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BaroFuse Operator's Manual

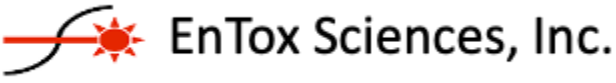
September 23, 2022

Overview

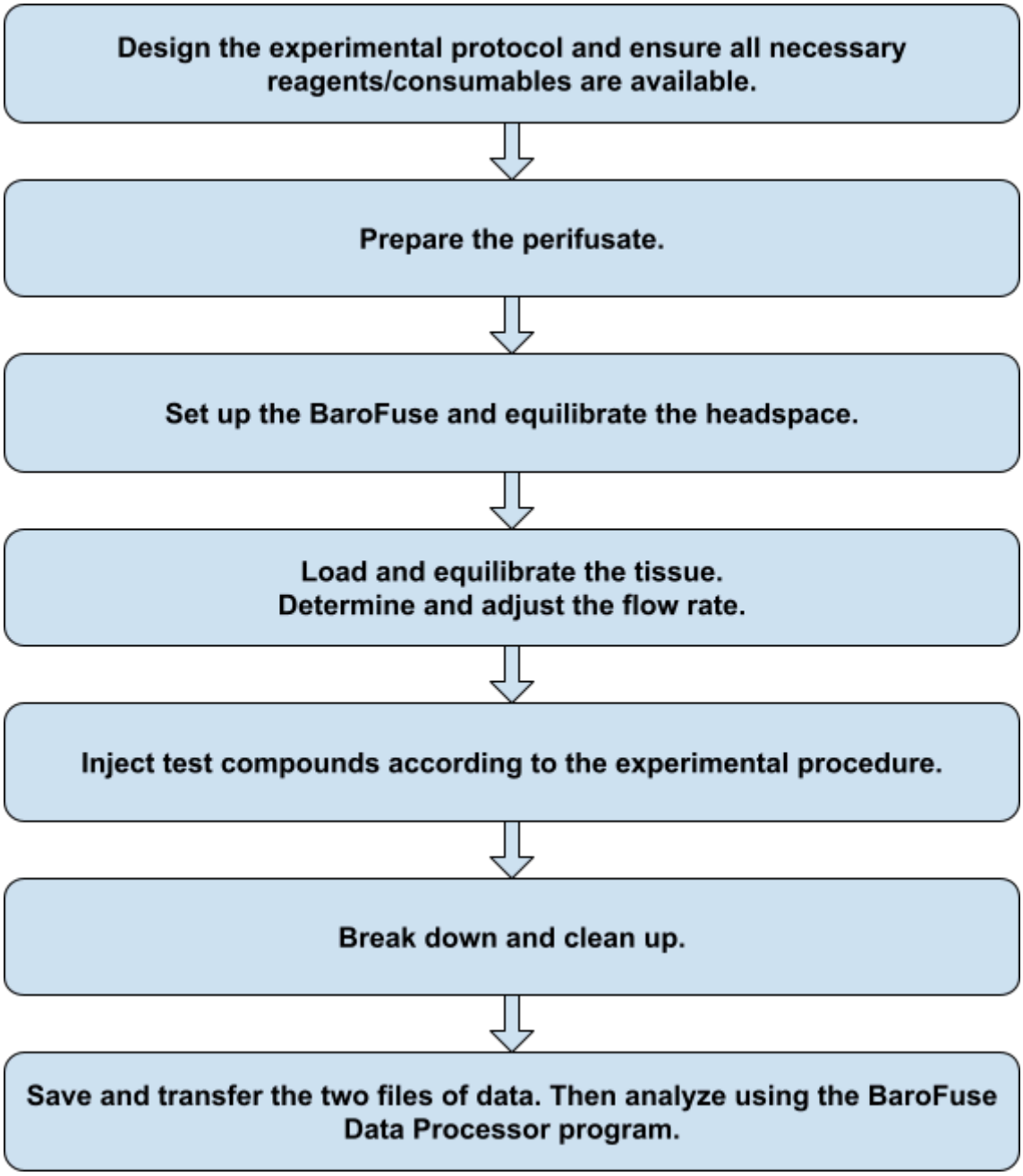
This Manual describes the steps involved with performing an experiment with the BaroFuse. It is divided up into the 7 sections that correspond to the different phases of the experiment: 1. Pre-Experimental Preparation; 2. Experimental Preparation; 3. Instrument Set Up; 4. Tissue Equilibration; 5. Experimental Protocol; 6. Instrument Break Down; and 7. Data Processing. Estimated time to complete each step is shown in parentheses. There are multiple appendices that follow the main sections that describe Training, Troubleshooting, and other procedures related to the BaroFuse (it is recommended to use the outline on the left of the document for navigation). For a first-time user, we recommend carrying out the protocol for quality control tests as described in the last Appendix section.

EnTox provides different software packages that are continuously updated and improved. The BaroFuse UI, which controls the Fraction Collector and Flow Monitor, and the EnTox Data Processor, which is used to analyze oxygen data, can be found on the EnTox Sciences website on the Resources page.

For the first time starting up the EnTox Data Processor on a Mac, you must right click on the application and then click Open. The program will prompt you with a warning and then you can click open again. Alternatively when you try to open the application you can open the "Security & Privacy" section in Settings of your Mac and allow the program to run.



Experimental Flow



1. PRE-EXPERIMENTAL PREPARATION

It is recommended that the following tasks be completed at least a day in advance of the experiment.

a. Design the experiment protocol. Typically this involves specifying
   i. What and how much tissue/cell sample will be placed in each of the 8 channels
   ii. Flow rate and total volumes of buffer/media in the two chambers of the MRM. NOTE: It is better to finish the experiment with extra media rather than run out during the experiment due to unexpected delays or addendums to the planned protocol. Typically, 70mL are added into each trough, but the amount is determined by the flow rate and the length of the experiment.
   iii. Timing and concentration of test compounds. Also ensure that all test compounds are available in sufficient quantities. View Appendix G for more information regarding reagents.

b. Set out the consumable parts. These include
   i. Tissue Chamber Assemblies (pack of 8)
   ii. Outflow Tubing Assemblies (pack of 8 tubes)
   iii. Test Compound Injection Tubing (2)

c. Place clean forceps, hemostats (3), Media Reservoir Module (MRM), Stir Bars, and Purge Tubing Assembly in a Biological Safety Cabinet

2. EXPERIMENTAL PREPARATION (30 MINUTES)

Prepare the 200 mL of perifusate (buffer/media), put into tissue culture flasks and place in a 39°C incubator with 5% CO_2. (For more details regarding perifusate see **APPENDIX F**)

- Mix all components of the perifusate, except for the glucose, together the day before the experiment. Incubate overnight and add glucose in the morning.
- If using commercial KRB, the perifusate can be prepared the morning of the experiment. Mix all components of the perifusate and place no more than 100 mL in a T175 or T225 flask. Incubate the three flasks for at least one hour before placing the perifusate in the MRM.

If using a gas tank containing 5% CO_2, then a bicarbonate-based buffer/media must be used that is pre-equilibrated with 5% CO_2. NOTE: Cold solutions contain more dissolved gasses than warm, so if media warms up in the BaroFuse, it will degas and generate bubbles. This is a critical step!!

3. INSTRUMENT SET UP: EQUILIBRATION OF TEMPERATURE AND DISSOLVED GAS (90 MINUTES)

a. Add fuels to buffer

1. Remove perifusate from the incubator and add glucose and other fuels as needed.
2. Place back into incubator

b. Attachment of Tubing Assemblies to the Media Reservoir Module.

1. Place the MRM and a Fluidics Package onto the bench next to the BaroFuse. The hemostats (3), Stir Bars (2), and forceps should already be located in the Tool Tray.
2. Place a clean MRM Insert on each side of the MRM. Place a Stir Bar into each side of the MRM (See Fig. 1).

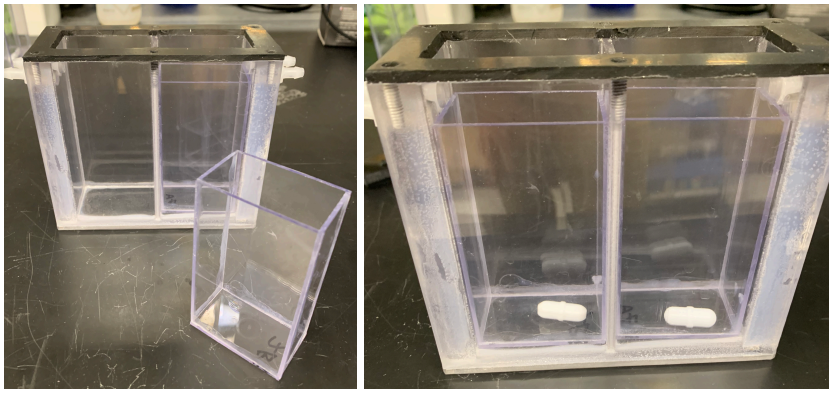


Fig. 1. View of the MRM with the MRM Insert (left) and Stir Bars (right) placed into the bottom.

3. Attach the Test Compound Injection Assemblies on injection ports on both ends of the MRM so that the end of the tubing is directly over the Stir Bar. The longer of the two Test Compound Injection Assemblies should be on the back.
4. Then attach the Gas Inflow Feed Line and Purge Tubing Assembly to the back and front vacant ports respectively (see Fig. 2).

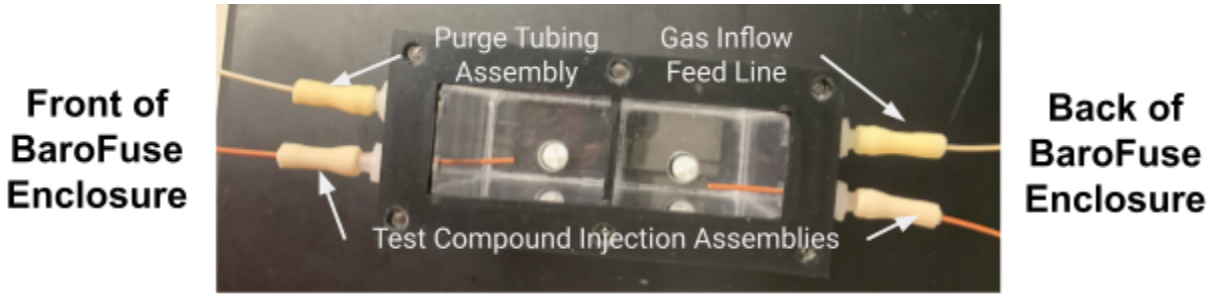


Fig. 2. Test Compound Injection Tubing Assembly and Purge Tubing Assembly attached to ports on the MRM.

c. Placing the MRM/Tubing Assemblies in the BaroFuse Enclosure.

1. Place the MRM (with the four tubing assemblies attached) into the MRM Heater (Fig. 3 Left).
2. Insert the four tubing assemblies into the grooves of the walls in the Base of the BaroFuse Enclosure (two on each side) so they will protrude through to the exterior of the Enclosure once the Middle Enclosure is put in place.
3. Secure the MRM between the clamps by tightening the two wheels on the BaroFuse stand.
4. Feed the longer Test Compound Injection Assembly protruding from the back of the BaroFuse through the two tube guides on the side of the BaroFuse so the opening of the tubes faces forward (Fig. 3 Middle).
5. Clamp a hemostat on each of the Test Compound Injection Assemblies (Fig. 3 Right).

NOTE:
When clamping the rubber pharmed tubing, do not clamp too close to the hinge of the hemostat. The tubing may tear if the flat part of the hemostat is not used.

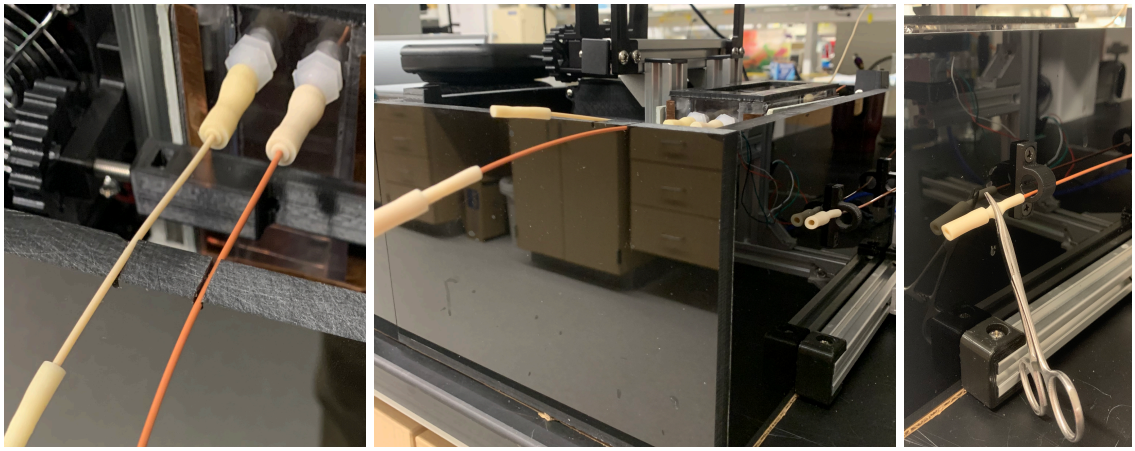


Fig. 3. The MRM left side's Test Compound Injection Assembly and Purge Tubing Assembly are placed in the groove of the BaroFuse Enclosure.

d. Assembling the BaroFuse Enclosure and Activating the Temperature Controllers.

1. Toggle the Power Strip to the On position. The MRM Temperature Controller should light up with the set value displaying 38°C (Fig. 4). If the number is different, reset to 38°C. The fan on the BaroFuse stand will also turn on at this time.
2. Turn on the stirrers to 60 RPM using the BaroFuse UI to ensure that the stir bars are rotating smoothly. Once proper stirring is observed, turn off the stirrers.
3. Place the middle section of the BaroFuse Enclosure on top of the base.
4. Connect the cable on the middle section of the BaroFuse Enclosure to the cable from the Electrical Box to allow power to the Ambient Temperature Controller Lever Switch.

5. Place the lid of the BaroFuse Enclosure on top. The display of the upper Temperature Controller (the Ambient Temperature Controller) should light up, and the set value should read 36°C (if not then reset it to the set value temperature). Start a 30 minute timer.

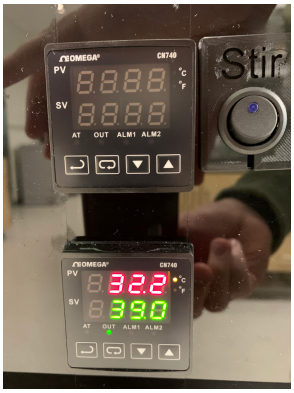


Fig. 4. Powering up the MRM Temperature Controller.

e. Inserting the Tissue Chamber Assemblies into the PCM.

