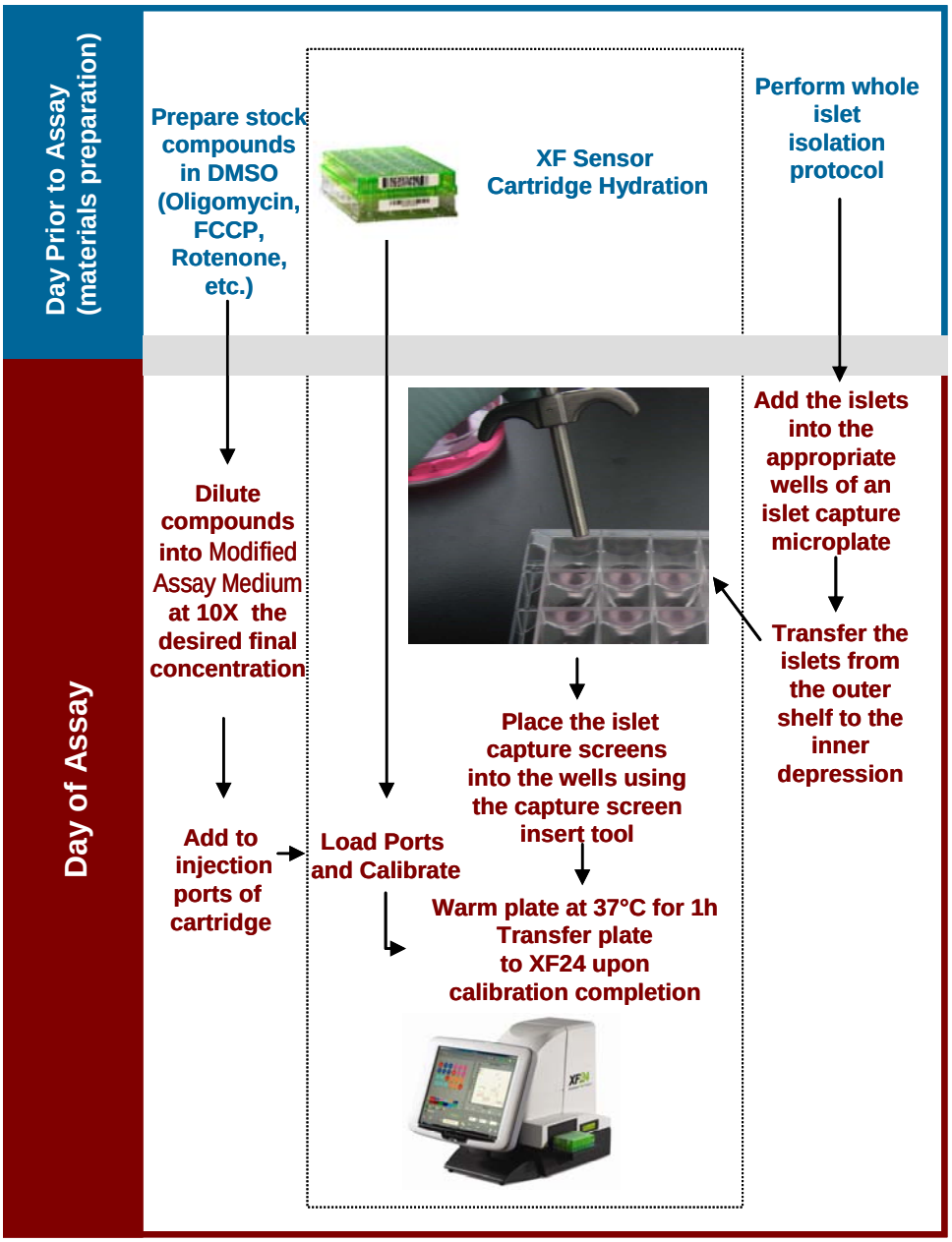
Islet Oxygen Consumption Assay using the XF24 Islet Capture Microplate

Shirihai Lab and Seahorse Bioscience Aug 6 2009

XF Analyzers are most commonly used with an adherent monolayer of cells attached to an XF tissue culture plate. Pancreatic Islets however are non adherent clusters of cells and their use in the XF24 analyzer required additional development. Seahorse Bioscience, in collaboration with our laboratory at Boston University School of Medicine, has developed the XF24 Islet Capture Microplate, to assess whole islet bioenergetics in vitro.

