The study of genetics depends heavily on the use of molecular biology techniques. In particular, genetics often requires that we isolate and manipulate DNA from our model organisms. Because the cellular environment is aqueous, many molecular biology techniques ultimately revolve around the manipulation and combination of small volumes of liquids that contain our molecules of interest (e.g., DNA). In this class we will practice pipetting because good pipetting technique is critical to the success of our experiments throughout this semester.

Background: Mutagenesis of *Breviolum minutum*

Breviolum minutum is a species of unicellular dinoflagellate algae and nutritional endosymbiont of corals, anemones like *Exaiptasia pallida* and other cnidarians. They play an active role in establishing and maintaining a symbiotic partnership with their cnidarian hosts, and because of this their genetics are also interesting to scientists studying coral bleaching. In this class we are attempting a **forward genetic screen** in which we cause random mutations with UV exposure and screen for a phenotype (here, resistance to 5-fluoroorotic acid or 5-FOA). This screening is accomplished with the use of selective media (plates containing 5-FOA) which should prove lethal to wildtype algae but is not lethal to *Ura3* null mutants.

Protocol for pipetting exercise:

Warm-up exercise:
What are the ranges of the pipettes at your table?
1)
2)
3)
4)

Select the correct pipette for desired volume:

1) 1.25µl	5) 9.8µl
2)165µl	6) 2.00µl
3) 200µl	7) 12.8µl
4) 20µl	8) 465µl

Exercises 1 & 2

Each student should receive four 1.5 ml tubes. Before pipetting label your tubes A, B, C, D.

Exercise 1: Adding & removing large volumes

1.Add 370µl Blue reagent to tube A.
2.Add 225µl of Red reagent to tube C.
3.Add 495µl of Yellow Reagent to tube B.
4.Add 160µl of Blue reagent to tube D.
5.Remove 155µl from tube A and dispose.
6.Remove 190µl from tube B and add it to tube D (do not mix).

Exercise 2. Adding vol. into vol. & common mixing techniques (Note before and after mixing).

1.Add 285µl of Red reagent to tube A and mix by pipetting up and down.
2.Add 195µl of Blue reagent to B and mix using inversion.
3.Add 275µl of Yellow reagent to tube C and mix using flick/tap method.
4.Add 150µl of Red reagent to tube D and mix by vortexing.

Exercises 3 & 4

Each student should receive four 200ul tubes & label them E, F, G, H.

Exercise 3: Adding small volumes
1) Add 20µl of Blue reagent to tube E.
2) Add 11µl of Red reagent to tube F.
3) Add 2.5µl of Yellow reagent to tube G.
4) Add 1µl of Blue reagent to tube H.

Exercise 4: removing & mixing small volumes
1.Remove 10µl from tube E and dispose of it.
2.Remove 1µl from tube F and dispose of it.
3.Add 7.5µl of Red reagent to tube G and mix by pipetting up and down.
4.Add 9µl of Yellow reagent to tube H and mix by pipetting up and down.

Exercise 5. Making a master mix. (1-1.5 ml tube per person)
Often to save on time and to reduce variation between reactions, it is helpful to make a master mix when doing multiple reactions. Master mixes are made by making reaction mixes that are larger in total volume but contain the same ratios of reactants as 1x. It is often customary to make master mixes larger than what is desired to account for pipetting error.

Example: Desired=5x, Make = 5.5x
Desired =20x, make=22x

1x Reaction	Desired=9x	Make=__x reaction
10µl Yellow reactant.	__Yellow Reactant.	__Yellow Reactant
9µl Blue reactant	__Blue Reactant	__Blue Reactant
1µl Red reactant	__Red Reactant	__Red Reactant
20µl Total	__Total	__Total

Protocol for *Breviolum* mutagenesis

Note to scientists in training:

As a rule, read all protocols from beginning to end before starting. Ensure that you have access to all required materials and equipment. Make sure that you understand both how to perform each step and why you're performing the step. Understanding why you're doing something is important for avoiding mistakes AND critical for troubleshooting. If there is any confusion, resolve it before beginning.

We will be mutagenizing *Breviolum minutum* strain SSB01 using ultraviolet light. Each table will be administering a different dose of UV to randomly induce mutagenesis in a large population of cells. In the following weeks, we will select for mutants which possess the phenotype of interest. This protocol has been carefully designed to eliminate any UV exposure to experimenters

Each table/group is assigned a UV exposure time: 0 min (negative control), 15s, 30s, 1min, 2min, 4min.