Use the EnTox TCA Insertion Tool to insert each of the 8 TCAs into the PCM holes and, press on the adaptor so that the top of the tubing sleeve wrapped around the Tissue Chamber touches the surface circumscribing the holes in the PCM (Fig. 5). Completely insert one TCA before inserting the next one. This will take a bit of pressure to get the TCAs to rest against the PCM surface. Set the partially assembled PCM aside next to the PCM Brace and 6 screws.

Click here for a video demonstrating the use of the Tissue Chamber Insertion Tool to insert the Chambers into the Perifusion Chamber Module.

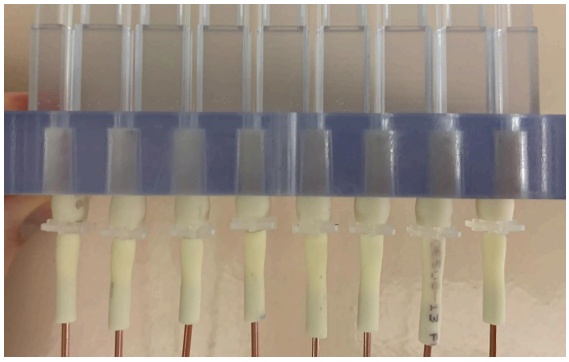


Fig. 5. 8 TCAs inserted into the PCM..

f. Filling the two troughs in the MRM with pre-equilibrated perifusate.

1. Ensure 30 minutes has passed since the BaroFuse Enclosure was assembled before continuing with this step.
2. Transfer the prepared (and pre-heated) perifusate into the pre-heated MRM. Either by using 50-mL pipette or directly pouring into the MRM. Add the perifusate along the sides of the insert to avoid creating any froth/bubbles.

Immediately carry out the next step to avoid transfer of gas between the fluid in the MRM and the atmosphere.

g. Assembling the MRM/PCM to create a gas-tight seal and placing the O_2 detector.

1. Place the PCM onto the MRM by Inserting the TCAs emanating from the PCM into the MRM, 4 on each side of the MRM Divider. The PCM fins should be closest to the O_2 Detector.
2. Secure the PCM and the PCM Support Brace with the 6 Screws using the Electric Screwdriver.
3. Secure the TCAs with the Rubber Band provided by stretching it around the fins of the PCM at the level of the rubber gaskets (Fig. 6).
4. Position the O_2 Detector on the BaroFuse Stand so that the face of it is snug against the PCM. The LED/Photodetector pairs should now be lined up with the O_2 sensors in the Tissue Chambers.
5. If needed, the oxygen detector lateral guides can be adjusted after loosening the screws to allow the O_2 detector to be moved.
6. Place the lid of the BaroFuse Enclosure on top.

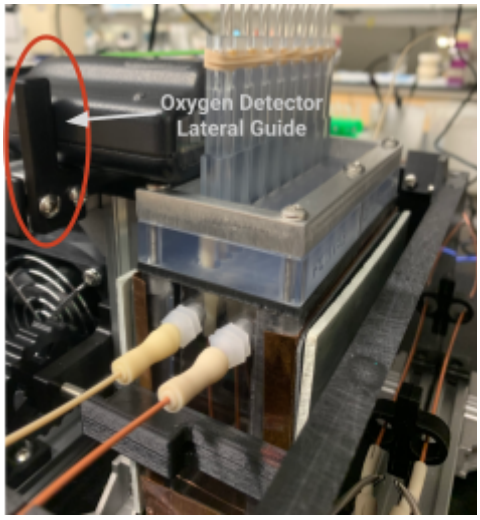


Fig. 6. Positioning of the Electric Stir Box, the MRM Heater, the MRM, the PCM and O_2 Detector on the BaroFuse Stand.

h. Starting the O_2 measurements.

1. Activate the O_2 Detector Software by clicking on the icon that says “Oxygen Detector”.
2. Once the program opens (Fig. 7), ensure the correct COM port and settings are selected. If needed, the COM port may be identified by unplugging and plugging the O_2 detector from the computer so that the port number is displayed. If the COM port is unplugged while the application is running, then the application must be closed and reopened before use.

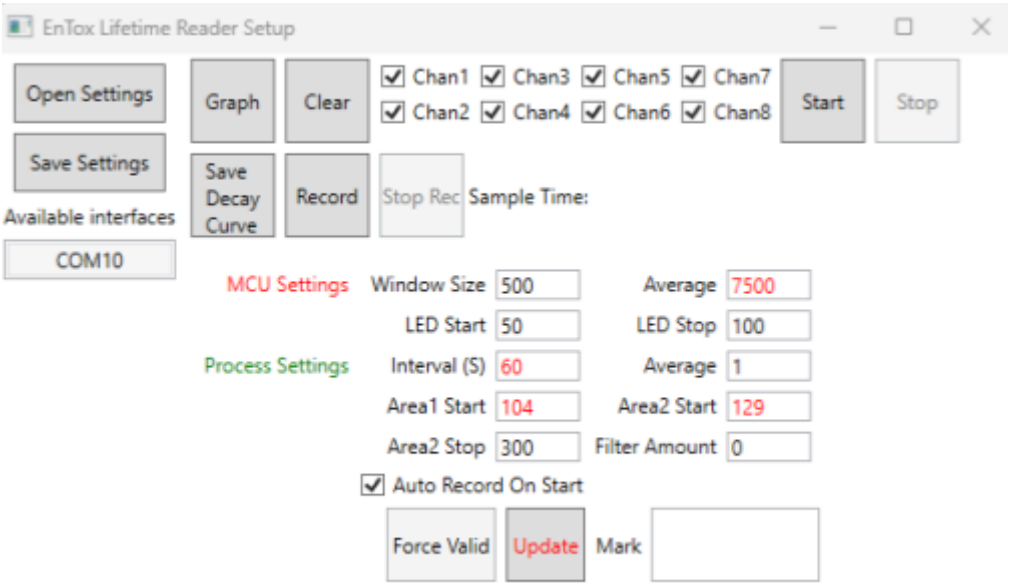


Fig. 7. GUI of the startup window of the O_2 Detection Software.

3. Click "Start" and save the data in the desired folder -> Click "Graph".
4. Change the Average value on the bottom left to 5. After one minute has passed and the first data point shows on the graphing screen, click "Auto Scale".

i. Equilibrating the head space in the MRM with the perifusate.

1. With the High Pressure Valve fully secured and closed, open the gas tank valve by turning the cylinder valve on top of the tank counterclockwise.
2. Adjust the high-pressure regulator to a pressure of 10 psi using the knob on the regulator.
3. Pressurize the MRM by setting the Low-Pressure Pressure Valve to 1.0 psi (Fig. 9).



Fig. 9. High and low pressure regulators on the gas tank.

4. Allow the gas from the tank to replace the air in the MRM headspace with the purge tube unclamped (and the test compound injectors still clamped). Submerge the end of the purge tube into a beaker of water to confirm flow.
5. After 15 minutes, turn the stirrer on at 60 RPM and leave running for the rest of the experiment.
6. After 15 minutes has passed, clamp the Purge Tubing Assembly shut with a hemostat (Fig. 10 Right).
7. Lower the pressure on the low-pressure regulator to the operating pressure for the experiment (usually between 0.5-0.7psi). If the flow rate is above 20uL/min temporarily set the pressure to 0.3 psi to allow time to load the frits and tissue without the chambers overflowing. This is not necessary if the tissue will be loaded within 15 minutes of the clamp being placed. (NOTE: do not let media run down the outside of the tissue chamber, since the fluid can interfere with O_2 sensing.)

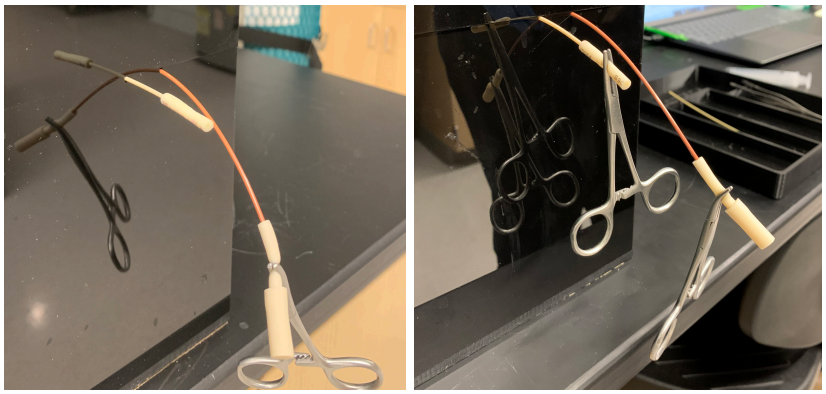


Fig. 10. Closed BaroFuse system with Purge Tubing Assembly on the left of each image and the MRM left side's Compound Injection Assembly on the right of each image.

3. TISSUE LOADING AND EQUILIBRATION PERIOD (90 MINUTES)

a. Positioning the Frits in the Tissue Chambers.

1. Remove the Lid and Middle sections of the BaroFuse Enclosure.
2. After the perifusate in the Tissue Chambers have risen above of the top of the pre-positioned frit, push the frit down with the Frit Cue by lightly tapping on the top of the frit in order to remove any air bubbles that formed below or within the frit.
3. Position frits about 0.25 inches above the bottom of the Tissue Chamber. Do not let perifusate run down the outside of the tissue chamber as the fluid can interfere with O2 sensing.

b. Loading Tissue into the Tissue Chambers.

See ***APPENDIX E*** for handling tissue prior to loading it into the chambers. Tissue is loaded into the chamber once the level of media is ½" from the top. For retina, use fine point forceps to place the tissue into each chamber while using a Kim-Wipe to prevent liquid from the tissue chamber dripping onto the O_2 sensor. Observe tissue sinking toward and onto the frit.

c. Attaching the Outflow Tubing Assemblies to Tissue Chambers.

1. Remove the Outflow Tubing Assembly Pack and then place the Outflow Tubing Enclosure Guide on the middle section of the enclosure edge with the Outflow Tubing Adapters on the inside of the Enclosure.
2. Attach the Outflow Tubes Adapters onto the TCAs (Fig. 11). Be careful not to push too hard on the TCAs or they will come loose from the MRM.
3. Replace the Middle of the BaroFuse enclosure and reconnect the Ambient Temperature Control cable.
4. Replace the Lid of the BaroFuse Enclosure.
5. Feed the eight Outflow Tubing Assemblies through the Fraction Collector guide arm.



Fig. 11. Picture of Outflow Tubing Assemblies attached to the Tissue Chambers.

d. Activating the Fraction Collector.

1. Make sure the Fraction Collector is centered with respect to the wall of the BaroFuse Enclosure and Outflow Tube Holders: the left support of the Fraction Collector base should be running along the BaroFuse Enclosure edge.
2. On the laptop, click the ”BaroFuse UI” shortcut and the “Experiment Info” page will open (Fig. 12 Top).
3. Fill in all available information on the Experimental Info page (this can be done prior to the experiment start) and then click the “Flow & Fraction Collector” page on the top (Fig. 12 Bottom).

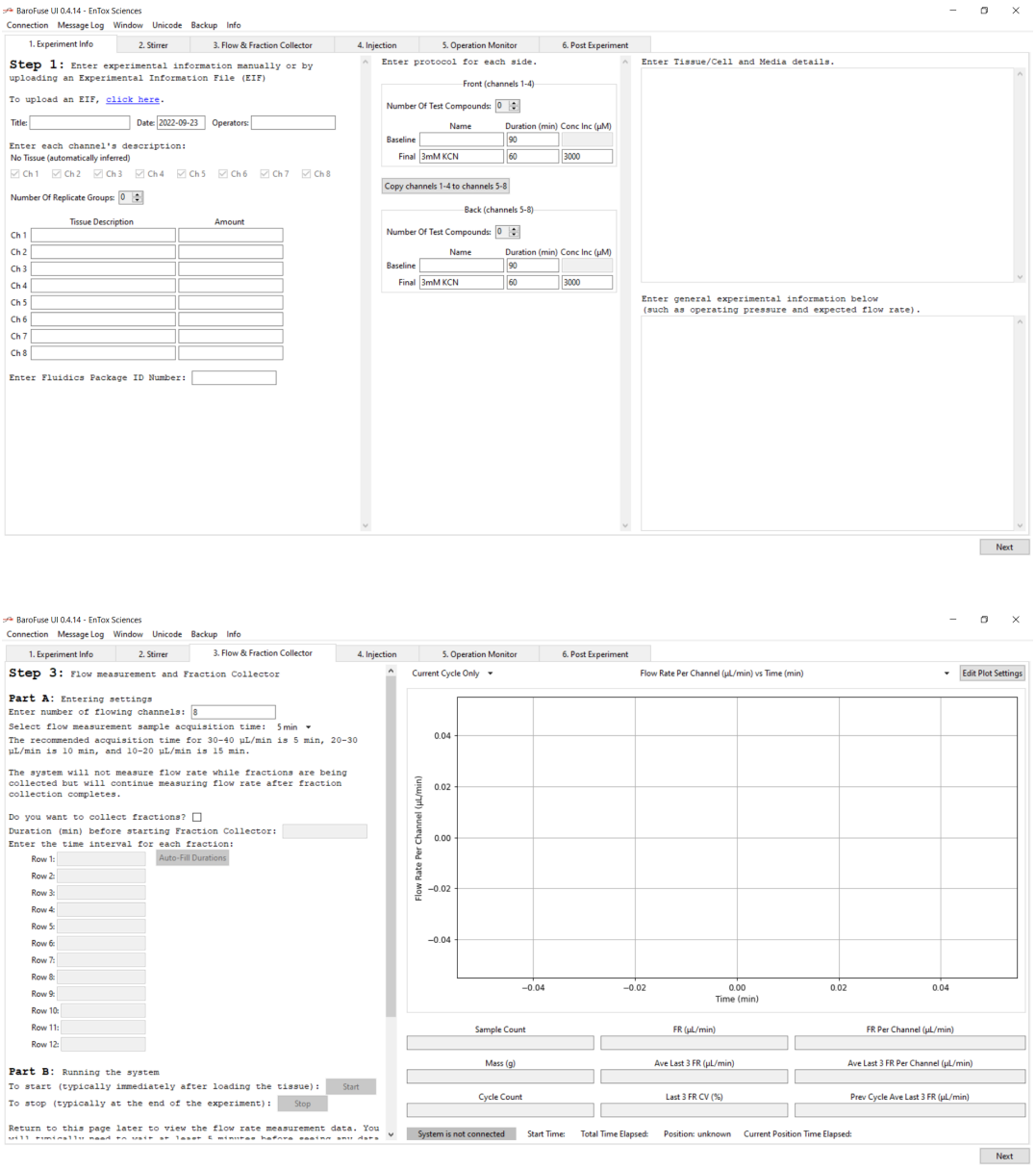


Fig. 12. GUI for Fraction Collector settings.

NOTE:
The slower the flow rate, the longer the sampling rate has to be in order to resolve the changes in mass accurately.

e. Measuring Flow Rates Using the Flow Monitor.

1. To set parameters controlling the flow rate measurement, check the “Read flow” box, and select an appropriate time in the ”Sampling rate” drop-down menu on the top middle.
2. Then click “Start” if no fractions will be collected in this experiment. If fractions are going to be collected then continue to the next step before clicking “Start”.

f. Collecting Fractions.