Assay Flow Chart



I. Reagents, Materials, and Injected Compounds

The whole islet protocol described below is a modification of the XF24 Analyzer Protocol described in the XF24 User Manual (Version 1). Please feel free to modify the protocol to realize your intended research goals.

Modified XF Assay Media (MA Media): Supplement XF DMEM assay media with 3 mM glucose and 1% FBS to run whole islets. (FBS is needed to prevent the islets from becoming too adherent).

I.1. Components/Formulation of Modified XF Assay Media

Compound	Brand	Catalog Number	MW or Molar Concentration	Final Concentration	Grams or ml for 500 ml of XF Assay Media
Glucose	Sigma	G7528	180	3 mM	0.27 g
FBS	Hyclone	SH30070.03	100%	1%	5 ml

I.2. Components/Formulation of compounds to affect mitochondrial function

It is recommended that all compounds to be added or injected are diluted into MA Media as described in section I.1.

Compound	Brand	Catalog Number	Final Concentration	Dissolve in:
Rotenone	Sigma	R8875	5 µM	Stock 1000X in DMSO Dilute to 10X in MA Media
Oligomycin	Sigma	O4876	5 µM	Stock 10000X in DMSO Dilute to 10X in MA Media
FCCP	Sigma	C2920	1 µM	Stock 10000X in DMSO Dilute to 10X in MA Media
Glucose	Sigma	G7528	20 mM	Stock 1000X in DMSO Dilute to 10X in MA Media
Myxothiazol	MP Biomedicals	155765	5uM	Methanol
Note: Oligomycin, FCCP, rotenone, and myxothiazol should be freshly diluted in MA Media for each experiment. Stock solutions in DMSO may be stored at -20°C.				

I.3. Other items needed

XF24 Biosensor Cartridge
XF24 Islet Capture Microplate
Islet Capture Screens
Capture Screen Insert Tool
Calibration buffer (Seahorse Bioscience)
Dissecting microscope
Open faced bio-hood
Multi-channel pipettes and tips

Eppendorf and 15/50 ml Falcon tubes

II. Preparation of XF Assay Templates, XF Cartridges, and whole islets (These steps should be performed the day before the assay is run).

II.1. Prepare an XF assay template (via the Assay Wizard) using the XF24 operation manual as a guide and incorporating proper experimental design. Upload the assay template to the XF24 Analyzer before starting the assay. The experiment outlined below is an example of how to obtain the various mitochondrial respiration states using the XF24.

Use the following table as a guide to program the Mix, Wait, Measure and Injection protocol.

Command	Time (minutes)	Port
Calibration	-	-
Equilibrate	12*	
Mix	2	
Wait	2	
Measure	3	
Mix	2	
Wait	2	
Measure	3	
Mix	2	
Wait	2	
Measure	3	
Inject		A
Mix	2	
Wait	2	
Measure	3	
Mix	2	
Wait	2	
Measure	3	
Mix	2	
Wait	2	
Measure	3	
Inject		B
Mix	2	
Wait	2	
Measure	3	
Mix	2	
Wait	2	
Measure	3	
Mix	2	
Wait	2	
Measure	3	
*Default Equilibrate command consists of 2 min Mix, 2 min Wait repeated 3X. The same pattern could be followed for more injections.		

Note that the Measure times indicated are guidelines only. Typical Measurement times are 3-5 minutes. It is advised to empirically determine the optimal Mix, Wait and Measure times for your desired application and experiment.

II.2 Prepare the XF sensor cartridge

II.2.1. Hydrate the XF sensor overnight in XF Calibration Buffer at 37°C, without CO_2.

II.3 Prepare whole islets by the standard protocol(s) used in your laboratory. For the protocol described here, ~8 mice were sacrificed to obtain ~1400 islets – enough for 20 wells at 70 islets/well. Incubate whole islets in a petri dish overnight under standard conditions for islet culture. (For the data shown, islets are cultured in RPMI media with 11mM glucose, 10% FBS, and 1% pen/strep).