1. Each table will receive a 6-well plate. Each student will label their own well accordingly, with the following information:
    a. Table #
    b. Section #
    c. Experimenter name
    d. Duration of exposure to UV
    e.g. T6, S07, Josh, 32 min
2. Locate the T75 culture flask containing a dense culture of SSB01 on your table and swirl to homogenously suspend cells. Density is approximately 5×10^5 cells/ml.
3. Each student must transfer 1ml of SSB01 culture suspension into one well of the plate (each table will use four wells total). If the culture has settled, swirl before pipetting. Gently swirl plate to distribute culture across bottom of well.
4. When all tables are ready, students can begin carefully putting their plates into the UV chamber under the supervision of their TA. Each table should leave their plate in the chamber with the UV source on for the appropriate time before moving to the next step.
5. As soon as a sample's exposure is complete, add 8 ml of the provided recovery medium. Recovery medium for this particular screen is Daigo's IMK medium supplemented with 1g/L uracil.
6. Incubate at 25°C under a 12:12 light cycle, with 10-50µE/m^2s^1 PAR for 7 days.

TAs: Ensure that the UV source is OFF before leaving.

For your lab notebooks:

As a rule, when you are provided with a protocol, you do **not** need to copy the entire protocol into your lab notebook. It is perfectly fine to write something along the lines of "we performed the mutagenesis according to the protocol we were given in class." However, it **is** important to include any mistakes or deviations from the protocol if any occur, as well as all data and everything listed in the lab manual for the week. You should also include the date and title of that week's lab at the beginning of every entry. Lastly, you should not use a notebook that you are using for another course as your lab notebook, as your TA may need to keep it for a week or more when grading it. For this week, you should include the following information and answer the following questions:

1) Include photographs in your notebook or draw pictures of the symbiotic and aposymbiotic anemones you've observed. Note the differences in pigmentation. Explain what accounts for the differences in appearance you observe.
2) Write in your notebook the answers to the warmup exercises and exercise 5. For exercises 2-4, write the final volumes that each tube should have had. Did your tubes have those volumes when you were finished with the exercise?
3) For this experiment, each table will perform one treatment, and the experiment as a whole will be completed by the class. Because of this, it's important that you write down all data. Record in your notebooks the exposure times that each table used. Which exposure time did your table use? Write your predictions of which tables (if any) will generate mutant algae, and which will generate the most.

Week 2

Background: plating of *Breviolum minutum*

Previously, we mutagenized cultures of the symbiotic algae *B. minutum* (strain SSB01) with ultraviolet radiation. Now, we hope to screen these cultures of randomly mutagenized cells for a specific phenotype: resistance to the compound 5-fluorooritic acid (5-FOA). 5-FOA is broken down by action of the protein Ura3 into the toxin 5-fluorouracil (5-FU). *Ura3* knockout mutants will not metabolize 5-FOA into 5-FU and as such will be immune to its deleterious effects. We're plating our mutagenized *B. minutum* cells onto media containing 5-FOA to screen for *Ura3*$^-$ (i.e., knockout) mutants.

Protocol for plating of *Breviolum minutum*

One week ago, you irradiated your *Breviolum* with ultraviolet light to cause DNA damage and induce random mutations in the cells. We're performing a mutant screen for tolerance to 5-FOA. This week, you will plate these mutagenized cells on agar plates containing 5-FOA at a concentration of 10ug/ml which is lethal to wild type cells. Your plates also contain 1g/l uracil added to the medium. SSB01 cells grow slowly, so it will take several weeks for any mutant colonies to become visible. Because of this long incubation time, cultures are susceptible to mold and bacterial growth, so **it is critically important that you work with good sterile technique**.

1. Retrieve your cells from the incubator.
2. Obtain a 2ml tube and label it with your name.
3. Transfer 2ml of the liquid culture (from your well in your table's 6-well plate) into a 2ml tube labelled with your name.
4. Centrifuge at 10,000g for 1 min.
5. Be mindful of where the pellet is and be sure to not disturb it. **If you disturb the pellet, repeat the centrifugation**. Using a pipette, remove 1.5ml supernatant and dispose of it in the waste cup provided on your table.
6. Add 1.5 ml of the culture to fill up the tube back up to 2ml.
7. Repeat step 4, 5 and 6 until you have no liquid culture remaining and all cells are collected in a pellet in 0.5ml volume in your 2.0ml tube. Resuspend your cells in ~0.5ml volume. The suspension should be dark brown in color because the cells are now densely concentrated
8. Obtain a media plate from your TA and label appropriately. **The label must include your name, your section, your table, and treatment (0, 2, 4, 8, 16, or 32 min)**.
9. Pipette 0.5ml of the concentrated culture on to the center of the plate.
10. Use the sterile plastic spreader to spread the cell suspension evenly.
11. Cover plate and do not move for a few minutes to allow the liquid toabsorb.
12. Cut a 1cm wide strip of Parafilm. Carefully wrap your plate with Parafilm to prevent evaporation and contamination. **Note that if you do not wrap your plate properly, your media will dry and your cells will die.**
13. Turn your plate upside down so that condensation does not drip onto the plate and interfere with the cells.
14. Incubate at 25^{o}C under 12:12 light cycle (~10-40μE m-2s-1PAR).
15. Inspect in 4-6 weeks.