1. Check the “Collect fractions” box next to the “Read flow” button.
2. Enter the amount of time to wait until fractions begin in the “Waste 1” section.
3. Enter the duration of each collection into “Row 1” in order to set the Time interval for advancing the Fraction Collector.
4. Ensure the “Waste 2” section value is 9999 and then click “Run”.

g. Measuring Flow Rates Manually. (Optional)

Another method to measure the flow rate is to use the Fraction Collector’s Microcentrifuge Tube Holder.

1. Weigh eight microcentrifuge tubes and record their weight.
2. Set the Tube Holder on the Carriage with eight microcentrifuge tubes in it. Click “Measure Flow Rate Manually” in the Other utilities section at the bottom of the screen. Select the duration for measurement and then click “Generate Template”. Close the window and click “Start”
3. The Fraction Collector will collect fluid from the Outflow Tubing Assemblies for the input time and then return to its home position.
4. Weigh the microfuge tubes after collection and use the difference in weight to calculate the flow rate.

NOTE:
The pressure should be kept within 0.4 - 0.7 psi during standard flow operations.

The last time pressure can be adjusted is 30 minutes before the first injection is given.

h. Adjusting Flow Rate.

Adjust the pressure as needed to fine-tune the desired flow rate (for instance, if flow rate is 10% higher than wanted, then lower the pressure by 10%). Density of most buffer/media is close to that of water, so 10 mg of buffer/media would be 10 uL in volume.
The last time pressure can be adjusted is 30 minutes before the first injection is given.

i. Baseline Stabilization.

Prior to the first injection, it is important to allow the tissue to reach a steady state. This is a critical step in generating reproducible and high-resolution results, as tissue takes time to recover from the trauma of harvesting, and perturbations in tissue function and metabolic rate by changes in temperature and gas dissolved in the perifusate. In addition, responses to test compounds can best be resolved if the slope of the baseline is small relative to the change. It is also important to measure flow rate with enough accuracy so it can be used to calculate the volume left in the MRM. At least 30 minutes after an adjustment in pressure,

enter the “Ave last 3 FR per channel” value into the Injection page for preparing Test Compound Injections. Ideally this is between 20-30 minutes prior to the first injection

4. EXPERIMENTAL PROTOCOL (2-6 HOURS)

a. Preparing Test Compound Injections.

1. Complete the table in the BaroFuse UI on the Injection page to determine the volumes for the injections (Fig. 13). To calculate how much stock solution to inject to achieve the correct concentration, the program uses the flow rate, the time of the protocol, the starting volume of fluid in the MRM, and the concentration information.
2. To calculate how much media and stock are needed for the injection fill in the white boxes and click “Compute”.
3. Prepare the first test compound by mixing the stock solution and buffer/media. Load the syringes for the first injection.

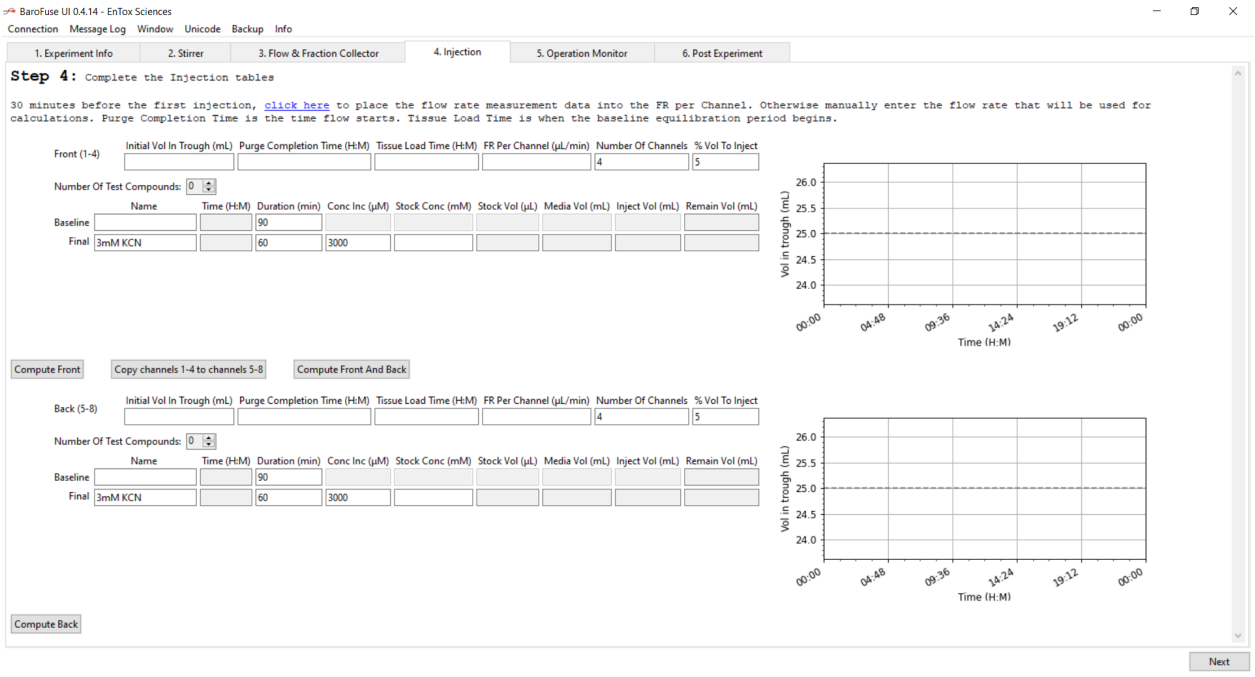


Fig. 13. Injection page which is to determine injection volumes.

WARNING:
If the injection is performed too quickly then it is possible that the Test Compound will not go into the MRM Insert.

b. Injecting Test Compounds.

Connect the syringe with the compound to the injection ports (Fig. 14 Left). Unclamp the hemostat and very slowly inject the compound (Fig. 14 Middle); injection rate should be about 3 mL/min. Re-clamp the Pharmed (soft walled) tubing on the injection port and remove the syringe (Fig. 14 Right).

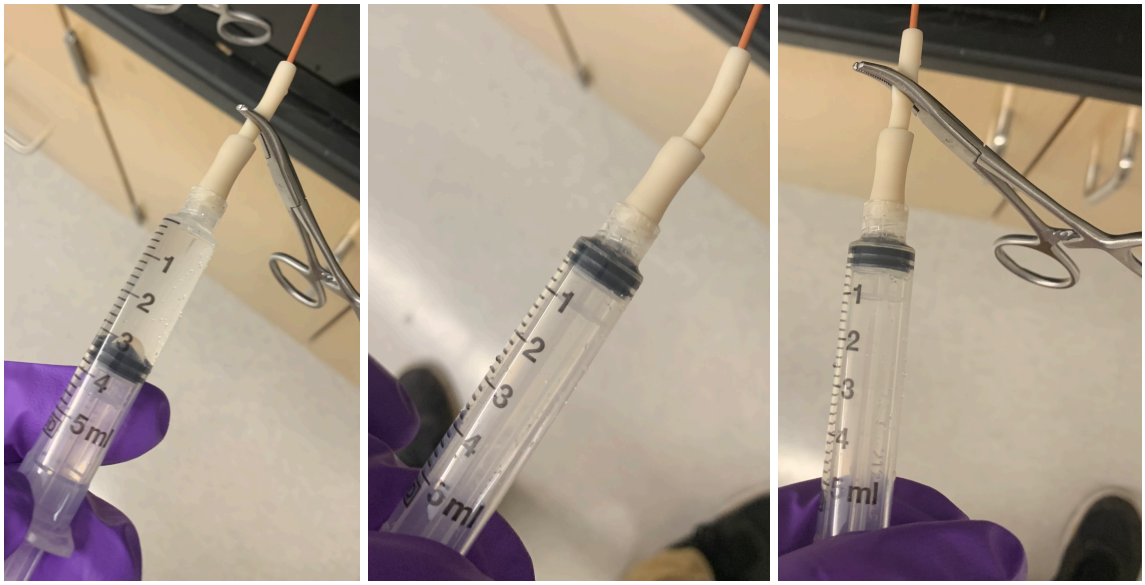


Fig. 14. Injecting a test compound using a 5 mL syringe.

After injecting the test compounds, prepare each subsequent test compound to be injected according to the BaroFuse UI Injection page.

c. Determining Inflow O_2 Signal for each channel.

O_2 Consumption Rate is calculated as the Flow Rate times the difference between Inflow and Outflow O_2. Because the Inflow O_2 is constant throughout the experiment, it is measured once at the end of the experiment. To do this, we consider that the Outflow O_2 signal is equal to the inflow O_2 tension when O_2 consumption by the tissue has ceased. At the end of every BaroFuse experiment, respiration is completely inhibited by adding 3 mM KCN, and the Inflow O_2 signal value is determined after it has reached a steady state (usually about 60 minutes after the KCN injection depending on the flow rate).

5. BREAKING DOWN AND CLEANING THE SYSTEM (30 MINUTES)

a. Saving the Oxygen Data.

Click the Save button on the top left of the graphing window on the Oxygen Detector Software; name the file and store it into the folder where the file will be kept. It is recommended to name the file descriptively and by date. The main file and the backup file contain the same information. If processing the data on a different computer, transfer the .csv file as needed.

b. Saving the BaroFuse UI "Experiment Info File".

Click the "Save profile" button on the top left of the BaroFuse UI's General page; name the file and store it into the folder where the file will be kept. On the Fraction & Flow page of the BaroFuse UI click the "Tools" drop down menu and then "Save"; Keep the generated name or choose your own and store the file where desired.

If necessary, there are backup files which can be accessed and saved. To access the backup directory, open the BaroFuse UI and click "Info" in the top action bar. Then select "Backup Directory" which will bring you to a folder in your computer's Document section. Select "fraction-collector" for flow rate data from the "barofuse-ui-profile" to access protocol information.

c. Breaking down the BaroFuse.

Since KCN is volatile, dispose of fluidics assemblies in a fume hood; pour media from MRM and FC waste tray into a waste container, and rinse the MRM Inserts and Stir Bars thoroughly with water. Pour the contents of the waste container (containing KCN) into a labeled chemical waste container for subsequent disposal by chemical safety. Prior to the next experiment, thoroughly clean the Stir Bars and forceps.

6. DATA PROCESSING (15-45 MINUTES)

1. Open the BaroFuse Data Processor application on a Mac or PC computer.
2. Select the .csv data file generated by an experiment. If this experiment's protocol is similar to a previously analyzed experiment then select that settings file and click "Next Step". Otherwise just click "Next Step" to begin entering the experiment's settings.
3. Fill in the various settings of the experiment. On the "Determination of Reference Time Point" select the time point directly before the KCN effect went into effect and the Lifetime values decreased. This point can be selected with the slider or by typing the time value into the box.
4. Click "Calculate" to generate OCR graphs. The Graphs can be saved as png's by pressing the "Export As PNG" button to the upper right of the graph.

APPENDIX A: TROUBLESHOOTING

- Bubbles in the Tissue Chamber prior to or during loading
    - Tap the frit more if the bubble is located underneath the frit
- Unexpected increase to the oxygen lifetime Ratio during the experiment
    - Ensure no great decreases or stopping of flow rate in that channel
    - Evaluate whether perifusate was prepared properly
    - Ensure no decrease to temperature
- Unusual lifetime ratio values (outside 0.5-0.7)
    - Check to see that the temperature on the controller is the expected value
    - A high value of lifetime Ratio could be caused by bacteria growth
- No flow
    - Check pressure regulator to ensure no leaks and that it is functioning properly
    - If it's only one or two channels then use a syringe to suck from the end of an outflow tube
    - If more than two channels have flow issues check MRM/PCM for leaks
- Temperature values not reaching set value
    - Check to see if the heater is getting power sent to it by checking to see if the green light comes on below the "Out" of the Temperature Controller.
- Oxygen data drift prior to tissue in the system
    - Temperature has not reached steady state

APPENDIX B: PRE-EXPERIMENT CHECKLIST

- ☐ Determine tissue model and quantity for each channel
- ☐ Determine the flow rate and starting perifusate volume
- ☐ Ensure test compound stocks are available and determine stock concentrations
- ☐ Prepare the perifusate the day before the experiment and place it in a 40°C incubator with 5% CO_2. (Remember to add fuels and other additions the morning of the experiment before placing into the MRM)
- ☐ Set out the fluidics package to be used in the experiment
- ☐ Ensure the Forceps and Stir Bars are clean

APPENDIX C: Protocol Changelog

Date	Section	New	Old
8/9/22	Instrument Set-Up	Stirrer speed is 60 RPM. Stirrer turned off at the end of the purge process. Stirrer turned back on 45min after tissue loaded.	Stirrer speed is 250 RPM. Stirrer turned on 15 min into purging and left on for duration
8/16/22	Instrument Set-Up	Includes TCA Insertion Tool and video.	Directs user to use hands to push in TCAs
8/18/22	Tissue Loading and Equilibration	Enumerated and streamlined FC usage	
8/22/22	Instrument Set-Up	Stirrer is left on after it is turned on during the purging process until the end of the experiment.	Stirrer turned off at the end of purging. And then turned back on 45min after tissue loading.
8/22/22	Overview	Added an “Experimental Flow” diagram on page 2	
8/22/22	Overview	Added a few paragraphs about software (UI and EDP)	
8/25/22	Instrument Set-Up	Specified that users should insert TCAs one at a time into the PCM	
9/23/22	Experimental Protocol	Adjusted entire FC section to reflect changes from v0.3 to v0.4	
10/4/22	Instrument Set-Up	Change purge pressure to 1.0psi	Purge pressure was 1.5psi
10/4/22	Instrument Set-Up	Added a note to not clamp too close to the hinge	
10/4/22	Instrument Set-Up	Frits need to be submerged in buffer then centrifuged	Frits were sent submerged in PBS beforehand
10/4/22	Instrument Set-Up	MRM heater and ambient heater operating temperatures are 38C and 36C, respectively.	MRM heater and ambient heater operating temperatures were 39C and 37C, respectively.
10/4/22	Experimental Preparation	Added option of equilibrating buffer the morning of experiment	
11/3/22	Instrument Set-Up	Remove frit section	
	Instrument Set-Up	Update O2 detector SW section for v1.3	More buttons had to be pressed on old software

APPENDIX D: Determination of Amount of Tissue/Cells to Load

In general, the BaroFuse functions best at flow rates between 40 - 100 uL. However, it can generate reliable data down to 9 uL/min, which is used when tissue availability is limited. The user will want to optimize conditions for their application, which will not only depend on how much tissue is available, but also the magnitude of the changes that are to be resolved. In general, the more tissue (and the higher the flow rate) the crisper the data will be, since the contributions of the delay and dispersion from the fluidics system relative to the kinetics of the biological effects become smaller. Overall, there is a tradeoff between the ratio of tissue/flow rate: the higher the ratio, the bigger the difference between inflow and outflow and the higher the S/N will be; conversely, the bigger the difference between inflow and outflow results in potentially hypoxic effects. The recommendations in the Table are based on keeping the outflow O2 no less than 75% of the inflow (corresponding to an outflow of equilibrium with 15% O2 with an inflow O2 of 21% and a change in Lifetime Ratio of 0.03). If the tissue or cell model is not overly sensitive to the O2 levels in that range, the ratio of tissue to flow rate can be increased so that the outflow is close to 12.5% (or a Lifetime Ratio of 0.045).