III. Add whole islets and capture screens to the wells.

III.1 Aspirate islets from Petri dish and dispense into a 50 ml tube.

III.2 Wash 1X in MA Media.

III.3 Remove supernatant and re-suspend in 2 ml MA Media.

III.4 While creating turbulence in the tube with a 20 μl pipettor, take 20 μl aliquots and place as a drop on a culture dish – make 3 drops total (this gives you ~3% of the islets).

III.5 Count islets under a dissecting microscope.

III.5.1 This will give you an average amount of islets per volume from which you can estimate the total number of islets.

III.6 Determine the count of the islets, and adjust volume so you get ~70 islets for every 100 μl of media (700 islets/ml).

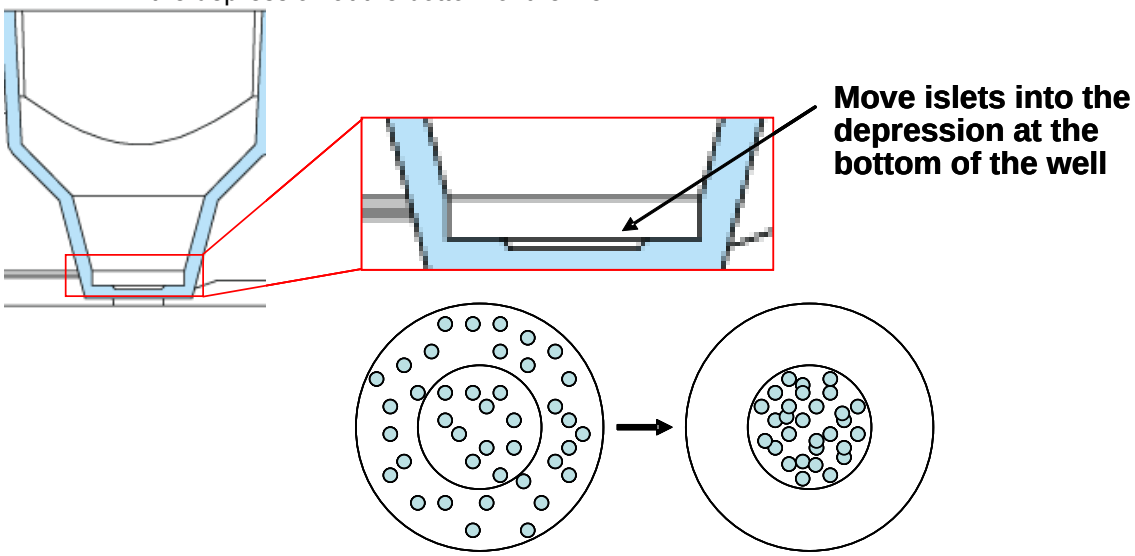
III.7 Add 400 μl MA Media to each well of the XF24 Islet plate.

III.8 Add 50 μl of the islet suspension to each well, and repeat so each well gets a total of 100 μl of the islet suspension.

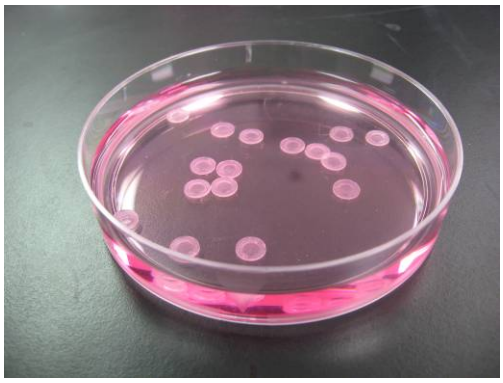
III.8.1 Final volume should be 500 μl per well.

III.9 When islets are seeded use a 20 μl pipette to move all of the islets into the depressed chamber in the bottom of the well

III.9.1 This step is tedious – use a dissecting microscope to be sure all of the islets are in the depression at the bottom of the well.