For your lab notebooks

It is not important to copy the entire protocol down into your lab notebook, unless you feel compelled to do so. Instead, you may summarize what you've done in class today. More importantly, state clearly *why* we did this protocol—what do we hope to achieve? If necessary, write down any mistakes or deviations from the protocol, what you expect the outcome of those deviations will be, and what you've done to keep the protocol on track. Finally and most importantly, what do you think will be the outcome of this experiment? Write down in detail what you predict the results will be across the whole experiment (remember, each table is one treatment, so the experiment is all of the groups together). Why do you think this will be the case?

Week 3

Background: anemone stress challenge

Coral bleaching is the breakdown of symbiosis between the coral animal and its endosymbiotic algae. The loss of the pigmented algae results in the white coral appearing to have "bleached." Other cnidarians including the anemone *Exaiptasia pallida* and the jellyfish *Cassiopea xamachana* also engage in symbiosis with photosynthetic dinoflagellates and can be

used as models to study coral bleaching. This week, we will begin the second major experiment in this course: the anemone stress challenge. We will be quantifying a phenotype (tolerance to thermal stress or menthol exposure) and then spend the rest of the semester seeing if variations in this phenotype correspond to differences in genotype. Little is known about the mechanisms underlying coral bleaching, so understanding genes involved in this process is of great value to the scientists who study coral bleaching and hope to prevent the loss of coral reefs to climate change.

Every table will have one **genet** of anemones (a clonal colony propagated asexually from a single individual). Each anemone in a genet is called a **ramet**. Students will place one ramet in each of four different treatments: 25^{o}C seawater, 32^{o}C seawater, 34^{o}C seawater, and 25^{o}C seawater with 0.19mM menthol. Menthol is known to bleach anemones, although the mechanism is unknown. These treatments then represent, respectively, a normal temperature they would experience in nature and a **negative control** treatment, mild heat stress, more extreme heat stress, and a **positive control** treatment. Each anemone will be left in the appropriate temperature for one week.

Before going into the incubator, students will measure the color of each anemone using the coral health chart. This is an indirect measure of symbiosis, because more algae in an anemone's cells means more pigment and a darker color. They will then be measured at the end of one week, and loss of pigment (and therefore algae) can be discerned as the difference between color before and after the stress test.

Tolerance to thermal stress of menthol can here be defined as the difference in color of an anemone before and after a stress treatment compared to the difference seen in a control anemone in the same genet. A genet in which a thermally stressed anemones go from very dark to completely bleached is less tolerant to thermal stress than a genet in which thermally stressed anemones go from very dark to only partially bleached (assuming their respective controls behave consistently).

Protocol: anemone stress challenge

Each table will receive one genet (clonal population) of at least 4anemones.

1) Before transferring anemones, score the color of each individual using the coral health chart provided. Take photos of your anemones with reference chart in background.
2) Label your Falcon tubes with genet ID (location), section number, table number, student name and treatment (e.g. ARB31/U02/T3/Josh/Menthol)
3) Each student will transfer ~40ml of seawater into 50ml calibrated Falcon tube. Note: for anemones in the menthol treatment, use 40ml of pre-made mentholated seawater instead.
4) Each student will transfer one anemone using a cut-off transfer pipette to a Falcon tube containing 40ml of seawater or mentholated seawater. Note: the anemones like to adhere to their cups, and sometimes work themselves into corners and are difficult to remove. A firm but delicate touch is best, and if you are afraid you are causing significant damage to your animal, don't hesitate to call your TA over to help. Remember, although they are simple, they are animals and should be treated with respect and care!
5) Loosely cover the top of each tube in plastic wrap and place each tube in the appropriate incubator for seven days.

Notes: if a table has less than four students or a student is absent, each group should still put an anemone in each of the four treatments. Return any remaining anemones to your TA to preserve the lineage.

For your lab notebooks

Write a brief overview of the whole experiment. Why are we doing what we're doing, and what do we hope to achieve? Record the genet ID of your anemones, and write down the coral health chart score for each individual. How do you predict each of your four anemones will fare? Which treatments will see the most bleaching, and which will see the least? Do you expect to see variation in tolerance among different groups, and if so do you think that there may be a genetic basis for that variation? Why or why not?