TABLE 1. Recommended Operating Specifications for Different TIssue and Cell Models.

TISSUE/CELL	AMOUNT TO LOAD	FLOW RATE	CHAMBER ID (mm)
Retina	1	40	3.4
Hypothalamus	1/4	40	3.4
Liver	12 mg	40	3.4
Heart	6 mg	40	3.4
Cells on Culture Beads	0.5-1 x 10^6 Cells	40	3.4
Muscle	12 mg (4 x 3 mg)	20	3.4
RPE Cells on Transwell Membranes	240,000 Cells (6 x ⅓ filter strips)	12	2.4
Islets	100	9	1.5

APPENDIX E: Preparation, Loading and Stabilization of Tissue/Cells in the Perfusion Chamber

a. Tissue: Hypothalamus, Retina, Liver, Heart, Muscle, Cerebellum

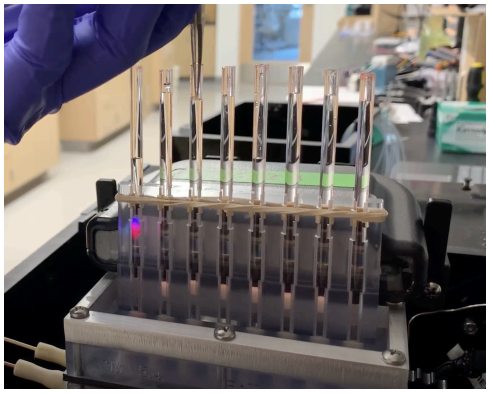
Preparation.

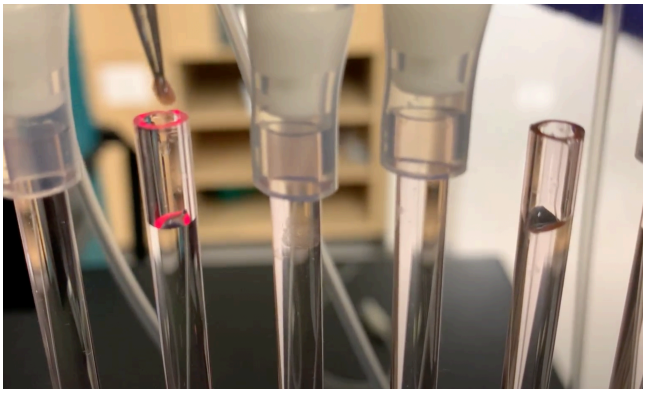
Maintaining tissue viability after harvesting tissue but before loading into the chamber. It is critical that tissue in a bicarbonate based buffer/media should not be left out of the incubator for more than 10 minutes. If it is more than this, the tissue should be placed in Hank's Buffered Salt Solution. In addition, there needs to be enough buffer/media to keep the tissue from becoming hypoxic. A guideline is 10 mL/25 mg of tissue.

Loading.

Hypothalamus, liver, heart, muscle, cerebellum samples should be cut into 3 mg pieces before loading. Retina should be left as whole pieces. Most tissue samples are loaded using thin forceps.

Click on the videos below to see an example of loading retina into tissue chambers.





b. Tissue: Isolated Pancreatic Islets

Preparation.

Maintaining tissue viability after harvesting tissue but before loading into the chamber. It is critical that tissue in a bicarbonate based buffer/media should not be left out of the incubator for more than 10 minutes. If it is more than this, the tissue should be placed in Hank's Buffered Salt Solution. In addition, there needs to be enough buffer/media to keep the tissue from becoming hypoxic. A guideline is

35 mm Petri dish, 2 mL media/buffer and < 300 islets,

60 mm Petri Dish, 5 mL media/buffer and < 750 islets,

100 mm Petri Dish, 14 mL media/buffer and < 1600 islets,

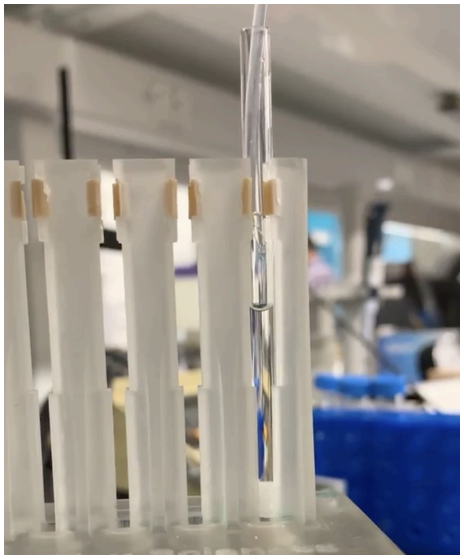
Selection of Tissue Chamber and Flow Rate.

The size of tissue chambers used for islets depends on the number of islets being perifused. For 200 islets or more, use the standard Tissue Chamber Assembly (ID = 3.4 mm, and flow rate = 25 -30 uL.min). For less than 200 islets, the ID of the tissue chamber is 1.5 mm. To measure OCR for either chamber size , the flow rate will be approximately FR in uL/min = # of islets/10.

Loading islets into tissue chamber with ID = 3.4 mm.

1. Using a P-200 pipet (with a tip that has been beveled slightly with a razor blade), pick the amount of cultured islets needed into a petri dish containing 3 mM KRB (with 0.1 wt/v% BSA). Note do not leave KRB out of an incubator for more than 10 minutes.
2. Insert frits into tissue chambers so that they are submerged into the buffer/media.
3. Pick and pipet islets from the petri dish into a 1.7 mL microcentrifuge tube. The number will equal how many will be in a chamber, and the volume of the buffer will equal the number of islets per channel. So for 300 islets, there should be a total of 300 uL.
4. Attach the Islet Loading Tubing (7 inches of PE205 tubing attached to P-1000 tip) to a P-1000 pipet, and take up no more than 300 uL of the islet suspension from the microcentrifuge tube into the PE205 tubing. Make sure there are no bubbles in the tubing and that the islet suspension does not get sucked into the pipet tip.
5. Gently submerge the tip of the PE tubing into the fluid in the TIssue Chamber just beneath the surface of the top of the fluid column. If the tip of the PE tubing is too deep, then a bubble will be generated when the islet suspension is pipetted.
6. Gently press the plunger so the islet suspension transfers from the tubing and into the TIssue Chamber. Make sure that islets are being pipetted into media/buffer and not into air, and that the fluid in the tissue chamber doesn't overflow. Note that 300 uL will raise the fluid by about 1.33 inches so add islets before the Tissue Chamber is too full.
7. Place the Outflow Tubing Assembly onto the top of the Tissue Chamber.

Click on the video below to see an example of loading islets into a tissue chamber.



Loading islets into tissue chamber with ID = 1.5 mm.

Loading 200 islets or less into a 1.5 mm ID tissue chamber is very similar to loading into the 3.4 mm ID except for 12" of PE50 tubing is used to transfer the islets onto the frit in the tissue chamber, only 75 uL of buffer are used to transfer islets into the Tissue Chamber, and a P-100 pipet is used instead of a P1000 pipet. Thus the steps are as follows.

1. Using a P-200 pipet (with a tip that has been beveled slightly with a razor blade), pick the amount of cultured islets needed into a petri dish containing 3 mM KRB (with 0.1 wt/v% BSA). Note do not leave KRB out of an incubator for more than 10 minutes.
2. Insert frits into tissue chambers so that they are submerged into the buffer/media.
3. Pick and pipet islets from the petri dish into a 1.7 mL microcentrifuge tube. The number will equal how many will be in a chamber, and the volume of buffer will be 75 uL regardless of the number of islets being loaded per channel.
4. Attach the Islet Loading Tubing (12 inches of PE50 tubing attached to a P-100 tip) to a P-100 pipet, and take up no more than 75 uL of the islet suspension from the microcentrifuge tube into the PE50 tubing. Make sure there are no bubbles in the tubing and that the islet suspension does not get sucked into the pipet tip.
5. Gently submerge the end of the PE tubing into the TIssue Chamber and position it just above the frit and beneath the surface of the top of the fluid column.

6. Gently press the plunger so the islet suspension transfers from the tubing and into the TIssue Chamber. Make sure that the islets are being pipetted into media/buffer and not into air, and that the fluid in the tissue chamber doesn't overflow. Note that 75 uL will raise the fluid by about 1.75 inches so add islets before the Tissue Chamber is too full.
7. Place the Outflow Tubing Assembly onto the top of the Tissue Chamber.

Loading islets into tissue chamber with ID = 3.4 mm for measurement of Insulin Secretion but not OCR.

If it is desired to use the BaroFuse to measure insulin/glucagon secretion from islets, without OCR, then only 10 islets are needed per chamber, and the flow rate is typically 100 uL/min. This is fast enough to produce first phase insulin secretion from a rapid change in glucose concentration if desired. Up to 90 mL can be placed into the MRM Inserts, so the maximum length of the protocol would be about 3 hours.

1. Using a P-200 pipet (with a tip that has been beveled slightly with a razor blade), pick the amount of cultured islets needed into a petri dish containing 3 mM KRB (with 0.1 wt/v% BSA). Note do not leave KRB out of an incubator for more than 10 minutes.
2. Insert frits into tissue chambers so that they are submerged into the buffer/media.
3. Pick and pipet 10 islets from the petri dish into a 1.7 mL microcentrifuge tube containing 50 uL of buffer.
4. Attach the Islet Loading Tubing (6 inches of PE50 tubing attached to a P-100 tip) to a P-100 pipet, and take up 50 uL of the islet suspension from the microcentrifuge tube into the PE50 tubing. Make sure there are no bubbles in the tubing and that the islet suspension does not get sucked into the pipet tip.
5. Gently submerge the end of the PE tubing into the TIssue Chamber and position it just above the frit and beneath the surface of the top of the fluid column.
6. Gently press the plunger so the islet suspension transfers from the tubing and into the TIssue Chamber. Make sure that the islets are pipetted into media/buffer and not into air, and that the fluid in the tissue chamber doesn't overflow. Note that 50 uL will raise the fluid by about 0.25 inches.
7. Place the Outflow Tubing Assembly onto the top of the Tissue Chamber.

c. Cells Attached/Grown on Culture Beads

The final ratio of beads to seeded cells should be 2.5 mg per million cells and will be prepared by transferring and washing beads stored as a stock solution. To attach cells to culture beads, you will first need to prepare a stock solution of culture beads in silanized glass bottles and determine the final stock concentration (see Appendix I for silanization and culture bead stock solution preparation).

Preparing the Culture Beads.

To transfer the desired number of beads from the stock solution, first swirl the contents of the bottle to evenly disperse beads and before the beads can settle, pipette the desired volume with a 25 or 50 mL pipette. The volume of beads to use is calculated as:

$$Volume\ of\ bead\ stock\ =\ Desired\ no.\ of\ cells\ x\ 2.5\ mg/million\ cells \div 15\ mg/mL$$

Then transfer to a 50-mL conical without touching the sides. Allow beads to settle, aspirate the PBS, and then wash with ~10 mL culture media a total of 3 times. Allow the beads to settle and then aspirate as much media as possible without aspirating the beads (this will leave a final bead volume of ~1.25 mL in the conical).

Harvesting and Counting the Cells.

Next, trypsinize and harvest the cells in the flask and place in a 15-mL conical. Then spin the cells down and reconstitute them in 4mLs of media. Dilute 100uL of the cell volume in 1900 uL media (1:20) and count in a hemocytometer. Calculate the concentration of cells/mL and volume to add to beads using the following equation:

$$Cell\ concentration\ =\ Avg.\ cells/grid\ x\ 10000\ x\ 20$$

$$Volume\ of\ cells\ to\ add\ to\ beads\ =\ Desired\ no.\ of\ cells \div Cell\ concentration$$

Combining Cells and Culture Beads and Plating.

Using the volume of cells just determined, transfer cells with a P1000 pipette 0.5 mL at a time and mix with beads. Allow to sit for 5 additional minutes following the final transfer. Bring up the cell/bead mixture in media such that the desired number of cells/channel to be used are plated in 2.5 mL of media. For example, if 15 million cells are put on beads to run 6 channels, bring up the cell/bead to 16 mLs (2.5mL *6 + 1 mL extra to avoid bubbles while dividing). Subsequently, using a 10 mL pipette, triturate the bead/cell mixture (of 2-4 million cells) and divide in 2.5 mL volumes to non-treated 35 mm culture dishes or wells of a 6-well plate. Allow to attach overnight in a 37°C CO_2 incubator.

Loading into the BaroFuse.

Check the cells first thing on the morning of the experiment to make sure they are attached to the beads (12-20 cells/bead). Set aside 50 mL of media incubated overnight (with glucose) in a 37°C water bath. Five minutes prior to loading the cells, use a 10 mL pipette to transfer the cell/bead mixture divided the previous day from the culture dish/6-well plate to individual 15-mL conicals without touching the sides of the conical. Tilt the plate with the remainder of cells and dislodge any beads stuck to the bottom of the plate using 2 mL of 37°C KRB/media being used in the experiment. Then, transfer to the respective 15-mL conicals. Allow the beads to settle, aspirate media, and wash with 2 mL of warm KRB and aspirate. Repeat one more time so the supernatant is clear. Load using a P200 with a beveled tip.

APPENDIX F: Perifusate (Buffer/Media) Preparation

In general, BaroFuse experiments are carried out in either Krebs Ringer Bicarbonate (KRB), Culture Media, or Hank's Buffered Salt Solution (HBSS). The first two are bicarbonate based and require a gas supply that contains 5% CO_2. Using Hanks would require a tank of gas that does not contain CO_2.

Preparing KRB Using Stock Solutions

KRB is a commonly used perifusate for experiments with the BaroFuse. KRB should be prepared in two steps, the first step on the day before an experiment and the second step the morning of an experiment. Most components of the KRB are mixed together the day before an experiment and then stored overnight in a 39-40°C incubator with 5% CO_2. The morning of the experiment the fuels (i.e. glucose) should be added to the KRB before placing the buffer into the MRM. The stock solution recipes are found at the end of this section.