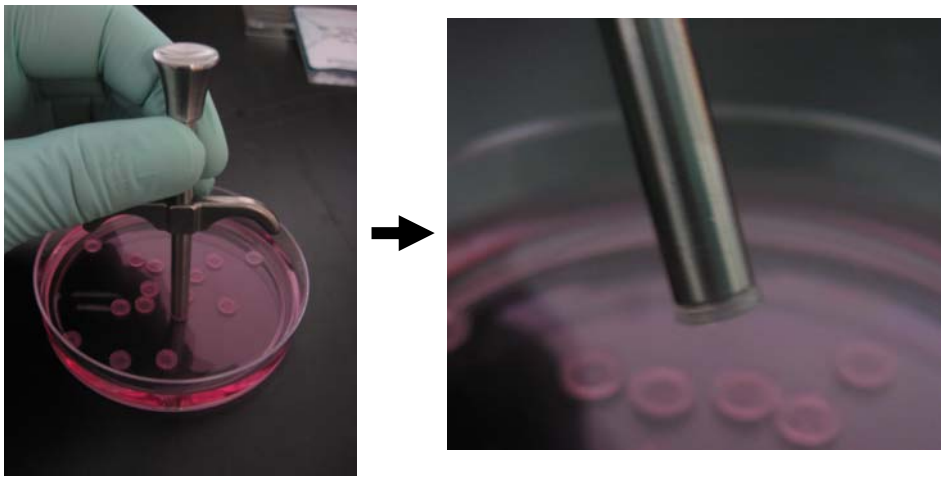


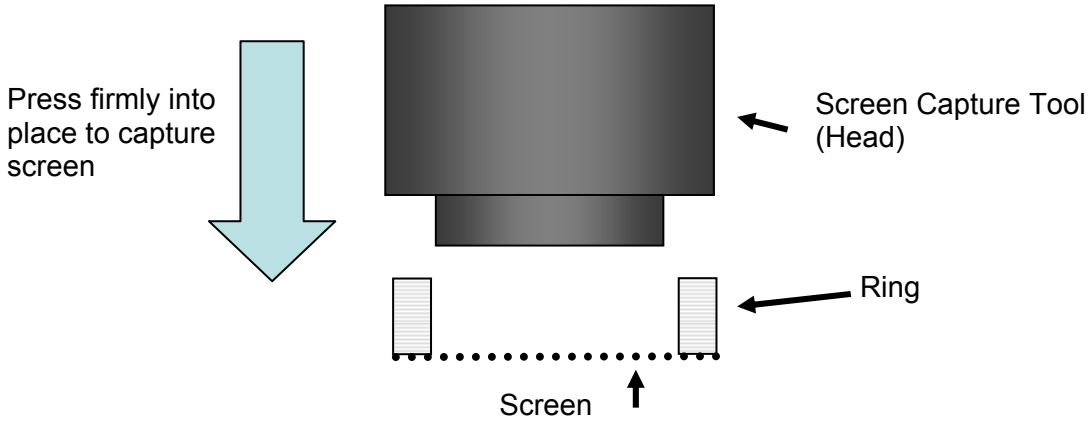
III.10 Add screens by pre-wetting them in MA MEDIA in a small Petri dish to remove any air bubbles.



III.10.1 Use a pair of sterile forceps to position the screens so that the ring is facing up.

III.11 Use the capture screen insert tool to pick up an islet capture screens from the petri dish.

