TRAINING PROTOCOL FOR THE OXYGRAPH SYSTEMS

You must work alone at a DO chamber today. It is essential that each of you be able to calibrate and operate the system yourself. For the mitochondria study next week you will work with a partner at one chamber, as part of a team of four that "shares" experiments. Today you will share a rack of reagents, two Hamilton syringes, respiration medium, and a two channel oxygen monitor with another person working at another chamber.

A teaching assistant will "walk" you through the calibration procedure.

Identify the system components

- Dissolved oxygen chamber, with stir plate, stir bar (stirring "flea"), Clark electrode
- Oxygen monitor - 2 channel YSI. Note digital display, selector should be on "air," calibration knobs with locking rings CCW in order to calibrate
- Chart recorder - there are two types, both Kipp & Zonen flatbed recorders. Note cables connecting oxygen monitor to recorders. During this training session we will ask you to work with both types of recorder.

Identify supplies

- Squirt bottle and transfer pipet for cleaning chambers (plastic, to avoid scratching insides of chambers)
- Bottle with respiration medium
- Micropipettor for filling chamber while measuring volume
- Glass stopper (fragile!)
- Hamilton syringes for adding reagents
- Reagents in eppendorf tubes in a plastic rack
- Fifty ml beaker with deionized water, for rinsing syringe and large (1L) beaker for waste

Your Hamilton syringes are color coded. Red is for poisons, blue is for substrates and ADP. You must be careful to use the proper syringe for the type of reagent that you add, so that poisons aren't inadvertently introduced into the substrates or ADP stocks. Now we'll take it one step at a time.

1. Clean out the chamber using the squirt bottle and transfer pipet.
2. Fill the chamber with a measured volume of respiration medium to the shoulder, making sure that fluid enters the stopper so that there is no vortex. Record the volume. The filling volume will change when electrodes are changed, so measure the volume each time. You must know the volume of fluid in the chamber in order to calculate oxygen content and determine rates of oxygen consumption.
3. There must be no bubbles or vortex when the stopper is placed in the chamber. Watch for any air bubbles trapped near the membrane. The stirrer can remain on. Make sure that the stir bar is moving fairly fast and steadily. It should not be jumping around in the chamber.
4. Leave the stopper out until the readout stabilizes. The chart recorder can be run at very low speed (e.g., 0.1 mm/sec) to keep track of drift. Because the temperature in the room is not constant, there will always be some drift.

5. It is necessary to equilibrate the solution with the stopper off and stir bar going, in order to facilitate the exchange of gases between the atmosphere and chamber medium.
6. Calibrate the oxygen monitor to 100% (+/- 1%).
7. The value 100% represents the amount of oxygen per unit volume (oxygen concentration) when the chamber is completely equilibrated (saturated) at the current temperature and pressure. The percentage will drop as oxygen is consumed. Later you will need to convert percentages to amounts of oxygen.
8. Turn on the chart recorder and zero it . Inspect both types of recorders so that you can work with either type. The zero suppress button grounds the input signal so that the recorder receives zero volts. We set the record to physically read zero, then release the zero button for recording.
9. Set the chart recorder to 1 volt full scale and release zero mode. The oxygen monitors deliver a 1 volt signal at 100% saturation. If the recorder scale does not agree with the monitor reading, check its calibration. A 1-2% error is okay.
10. Set the paper speed to 0.2 mm/sec and start the paper running.
11. Now it is time to add mitochondria. You have mitochondria that were frozen and thawed, therefore the membranes are damaged and they are incapable of making ATP. Previously frozen mitochondria are quite capable of electron transport, however, and will consume oxygen. We are going to conduct a brief experiment in which you add substrates that enter the electron transport system at different points. Following each substrate you will add a poison that blocks electron transport from that substrate.
12. Mitochondria suspensions are quite viscous. Using a micropipettor set to deliver 40 microliters, draw up some suspension. Release the micropipettor slowly and leave the tip down in the suspension for a few seconds to allow the pressure to equalize.
13. Deliver the suspension to the chamber by dipping the pipet tip under the surface and ejecting the contents.
14. Immediately triturate the chamber medium with a 1 ml micropipettor set to 0.5 ml or so. Trituration means to pipet up and down, mixing the contents. Trituration should disperse the mitochondria into the medium, so that they make a smooth suspension with no lumps. Try not to introduce air when you triturate the suspension.
15. Replace the stopper and watch the record for a minute or so. When you replace the stopper some of the medium must enter the capillary bore of the stopper in order to prevent a vortex from forming.
16. The mitochondria should bind up some oxygen right away. The binding sites for oxygen on the electron transport chain should have been mostly empty, since the mitochondria were stored as a very concentrated, oxygen poor suspension. After the initial binding you should see a steady decline in the oxygen content in the chamber. This means that mitochondria are consuming oxygen, probably using fatty acids as fuel. Low concentrations of fatty acids remain in the concentrated suspensions, which were prepared from liver, rather fatty tissue.

Substrates/poisons/order of addition may be changed

17. Now it is time to add the first substrate. Rinse a blue labeled Hamilton syringe by drawing water up and down into it. Wipe the needle tip and then draw up 20 microliters (μl) 0.5M succinate. Wipe the tip again. Insert the needle into the hole in the glass stopper all the way down, so that the needle tip enters the chamber. It may stop short. Just make sure that you get it down as far as it will go.

18. Eject the contents into the chamber without hesitation (push the plunger down all the way in one quick motion). Rinse the syringe again so that it is ready for the next reagent.
19. Watch the trace for a minute or two. You should see a faster rate of oxygen consumption (if not, continue anyway). The mitochondria are now in a steady state, using succinate as a source of electrons, transporting them to oxygen and converting oxygen to water. This process in intact mitochondria stores energy in the form of a gradient— energy that can be used to make ATP.
20. Rinse a red labeled syringe and draw up 10 microliters of antimycin. Deliver the antimycin to the chamber, wiping the needle tip at each step as before and rinsing when you are done. Watch the record. You should see a leveling off of the trace, because antimycin blocks electron transport from this substrate. If not, then continue anyway.
21. Use a blue syringe to deliver 20 microliters 0.5M ascorbate followed immediately by 20 microliters TMPD. In combination, these reagents deliver electrons to the system past the block by antimycin. Respiration should resume.
22. Now use the red syringe to add 10 microliters KCN (potassium cyanide). Cyanide binds the cytochrome oxidase complex, preventing oxygen from binding, and thus stops all respiration.
23. Stop the record, clean out the chamber, replace the medium with deionized water to the shoulder to cover the electrode. Empty the beakers and put all materials back where you found them. Electrode membranes will be ruined if they are left dry. Re-fill the DO chamber when finished with an experiment.

Keep your chart record. Study it as you prepare for the mitochondria lab. Understanding the results will be of immense help in understanding what you see in lab.