<u>General Recipe for 100mL of KRB.</u>

This is the general recipe for making KRB which requires 92mL of salt solution to be diluted with 4 mL of a 648mM bicarbonate solution and 4mL of other solutions. The last 4 mLs can vary depending on the experiment. Parts ***a*** and ***b*** should be mixed together the day before an experiment and stored in a 39-40°C incubator with 5% CO_2 and part ***c*** should be added the morning of the experiment before placing the buffer into the MRM.

a. 92 mL of salt solution
b. 4 mL of bicarbonate stock
c. 4 mL of other additions (substitute sterile water if no other additions desired)

<u>Recommended Recipe for 100mL of KRB.</u>

This is the recommended recipe for use with most BaroFuse experiments. Parts ***a-d*** should be added the night before and stored in a 39-40°C incubator with 5% CO_2 and part ***e*** should be added the morning of the experiment before placing the buffer into the MRM.

a. 92 mL of salt solution
b. 4 mL of of bicarbonate stock
c. 1 mL of sterile H_2O or 1 mL of Pen-Strep stock. If the experiment protocol is 8 hours or more then use PSF
d. 1 mL of 10 wt/v% BSA (see page 29 for stock details)
e. 500 uL of 1 M glucose stock + 1500 uL of sterile H_2O (for a 5 mM glucose solution)

Below is a list of alternative additions for part **e**.

a. Glucose solution at the desired concentration
    i. 20 mM = 2 mL of 1 M glucose stock
    ii. 5 mM = 500 uL of 1 M glucose stock + 1500 uL of sterile H_2O
    iii. 3 mM = 300uL of 1 M glucose stock + 1700 uL of sterile H_2O
b. Mitochondrial fuel mix with 5mM glucose as used in *Roles of Glucose in Photoreceptor Survival* article, published in the JBC in 2011
    i. 5 mM glucose
    ii. 10 mM pyruvate
    iii. 5 mM malate
    iv. 1 mM glutamine
    v. 1 mM leucine
c. Alternative Mitochondrial fuel mix with 5mM glucose
    i. 5 mM glucose = 500 uL of 1 M glucose stock
    ii. 0.5 mM pyruvate = 50 uL of 1 M pyruvate
    iii. 5 mM lactate = 500 uL of 1 M lactate stock
    iv. 1 mM glutamine = 500 uL of 200 mM glutamine
    v. 1 mM leucine = 450 uL of 220 mM leucine

Recipe for Salt Solution.

This salt solution is made in one liter of DI water and then filtered. The stock can be stored at room temperature in a glass bottle.

Solute	Grams per liter	MW (g/mol)	Stock Concentration (mM)	Final Concentration after dilution (mM)
NaCl	6.257	58.44	107.1	98.5
KCl	0.397	74.55	5.3	4.9
KH_2PO_4	0.178	136.09	1.3	1.2
$MgSO_4 * 7H_20$	0.321	246.47	1.3	1.2
HEPES	5.420	249.3	21.7	20
$CaCl_2 * 2H_20$	0.415	147.02	2.8	2.6

Recipe for Bicarbonate Stock.

This 648mM bicarbonate stock is made in 100mL of DI water and then filtered. The stock should be stored at 4°C in two 50mL conicals.

Solute	Grams per 100mL	MW (g/mol)	Stock Concentration	Final Concentration after dilution

			(mM)	(mM)
$NaHCO_3$	5.44	84.01	648	25.9

Recipe for BSA Stock.

This 10 wt/v% BSA stock is made in 100mL of DI water and then filtered. The stock should be stored at 4°C in two 50mL conicals.

Solute	Grams per 100mL	Stock Concentration (wt/v%)	Final Concentration after dilution (wt/v%)
Albumin	10	10	0.1

Recipe for Glucose Stock.

This 1M glucose stock is made in 100mL of DI water and then filtered. The stock should be stored at 4°C in two 50mL conicals.

Solute	Grams per 100mL	MW (g/mol)	Stock Concentration (mM)
Glucose	18.02	180.2	1000

Preparing KRB Using Purchased Solution

Alternatively, Krebs Ringer Solution can be purchased to reduce labor and number of steps in preparing for an experiment. See Appendix G for the recommended solution. The day before an experiment the Krebs-Ringer solution should be placed in a flask and then stored overnight in a 39-40°C incubator with 5% CO_2. The morning of the experiment, the fuels (i.e. glucose) and other additions should be added to the KRB before placing the buffer into the MRM.

Recommended Recipe for 100mL of KRB.

This is the recommended recipe for use with most BaroFuse experiments. Parts ***a and b*** should be added the night before and stored in a 39-40°C incubator with 5% CO_2 and part ***c*** should be added the morning of the experiment before placing the buffer into the MRM.

a. 98.61 mL of salt solution
b. 1 mL of 10 wt/v% BSA (see page 29 for stock details)
c. 392 uL of 1 M glucose stock (for a 5 mM glucose solution)

OR

a. 98.81 mL of salt solution
b. 1 mL of 10 wt/v% BSA (see page 29 for stock details)
c. 191 uL of 1 M glucose stock (for a 3 mM glucose solution)

APPENDIX G: Reagent Catalog

Perifusates

Name	Supplier	Catalog #
Krebs Ringer Solution (KRB)	Thermo Fisher Scientific	J67795
Hank's Buffered Salt Solution (10X)	Thermo Fisher Scientific	14065056

KRB Components

Name	Supplier	Catalog #	MW (g/mol)	Formula
Sodium Chloride	Sigma	S7653		NaCl
Potassium Chloride	Sigma	P9541		KCl
Potassium Phosphate Monobasic	Sigma	P5655		KH_2PO_4
Magnesium Sulfate (heptahydrate)	Sigma	M9397		$MgSO_4 * 7H_20$
HEPES hemisodium salt	Sigma	H7637		HEPES
Calcium Chloride Dihydrate, SigmaUltra	Sigma	C5080		$CaCl_2 * 2H_20$
Sodium Bicarbonate	Sigma	S5761		
Albumin, from Bovine Serum (BSA)	Sigma	A3059		
D (+) Glucose	Sigma	G8270		

Sources of Test Compounds

Name	Supplier	Catalog #	MW (g/mol)	Stock Concentration for Injection
Potassium Cyanide (KCN)	Sigma	207810	65.12	3 M
Oligomycin A	Cayman Chemicals	11342	791.06	10 mM in DMSO
FCCP	Sigma	C2920	254.2	1 mM in DMSO
Sodium Pyruvate (100mM solution)	Gibco	11360-070		
Antimycin A	Sigma	A8674		
Rotenone	Sigma	557368		
Lactate Oxidase	Sigma	L9795		
Sodium L-Lactate	Sigma	L7022	112	

To make these stocks:

Compound	Stock Conc, M	MW	Desired Vol, mL	Mass to add, g
KCN	3	65.12	10 *(H_2O)*	1.954
Oligomycin	0.01	791.06	5 *(DMSO)*	0.04
FCCP	0.01**	254.2	2 *(DMSO)*	0.005
Glucose	1	180.156	50 *(H_2O)*	9.008

If adding solvent directly to a purchased bottle with known mass:

Compound	Stock Conc, M	MW	Mass, g	Vol to add, mL
Oligomycin	0.01	791.06	0.005	0.63 *(DMSO)*
FCCP	0.01**	254.2	0.01	3.93 *(DMSO)*

**After the 10 mM stock of FCCP is made. Aliquot out samples in a 1:10 ratio of total volumes of 150 uL, which will result in a 1 mM stock.

APPENDIX H: Gas Tank and Accessories Catalog

Gas Tank Options

Gas Composition	Supplier	Catalog #	Description
21% O_2, 5% CO_2, and balance N_2	Airgas (Seattle)	X03NI74C3001309	CGA 590 outlet, Size 300
21% O_2, 5% CO_2, and balance N_2	Airgas (Boston)	Z03NI7422000083	CGA 500 outlet, Size 200 (H cylinder)
15% O_2, 5% CO_2, and balance N_2	Airgas (Boston)	Z03NI8022000097	CGA 500 outlet, Size 200 (H cylinder)

Gas Tank Accessories

Item	Supplier	Catalog #	Description
High Pressure Regulator	McMaster-Carr		CGA 590 inlet with ¼" NPT outlet
Bench Bracket for Gas Cylinder	Grainger	8AX37	Holds 1 gas cylinder of 4" to 14" diameter

APPENDIX I: Bottle Silanization and Culture Bead Stock Solution Preparation

Silanize bottles with Gel Slick Solution (Lonza, PO 50640) (from instructions)

1. If glass plates have been previously silanized, the silan must be removed before using Gel Slick® Solution. Follow the silane removal protocol.
2. Apply ~2.0 ml of Gel Slick® Solution onto the middle of one clean, dry glass plate (20 cm x 40 cm). One of the two plates should be left untreated.
3. Using a dry paper towel, spread Gel Slick® Solution evenly over the entire surface using a circular and overlapping motion.
4. Allow the coating to dry completely to a faint haze (approximately 5 minutes).
5. Remove the haze completely with soft paper towels or other soft wipes by buffing in a circular and overlapping motion. Use immediately to prevent airborne particles from depositing on the surface of the plate

Silane Removal From Bottles

To remove previous silanizing coatings or Gel Slick® Solution from glass plates, immerse the plates in a 2 N sodium hydroxide solution for 30 minutes. Caution: Wear gloves, eye protection and protective clothing when handling sodium hydroxide.

Rinse plates with cold water to remove sodium hydroxide solution. Wash with mild detergent.

Prepare Culture Bead Stock Solution

Prepare 0.5 g Cytodex 1 (GE Healthcare, PO 17-0448-01) by hydrating with 50 mL 1x PBS for at least 3 hours (preferably overnight) with occasional mixing in a 100-mL glass bottle that has been silanized to prevent the beads from sticking to the glass surfaces (the silanization protocol is described below). Aspirate PBS, wash by adding 30 mL PBS, aspirating, repeat wash and aspiration, then add 20 mL. Sterilize beads by autoclaving for 15 min at 115°C and 15 psi (1.1 bar).

** For Tuttnaeur table-top autoclaves, set time to 30min and temperature to one tick below 121°C **

After autoclaving, measure total volume to get an accurate concentration (0.5 g beads/mL PBS) using a large pipette to minimize surface area for beads to stick to. Add the required amount of sterile PBS to the solution to bring it up to a final concentration of 15 mg/mL. Store beads at room temperature.

APPENDIX J: Operating Principles and Data Processing

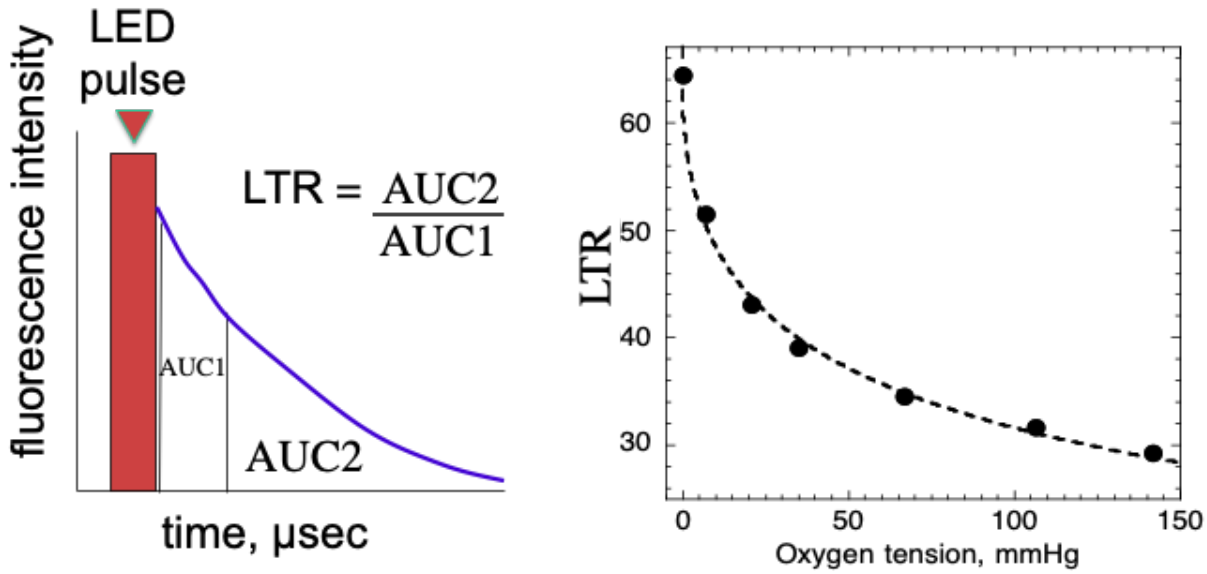
Aug. 1, 2022

Summary of Program Functionality

The BaroFuse is a fluidics instrument that simultaneously maintains tissue in flow culture while measuring O_2 consumption rate (OCR) of the tissue in response to sequentially injected test compounds. After the experiment, the BaroFuse Data Processing Program calculates and graphs OCR data from the time-dependent data generated during the experiment as well as user inputs that specify the timing of injections, flow rate and replicates.

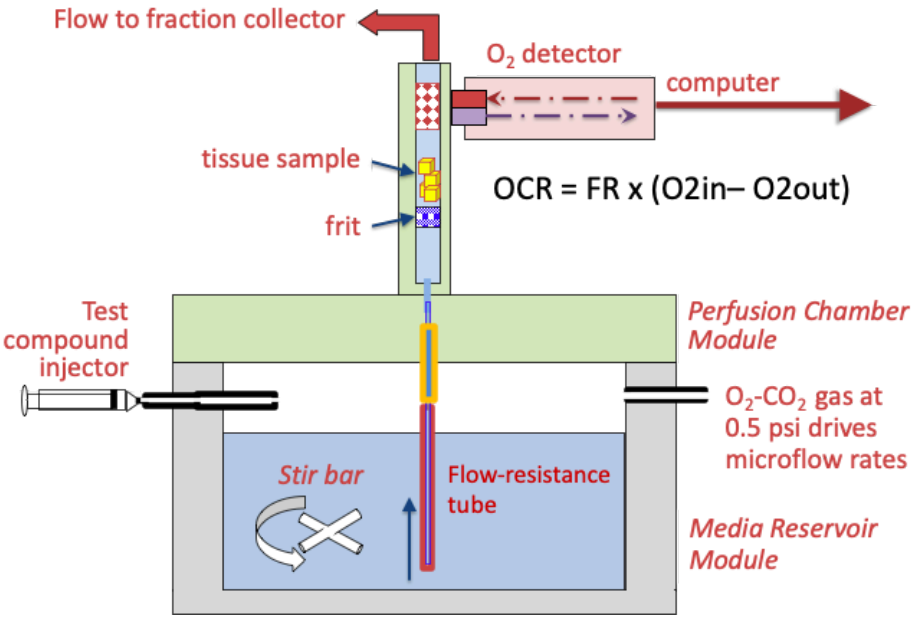
Measurement of a signal reflecting O_2: Lifetime Ratio

An O_2-sensitive dye painted on the inside of the tissue chamber downstream of the tissue is used to assess the O_2 in the outflow of the perifusion chamber. The operation of the sensor involves pulsed excitation of the dye (at 400 nm) and measurement of the time-dependent decay of the emission intensity (at 605 nm) after cessation of the excitation pulse (see Left Figure below). The rate of decay is quantified by the ratio of the area under the curve (AUC) at two intervals of time (AUC1 for time = 104 -129 usec, and AUC2 for time = 129-300 usec) where the Lifetime Ratio (LTR) = AUC2/AUC1. LTR is inversely proportional to the O_2 concentration, and a calibration curve can be used to convert LTR to O_2 levels (see Right Figure Below). The relation between LTR and O2 is linear between 10 and 21%, where the sensitivity (S) is a constant for all experiments done at a particular temperature. The remarkable stability of the approach is due to the ability to average large numbers of decay curves for each time point (typically 8000), and the fact that drift is virtually eliminated by using the decay rate of the signal as opposed to intensity.