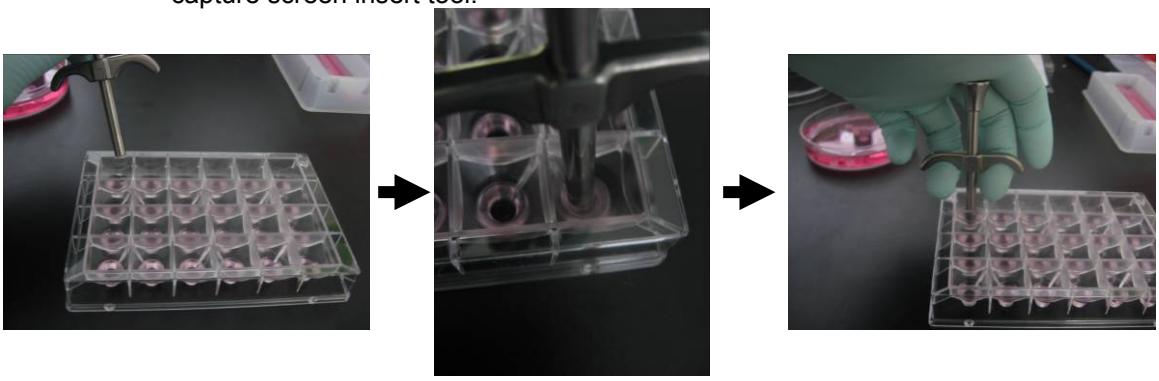




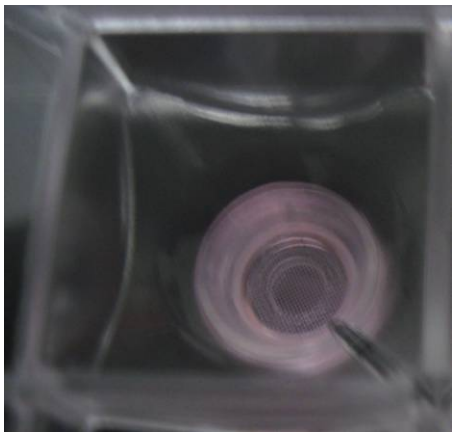
III.11 Carefully place the islet capture screens in the bottom of each well using the capture screen insert tool.

III.11.2 Be very careful during this step that don't cause too much turbulence so as to keep the islets resting in the depression at the bottom of the well.

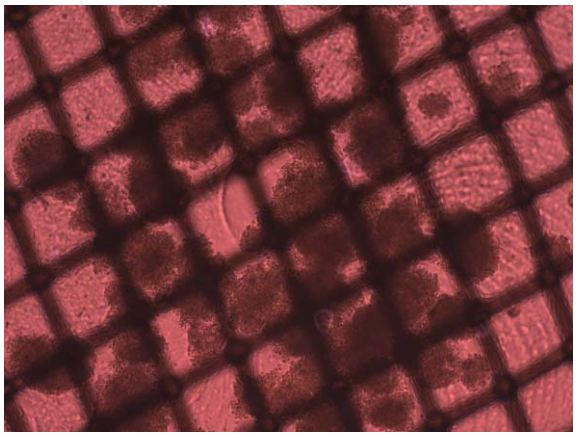
III.11.2 Release the islet capture screen into the well by pulling up on the T-lever on the capture screen insert tool.



III.11.1 Be sure the islets capture rings are stuck firmly at the bottom of the well. This can be confirmed by gently pushing the screens down with a blunt pipette tip.



Well with screen in place



Islets under screen

III.11.2 Make sure that there is an islet capture screen in each well, even if there are no cells in the well. A microplate without a full complement of screens will cause problems with the head on the XF24 unit.

IV. Run the Islet Capture Microplate on the XF24

IV.1 Place the microplate in an incubator set at 37°C, <u>without</u> CO_2.

IV.2 Store the microplate in the incubator for at least 1 h to equilibrate temp and adjust islet metabolism to 3 mM glucose.

IV.3 While plate is incubating, prepare cartridge with desired injections (See section V).

IV.4 After cartridge is filled with compounds for injection, load the cartridge and start program and calibration.

IV.5 When the XF24 calibration is complete, place the islet plate into the XF24.

IV.6 After program is complete you can normalize by counting the number of islets per well with the dissecting microscope. Islets may also be harvested for further downstream anylysis, e.g. protein.

IV.6.1 Some users have found that this step was not necessary, as basal rates were sufficient for normalization.

V. Prepare Biosensor Cartridge with Injections and Calibrate

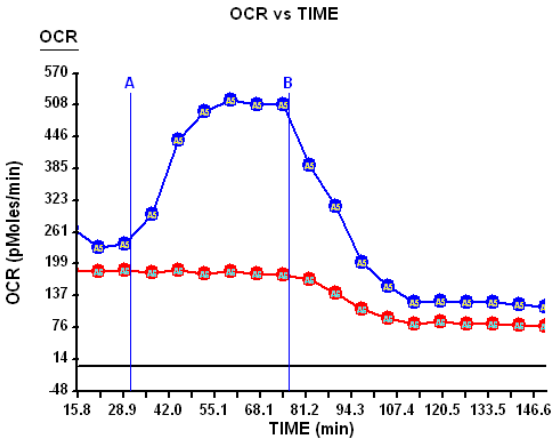
V.1 Before calibration, load the XF sensor cartridge injection ports with following compounds listed in the table below.