Calculation of OCR

A schematic of a single channel in the BaroFuse is shown below.



O_2 consumption rate (OCR) is calculated as

$$OCR = FR \times (O_2in - O_2out)/amount\ of\ tissue \quad (1)$$

where the FR (flow rate) is measured in uL/min and entered by the user for each channel and is assumed constant for the duration of the experiment. The instrument

software generates LTRout from 8 channels as a function of time in tabular form that is saved as a .csv file. The value of LTRin is determined by the measurement of LTRout at the end of the experiment after tissue respiration has been suppressed by the metabolic poison KCN and often is assumed constant for the entirety of the experiment. However, if there are channels that have no tissue, lifetime data from these channels are a measure of changes in the inflow LTR over time, and the data can be used to correct the inflow (the KCN value) for drift. The difference of (O_2in – O_2out) is calculated as (LTRout-LTRin)/S, where S is the sensitivity which = 0.000524 LTR/(nmol O_2/mL) at 37 degree C. Plugging these values into equation 1,

OCR = FR x (LTRin – LTRout)/S/amount of tissue (2)

where only LTRout changes as a function of time.

The difference (LTRin – LTRout) is an important measure to optimize and reflects the balance between the need for a signal large enough to measure, but not so large that the outflow is hypoxic. The program calculates LTRout(max)- LTRin from the peak LTR (calculated as the average of 10 time points) – LTR(KCN**).** The number will be near 0 for channels with no tissue but should be between 0.01-0.05. If the number is too low, then more tissue is needed and/or lower flow rate.

In practice, the amount of tissue is normalized in several ways dependent on the cell model. Here are 4 common examples

1. Amount of tissue as mg. Then the equation for OCR is
FR x (LTRin – LTRout) x S/mg of tissue in units of nmol/min/mg tissue
2. Amount of tissue as the number of tissue pieces such as the number of retina. Then the equation for OCR is
FR x (LTRin – LTRout) x S/number of retina in units of nmol/min/retina
3. Amount of tissue as the number of islets but normalized to 100 islets. Then the equation for OCR is
100 x FR x (LTRin – LTRout) x S/number of islets in units of nmol/min/100 islets
4. Amount of tissue as the number of cells but normalized to 100,000 cells. Then the equation for OCR is
100,000 x FR x (LTRin – LTRout) x S/number of cells in units of nmol/min/10^5 cells

Thus, the general OCR equation will be

OCR = Basis for Normalization x FR x (LTRin – LTRout) x S/amount of tissue in nmol/min/amount of tissue where the amount of tissue is in the same units as the Basis for Normalization**.**

Thus, the OCR is calculated by the equation and input for each of 8 channels from

1. LTRout from the .csv file

2. A calculation of LTRin from the average of the last 6 points of the LTRout from the .csv file
3. User-inputs
   a. FRs for each of 8 channels.
   b. Amount of tissue for each 8 channels.
   c. Basis for Normalization for each of 8 channels.

Calculating OCR as a function of time relative to a reference time

In general, the format for protocols will be a baseline of 90 minutes followed by a series of injections of test compounds at precise time intervals where the final injection of test compound will be KCN (the inhibitor of respiration that defines the LTRin). If the protocol involves sequential addition of 3 test compounds, The user will input the protocol information as

Baseline, 90 minutes
Addition of 'Test Compound 1', x minutes
Addition of 'Test Compound 2', y minutes
Addition of 'Test Compound 3', z minutes
Addition of 3 mM KCN, 60 minutes.

The user would input the names of the Test Compounds, and the time interval until the next injection (3 text variables and 3 numerical values). The 0-time point will typically be set at the exposure of tissue to the first test compound injected. Since there may be a biological delay in the response to this agent, the start of the effect of KCN is used to determine the relative time scale and is set to the time interval in minutes from the start of the injection of the first test compound to the time of KCN injection, in the case of the example this would be x + y + z minutes. The initial KCN response is identified by the sharp decrease in LTRout. However, there may be differences in the time this occurs between channels. If they are all pretty similar (say within 4 minutes of each other, then the KCN point is defined by the earliest responder (thus avoiding graphing data showing that the effect occurred prior to the exposure of tissue to KCN). If the data is quite variable or if no KCN effect is observed due to death of tissue prior to the addition of KCN, then the user selects the reference time point (individually or for all chambers) while looking at the lifetime and/or OCR data.

Representing Data as Fractional Changes

For each channel, the data is also represented as a fractional change from a reference value of OCR. The time that OCR will be normalized to 1 is defined by the user but the time will often be the last five minutes prior to the addition of the first test compound. Then, all values of OCR for that channel will be divided by the average of the values within the selected time window. It also may be the last 5 minutes prior to other test compounds whose calculation of the fractional change is desired.

Averaging replicates for comparison and evaluation of the effects of test compounds.

In general, experimental conditions for a given channel will be done in duplicate, triplicate or quadruplicate. The profiles will be grouped/averaged according to the replicates. The user will need to enter labels for each channel and specify if they are to be averaged. For example, the user would enter the following for two conditions done in triplicate

List of Conditions
Ch1 no tissue
Ch2 + drug
Ch3 + drug
Ch4 + drug
Ch5 control
Ch6 control
Ch7 control
Ch8 no tissue

Then calculations of replicates would include average and SE of Ch1 and Ch8, Ch2-4, and Ch5-7 as well as appropriate statistics. In general, mild smoothing is performed in the form of moving averages where the user would input the time window within which the averages are calculated.

Generating graphs that are appropriately labeled.

The graphs that are generated by the program are raw lifetime data, OCR and Fractional OCR for all channels as well as averaged for replicates (typically 5 graphs). The curves on the graphs need to be labeled with channel number and condition (as inputted by the user as in "List of Conditions" above, as well as annotating the graph to indicate times of injections.

Summary of User Inputted Info:

a. FRs for each of 8 channels (in uL/min).
b. Amount of tissue for each 8 channels.
c. Basis for Normalization for each of 8 channels .
d. Protocol Labels (text variable) and Timing (numerical variable)
    i. Addition of 'Test Compound 1', x minutes
    ii. Addition of 'Test Compound 2', y minutes
    iii. Addition of 'Test Compound 3', z minutes

If KCN effects are not distinct, then user will have to define the reference time.

e. List of Conditions for each channel (text variables) (example)
    i. Ch1 no tissue
    ii. Ch2 + drug

iii. Ch3 + drug
iv. Ch4 + drug
v. Ch5 control
vi. Ch6 control
vii. Ch7 control
viii. Ch8 no tissue

f. User needs to specify which conditions will be replicates, or the program can group those with the same names (but different channels)
g. Time window for baseline determination
h. Number of time points to average for smoothing.

APPENDIX K: Assay Kits

Lactate Assay

EnTox Sciences uses an Amplex Red Glucose/Glucose Oxidase Assay Kit from Fisher Scientific and replaces the glucose oxidase with lactate oxidase (purchased separately). It is recommended to purchase the following items and use the attached protocol:

Amplex Red Assay Kit
A22189 from Fisher Scientific

Lactate Oxidase
L9795 from Sigma

Sodium L-Lactate
L7022 from Sigma

Black Well Plates
655209 from Fisher Scientific

Reagent Preparation:

Calculations for 100 samples

1.1 Prepare a 10 mM stock solution of Amplex Red reagent.

Allow one vial of Amplex Red reagent (Component A, blue cap) and DMSO (Component B, green cap) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent in 60 µL of DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays, with a final reaction volume of 100 µL per assay.

1.2 Prepare 1X Reaction Buffer.

Add 1 mL of 5X Reaction Buffer (Component C, white cap) to 4 mL of deionized water (dH2O). This 5 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 100 µL each.

1.3 Prepare a 10 U/mL stock solution of horseradish peroxidase (HRP).

Dissolve the contents of the vial of HRP (Component D, yellow cap) in 1 mL of 1X Reaction Buffer. After the assay, any remaining unused solution should be divided into single-use aliquots and stored frozen at -20°C.

1.4 Prepare 100U/mL stock solution of lactate oxidase

Dissolve 100U of lactate oxidase in 1mL of 1X Reaction Buffer. After the assay, any remaining unused solution should be divided into 50 µL single-use aliquots and stored frozen at -20°C.

1.5 Prepare a 25 µM lactate stock solution.

Stock solution (2.5 mM) : 7 mg Sodium L-Lactate (FW = 112)/25 ml. Aliquot and freeze.
Then dilute 1:100 into the Reaction Buffer to yield 25 µM.
For cal curve, pipet 0, 4, 10, 20 and 50 µL (and additional Reaction Buffer to make up to 50 µL) to wells.
This yields a cal curve with 0, 2, 5, 10 and 25 µM

1.6 Prepare a working solution of 100 µM Amplex Red reagent, 0.2 U/mL HRP and 2 U/mL lactate oxidase.

Mix the following:

50 µL of 10 mM Amplex Red reagent stock solution (prepared in step 1.1)
100 µL of 10 U/mL HRP stock solution (prepared in step 1.3)
100 µL of 100 U/mL lactate oxidase stock solution (prepared in step 1.4)
4.75 mL of 1X Reaction Buffer

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be twofold lower in the final reaction volume.

1.7 Prepare Samples

Dilute samples appropriately in 1X Reaction Buffer.

Assay Procedure:

1. Pipet 50 µL of standards and samples into individual wells.
2. Add 50 µL of working solution (step 1.6) to each well containing standards, controls and samples.
3. Incubate at room temperature for 30 minutes, protected from light. Because the assay is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics of the reactions.
4. Use a microplate reader equipped for excitation at 560 nm and fluorescence emission detection at 590 nm.

Working Buffer Prep: (sufficient for 10 samples)	
Mixture:	Volume (uL):
10mM Amplex Red	5
1x Reaction Buffer	475
10U/mL HRP (Horseradish Peroxidase)	10
10uL lactate Peroxidase	10

Plate Prep:				
Mixture:		Sample/ Standard (uL):	1x Reaction Buffer (uL):	Working Buffer (uL):
Lactate Standard Curve	25uM Lactate	50 of 25uM Lactate	0	50
	10uM Lactate	20 of 25uM Lactate	30	50
	5uM Lactate	10 of 25uM Lactate	40	50
	2uM Lactate	4 of 25uM Lactate	46	50
	0 Lactate	0	50	50
Sample (Diluted appropriately in 1x Reaction Buffer)		50	0	50

Pyruvate Assay

EnTox Sciences uses the Pyruvate Assay Kit from Sigma-Aldrich. The catalog number and protocol notes can be found below:

Pyruvate Assay Kit
MAK332 from Sigma-Aldrich

10 uL of sample and standards in each well.
In a 50 mL conical tube add; 94 uL/sample of the enzyme mix, 1 uL/sample of dye mix
Add 90 uL of the mix to the samples in each well.
Shake for 30 minutes.
Measure on a plate reader with excitation at 530 nm; emission at 585 nm

Making aliquots: (so you don't have to repeatedly thaw and freeze)
- 1 mL of enzyme mix in 6 microcentrifuge tubes (11 total)
- 15 uL of dye mix in 5 microcentrifuge tubes (8 total)

Standards (make 1 mL of each standard in DI water):

Concentration [uM] (uL added)	Concentration in assay [uM]
0 (10 uL of H_2O)	0
3 (6 uL of 500 uM)	0.3
10 (20 uL of 500 uM)	1
30 (60 uL of 500 uM)	3
100 (200 uL of 500 uM)	10

APPENDIX L: Training Tests

For training purposes, it is advisable to perform these quality control tests without loading tissue since it takes practice to master the methodology. First prepare the perifusate and set up the instrument with the following steps.

PERIFUSATE PREPARATION

For the QC tests, perifusing with water will be adequate, although it is better to use KRB if you have a 5% CO2 tank driving the flow. These tests will be done at 40 uL/min and will require a fluidics package for 30-40 uL/min. To prepare the perifusate do the following.

1. Place two T75 tissue culture flasks (each containing 70 mL of water or KRB), in a 40°C CO_2 incubator for at least 60 minutes.

INSTRUMENT SET UP

b. Attachment of Tubing Assemblies to the Media Reservoir Module.

1. Place the MRM and a Fluidics Package onto the bench next to the BaroFuse. The hemostats (3), Stir Bars (2), and forceps should already be located in the Tool Tray.
2. Place a clean MRM Insert on each side of the MRM. Place a Stir Bar into each side of the MRM (See Fig. C1).

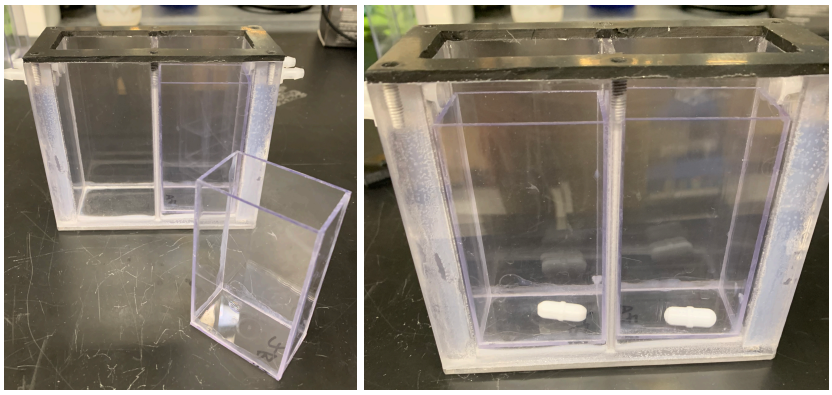


Fig. C1. View of the MRM with the MRM Insert (left) and Stir Bars (right) placed into the bottom.

3. Attach the Test Compound Injection Assemblies on injection ports on both ends of the MRM so that the end of the tubing is directly over the Stir Bar. The longer of the two Test Compound Injection Assemblies should be on the back.
4. Then attach the Gas Inflow Feed Line and Purge Tubing Assembly to the back and front vacant ports respectively (see Fig. C2).

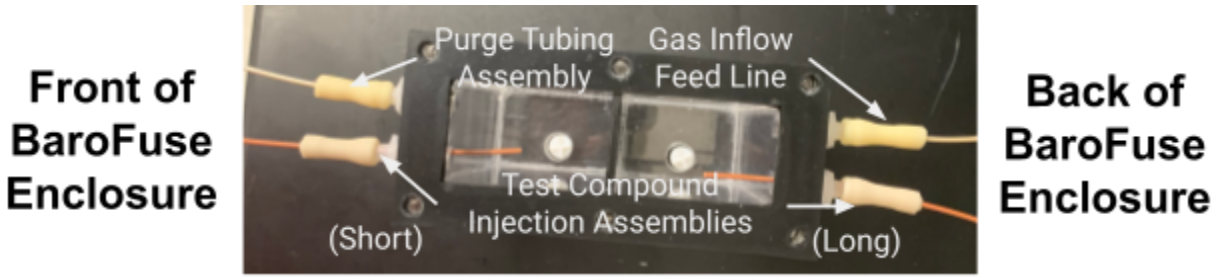


Fig. C2. Test Compound Injection Tubing Assembly and Purge Tubing Assembly attached to ports on the MRM.

c. Placing the MRM/Tubing Assemblies in the BaroFuse Enclosure.

1. Place the MRM (with the four tubing assemblies attached) into the MRM Heater (Fig. C3 Left).
2. Insert the four tubing assemblies into the grooves of the walls in the Base of the BaroFuse Enclosure (two on each side) so they will protrude through to the exterior of the Enclosure once the Middle Enclosure is put in place.
3. Secure the MRM between the clamps by tightening the two wheels on the BaroFuse stand.
4. Feed the longer Test Compound Injection Assembly protruding from the back of the BaroFuse through the two tube guides on the side of the BaroFuse so the opening of the tubes faces forward (Fig. C3 Middle).
5. Clamp a hemostat on each of the Test Compound Injection Assemblies (Fig. C3 Right).

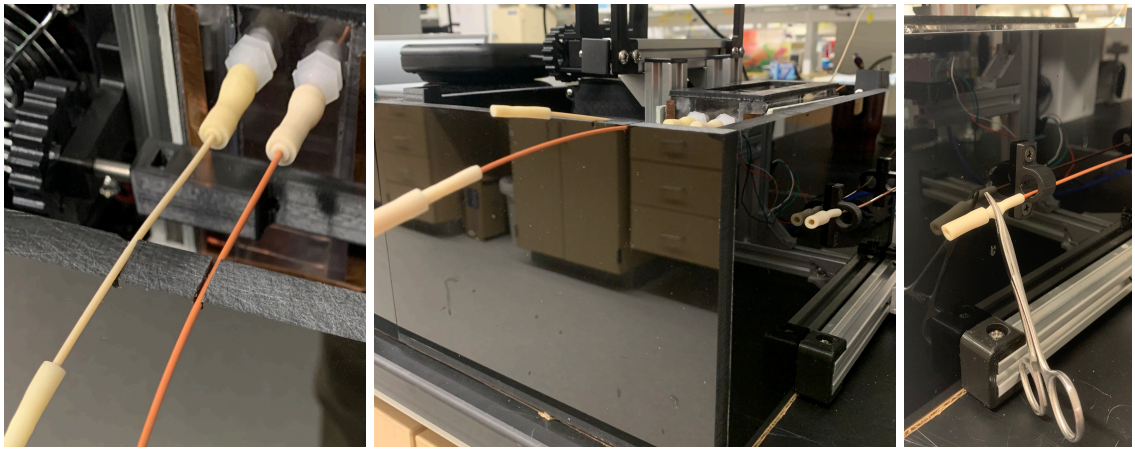


Fig. C3. The MRM left side's Test Compound Injection Assembly and Purge Tubing Assembly are placed in the groove of the BaroFuse Enclosure.

d. Assembling the BaroFuse Enclosure and Activating the Temperature Controllers.

1. Toggle the Power Strip to the On position. The MRM Temperature Controller should light up with the set value displaying 39°C (Fig. C4). If the number is different, reset to 39°C. The fan on the BaroFuse stand will also turn on at this time.
2. Turn on the stirrers to 60 RPM using the BaroFuse UI to ensure that the stir bars are rotating smoothly.
3. Place the middle section of the BaroFuse Enclosure on top of the base.
4. Connect the cable on the middle section of the BaroFuse Enclosure to the cable from the Electrical Box to allow power to the Ambient Temperature Controller Lever Switch.
5. Place the lid of the BaroFuse Enclosure on top. The display of the upper Temperature Controller (the Ambient Temperature Controller) should light up, and the set value should read 37°C (if not then reset it to the set value temperature). Start a 30 minute timer.

For instrument model #1, this is accomplished by pressing the switch near the temperature controllers



Fig. C4. Powering up the MRM Temperature Controller.

e. Inserting the Tissue Chamber Assemblies into the PCM.

Insert each of the 8 TCAs into the PCM holes and using your thumbs, press on the adaptor so that the top of the tubing sleeve wrapped around the Tissue Chamber touches the surface circumscribing the holes in the PCM (Fig. C5). This will take a bit of pressure to get the TCAs to rest against the PMC surface. Set the partially assembled PCM aside next to the PCM Brace and 6 screws.

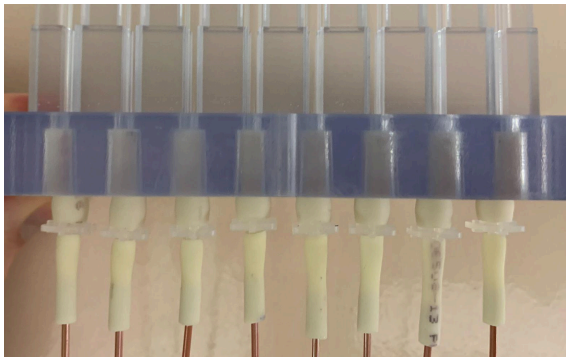


Fig. C5. 8 TCAs placed in the MRM.

f. Filling the two troughs in the MRM with pre-equilibrated perifusate.

1. Ensure 30 minutes have passed since the BaroFuse Enclosure was assembled before continuing with this step.
2. Transfer the prepared (and pre-heated) perifusate into the pre-heated MRM. Either by using 50-mL pipette or directly pouring into the MRM. Add the perifusate along the sides of the insert to avoid creating any froth/bubbles.

Immediately carry out the next step to avoid transfer of gas between the fluid in the MRM and the atmosphere.

g. Assembling the MRM/PCM to create a gas-tight seal and placing the O_2 detector.

1. Place the PCM onto the MRM by Inserting the TCAs emanating from the PCM into the MRM, 4 on each side of the MRM Divider. The PCM fins should be closest to the O_2 Detector.
2. Secure the PCM and the PCM Support Brace with the 6 Screws using the Electric Screwdriver.
3. Secure the TCAs with the Rubber Band provided by stretching it around the fins of the PCM at the level of the rubber gaskets (Fig. C6).
4. Position the O_2 Detector on the BaroFuse Stand so that the face of it is snug against the PCM. The LED/Photodetector pairs should now be lined up with the O_2 sensors in the Tissue Chambers.
5. If needed, the oxygen detector lateral guides can be adjusted after loosening the screws to allow the O_2 detector to be moved.
6. Place the lid of the BaroFuse Enclosure on top.

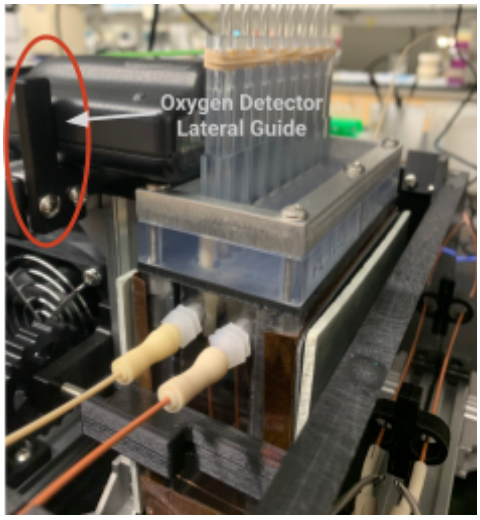


Fig. C6. Positioning of the Electric Stir Box, the MRM Heater, the MRM, the PCM and O_2 Detector on the BaroFuse Stand.

h. Starting the O_2 measurements.

5. Activate the O_2 Detector Software by clicking on the icon that says “Oxygen Detector”.
6. Once the program opens (Fig. C7), select the correct COM port. If needed, the COM port may be identified by unplugging and plugging the O_2 detector from the computer so that the port number is displayed. If the COM port is unplugged while the application is running, then the application must be closed and reopened before use.
7. Select all eight channels and set the numbers for the MCU Average, Process Interval, Process Area1 Start, and Area2 Start as shown below; then click “Update”.

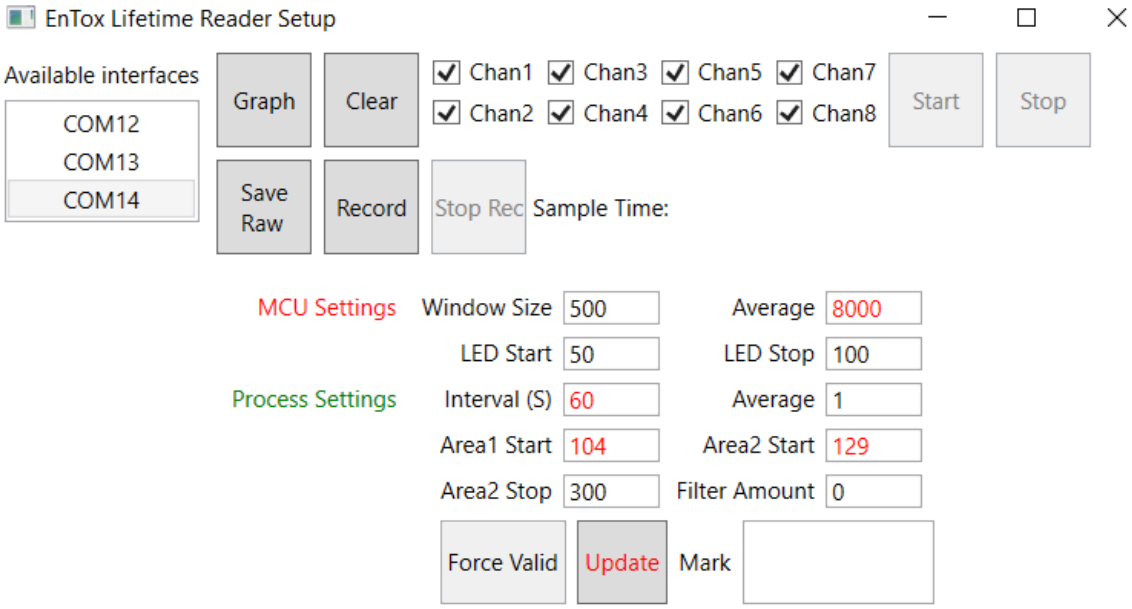


Fig. C7. GUI of the startup window of the O_2 Detection Software.

8. Click “Record” (and save the data in the backup folder) -> Click “Start” -> Click “Graph”.
9. Now in the graphing window, change Lifetime to Ratio on the top left menu (Fig. C8).
10. Then select all 8 channels. Change the Average value on the bottom left to 5. After one minute has passed and the first data point shows on the graphing screen, click “Auto Scale”.

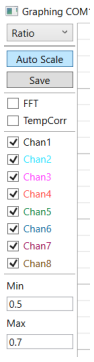


Fig. C8. GUI of the O_2 Detection software displaying the parameters that control the real time graphing of the O_2 signal.

i. Equilibrating the head space in the MRM with the perifusate.

1. With the High Pressure Valve fully secured and closed, open the gas tank valve by turning the cylinder valve on top of the tank counterclockwise.
2. Adjust the high-pressure regulator to a pressure of 10 psi using the knob on the regulator.
3. Pressurize the MRM by setting the Low-Pressure Pressure Valve to 1.5 psi (Fig. C9).



Fig. C9. High and low pressure regulators on the gas tank.

4. Allow the gas from the tank to replace the air in the MRM headspace with the purge tube unclamped (and the test compound injectors still clamped). Submerge the end of the purge tube into a beaker of water to confirm flow.
5. After 15 minutes, turn the stirrer on at 60 RPM and leave on for the remainder of the experiment.
6. After 15 minutes has passed since the stirrer was turned on, clamp the Purge Tubing Assembly shut with a hemostat (Fig. C10 Right).
7. Lower the pressure on the low-pressure regulator to the operating pressure. For 40 uL/min the operating pressure should be 0.6 psi.

For instrument model #1, this is accomplished by pressing the switch near the temperature controllers

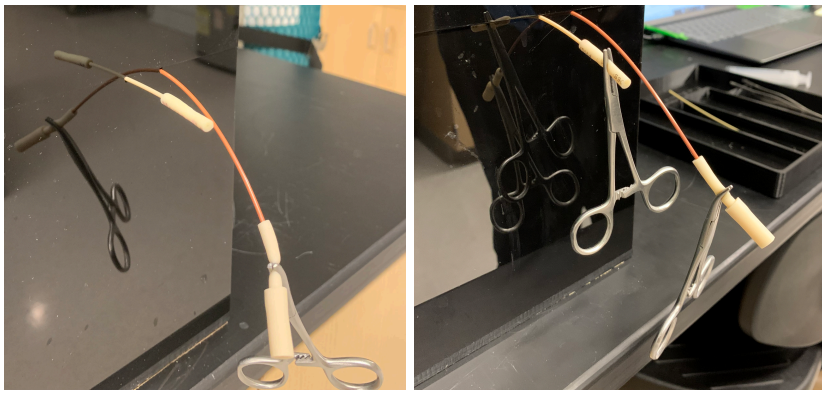


Fig. C10. Closed BaroFuse system with Purge Tubing Assembly on the left of each image and the MRM left side's Compound Injection Assembly on the right of each image.

INSERTING FRITS and OUTFLOW TUBING

a. Positioning the Frits in the Tissue Chambers.

1. Remove the Lid and Middle sections of the BaroFuse Enclosure.
2. After the chambers are halfway filled with media, insert the frits into Tissue Chambers (one frit per channel) using the Forceps provided. Frits may need to be squeezed with the forceps to allow for easy insertion into the Tissue Chambers.
3. Slowly push the frits down the Tissue Chamber using the Frit Cue (made of PEEK tubing) until they reach the perifusate and are halfway submerged.
4. Then continue to push the frit down by repeated, light tapping on the top of the frit in order to remove any air bubbles that formed below the frit. Position frits about 0.25 inches above the bottom of the Tissue Chamber. Do not let media run down the outside of the tissue chamber as the fluid can interfere with O_2 sensing.

b. Attaching the Outflow Tubing Assemblies to Tissue Chambers.

1. Remove the Outflow Tubing Assembly Pack and then place the Outflow Tubing Enclosure Guide on the middle section of the enclosure edge with the Outflow Tubing Adapters on the inside of the Enclosure.
2. Attach the Outflow Tubes Adapters onto the TCAs (Fig. C11). Be careful not to push too hard on the TCAs or they will come loose from the MRM.
3. Replace the Middle of the BaroFuse enclosure and reconnect the Ambient Temperature Control cable.
4. Replace the Lid of the BaroFuse Enclosure.
5. Feed the eight Outflow Tubing Assemblies through the Fraction Collector guide arm.



Fig. C11. Picture of Outflow Tubing Assemblies attached to the Tissue Chambers.

Now that the instrument is set up and the flow is activated, the following QC tests should be performed back to back and will take approximately 4-5 hours to complete.

QC TEST 1: Testing Flow Detection, Compound Injection, the Spinner System and the Fraction Collector.

a. Initialization and Set up of the Flow Rate Tracking and Fraction Collector UI Software.

1. Center the Fraction Collector with respect to the wall of the BaroFuse Enclosure and Outflow Tube Holders: the left support of the Fraction Collector base should be flush against the side of the BaroFuse Enclosure.
2. Place a transparent well plate in the carriage of the Fraction Collector with 1A in the back left.
3. On the laptop, click the ”BaroFuse UI” shortcut and the “General” mode window will open (Fig. C12 Top). Fill in all available information on the general page
4. Select “Fractions & Flow” in the top left (Fig. C12 Bottom).

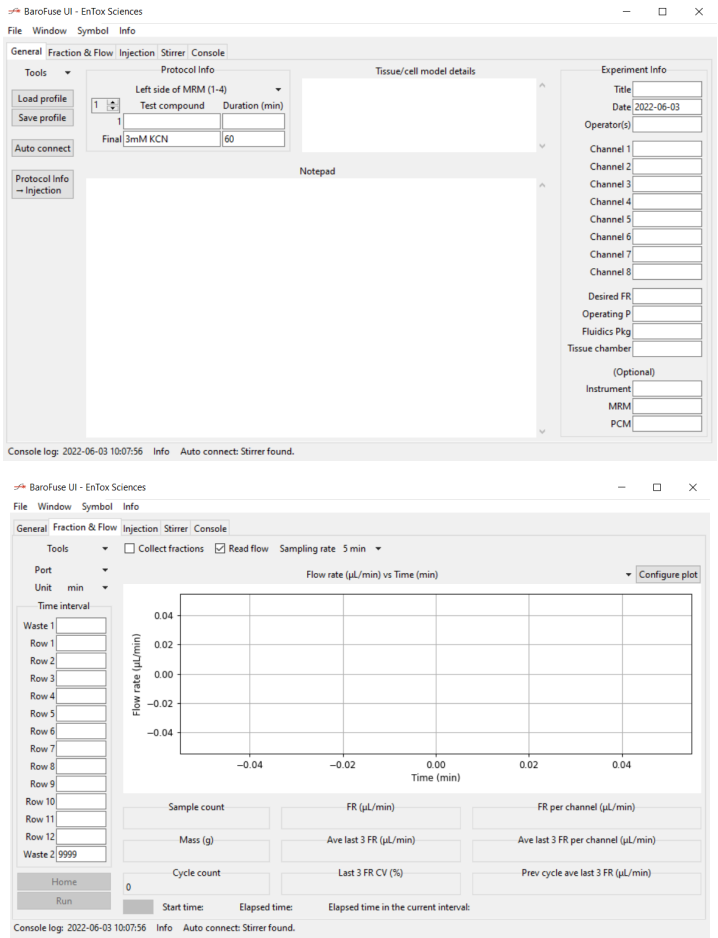


Fig. C12. GUI for Fraction Collector settings.

5. The sampling rate for flow measurement should be set to “5 minutes” for 30-40 uL/min per channel.
6. Click “Collect fractions” next to the “Read Flow” button.
7. Set the flow measurement collection time to 45 minutes by entering 45 into the “Waste 1” category.
8. Set the time interval for collecting fractions to 7 minutes/sample for 11 fractions by entering 7 into “Row 1” and 0 into “Row 12”. The 0 will cause the Fraction Collector to leave the last row empty.
9. Then click “Home” on the bottom left to return the 96-well plate Carriage to its Home position and click “Run”.

b. Evaluating Flow Rate Measurement System.

1. After 30 minutes manually measure the mass of one eppendorf tube.
2. Wipe the end of the desired outflow tube and then immediately hold the eppendorf tube under for 2 min.
3. After collecting perifusate for 2 minutes, measure the mass of the eppendorf tube.
4. Calculate the flow rate using the two mass values.
5. Repeat for additional channels and compare to the value from the BaroFuse UI.

Another method to measure the flow rate is to use the Fraction Collector’s Microcentrifuge Tube Holder. Set the Tube Holder on the Carriage with eight microcentrifuge tubes in it. Click “Tools” -> click “Measure FR manually” -> select the desired duration -> click “Generate” -> click “Run”. The Fraction Collector will collect fluid from the Outflow Tubing Assemblies for the input time and then return to its home position. Weight the microfuge tubes before and after collection.

c. Testing the Kinetics of the Test Compound Injection.

1. To quantify the time it takes for the injected material to reach the fraction collector, inject 0.4% Trypan Blue (1 mL of dye / 100 mL of perifusate) into the MRM.

The amount of Trypan Blue to inject is determined by the amount of fluid remaining in the MRM. Use the mass collected or the Injection page of the FC to determine how much dye to inject so that there is 1 mL of dye / 100 mL of perifusate (this should be ~0.65 mL of dye on each side).

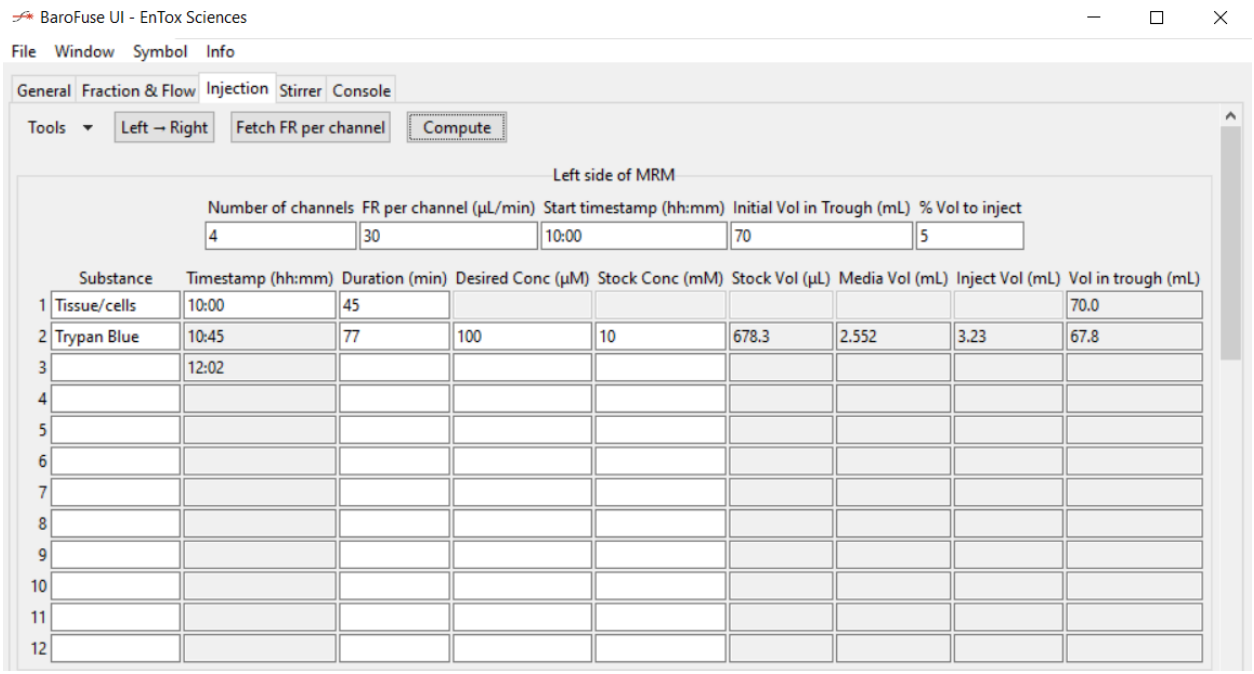
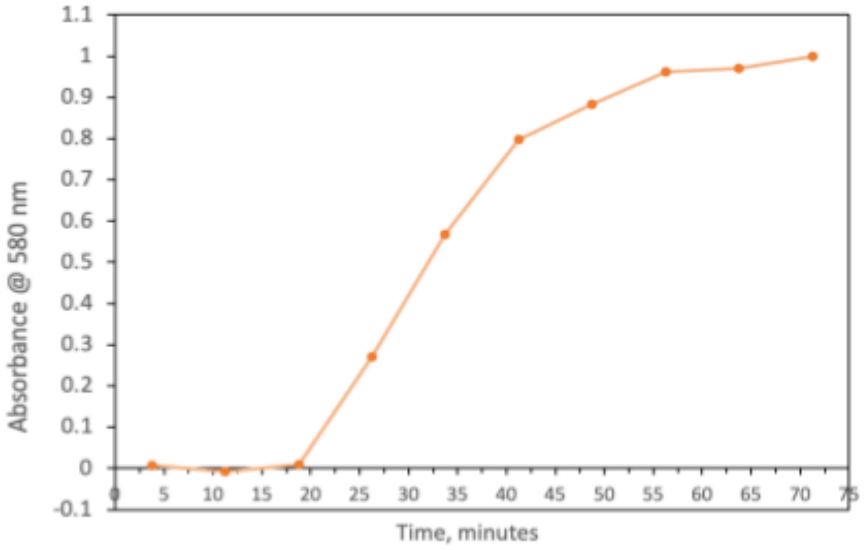


Fig. C13. GUI for Injection page with information filled in for the Trypan Blue Calculation.

2. Collect the outflow for 11 samples. In the 12th row of wells, pipette the same volume that is in the wells that have collected the fluid (which should equal 7 minutes x Flow Rate ~210uL). Take this perifusate from the MRM inserts after mixing them. Note: if you are proceeding to QC Test 2 this will be accomplished once the second test is completed.
3. Measure the absorbance of the wells in the plate using a spectrophotometer that reads 96-well plates at 580 nm.
4. Copy the data into the Excel Template and plot as shown below.



Average of eight channels with fractions taken for 10 rows at 7.5min intervals with a flow rate of 30uL/min (Data from 03/09/2022)

QC TEST 2: Testing of O_2 sensors and Temperature controllers.

a. Changing Temperature.

1. Open the temperature controller software (CN7-A). Press the "Graphing" button in the bottom right of the menu. Ensure the MRM and ambient channels are selected and that the sampling rate is 30 seconds. Press "Start Sampling".
2. Change the ambient temperature from 37°C to 32°C on the temperature controller.
3. Wait for the O_2 sensor to plateau again (this should be approximately 60 minutes) and then change it back to 37°C.
4. On the temperature controller software, press "Save Data" and save the .csv file in the desired location.

b. Changing to 15% O_2 (Optional Section).

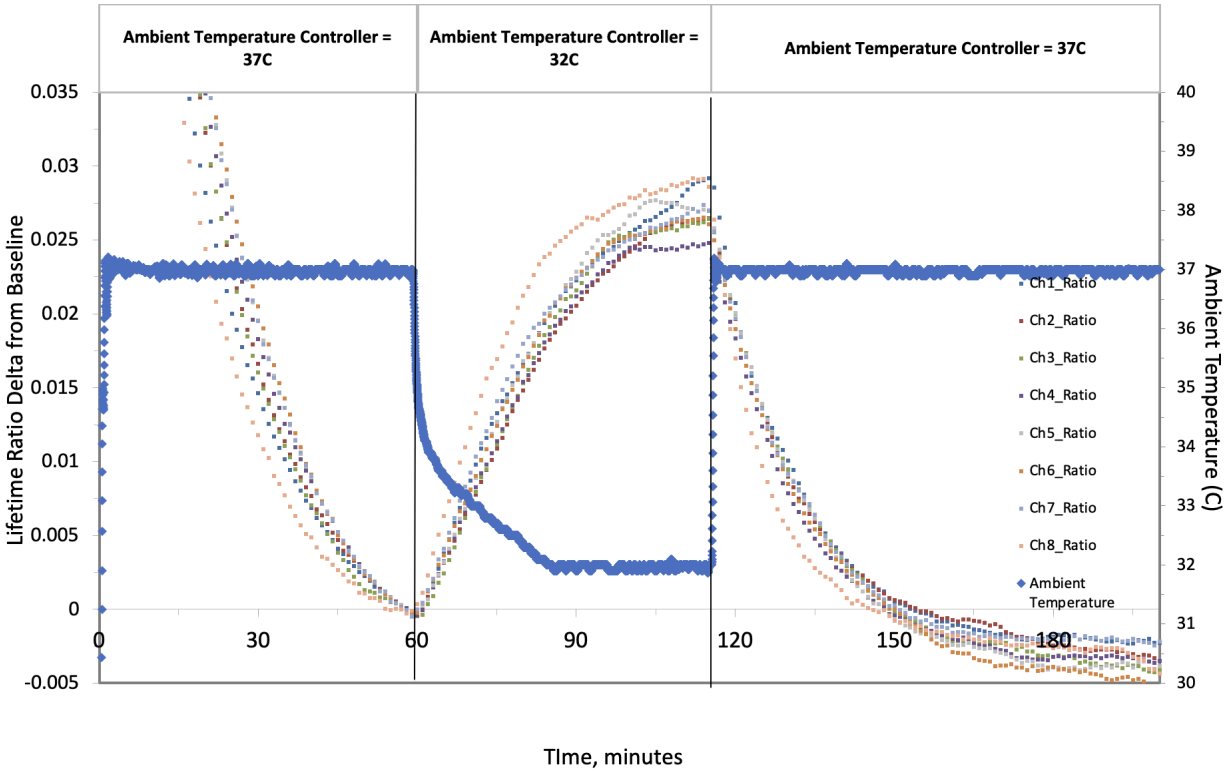
1. Turn the stirrer off
2. Remove the connection from the 21% O_2 tank and place it on the 15% tank.
3. Unclamp the MRM purge tubing.
4. Open the 15% tank and pressurize the MRM by setting the Low-Pressure Pressure Valve to 1.5 psi.
5. Allow the gas from the tank to replace the air in the MRM headspace with the purge tube unclamped (and the test compound injectors still clamped). Submerge the end of the purge tube into a beaker of water to confirm flow.
6. After 15 minutes, turn the stirrer on at 60 RPM.
7. After 15 minutes has passed since the stirrer was turned on, clamp the Purge Tubing Assembly shut with a hemostat.
8. Lower the pressure on the low-pressure regulator to the operating pressure for the experiment.

For instrument model #1, this is accomplished by pressing the switch near the temperature controllers

9. Wait for the O_2 sensor to plateau again (this should be approximately 60 minutes).
10. Swap back to the 21% O_2 tank by repeating steps 1-9 with the standard O_2 tank.

c. Analyzing the temperature and oxygen data.

1. In the Oxygen Detector UI, press “Save” in the top left corner of the graphing window.
2. To analyze the data, copy the temperature data into columns B-F of the “Raw Temperature Data” tab of the Excel Template and all of the oxygen data into the “Raw O2 Data” tab.
3. If the data time stamps do not line up then enter values into B46 and/or B47 until the data lines up. Adjust cells M48-T48 so that the range of cells it averages includes the first plateau at 37°C. The data below shows the expected results.



Alternatively, this test can be performed without gas tanks or liquid in the MRM. TCAs still must be placed inside the PCM but no Outflow Tubing Assemblies or Compound Injection Assemblies are needed.