Injection Ports	Volume	Concentration in Port	Final Concentration in Well
A: Glucose	50 µl	200mM	20 mM
B: Oligomycin	55 µl	50 µM	5 µM
C: FCCP	60 µl	10 µM	1 µM
D: Rotenone	65 µl	50 µM	5 µM
D: Myxothiazol	65 µl	50 µM	5 µM
Note: Vigorous mixing of the stock 20 uM oligomycin is required to prevent precipitation. Rotenone and Myxothiazol are mixed together in the appropriate concentrations for injection.			

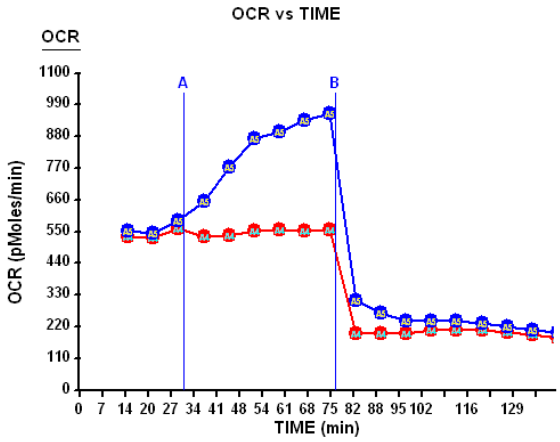
V.2 Calibrate the sensor cartridge (loaded with desired compounds) as described in the XF manual.

VI. Example of results and data analysis

The results below were obtained using 70 mouse islets/well or isolated beta cells.



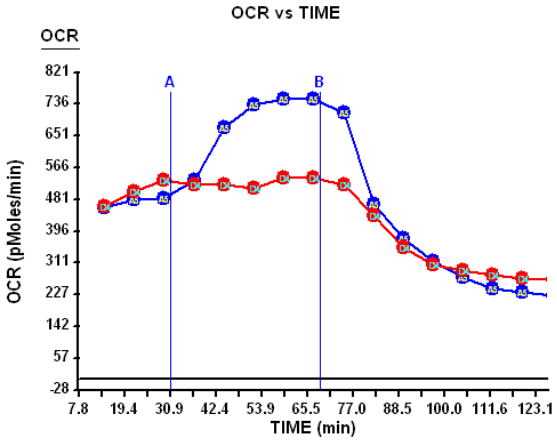
OCR vs. time for Whole Islets
Shirihai Lab unpublished data



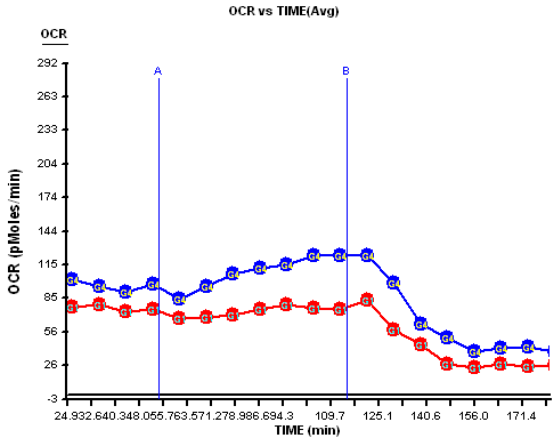
OCR vs. time for Beta Cells
Shirihai Lab unpublished data

Whole pancreatic islets show a similar response to glucose addition as clonal INS1 beta cells run in a standard XF culture plate. Red lines - Blank injection at A; oligomycin at B. Blue lines - 20mM glucose injection at A; oligomycin at B.

The figure below shows a direct comparison between normal human islets and diabetic human islets. Note that the basal OCR readings for the normal islets are 4X higher than that of diabetic islets and the response to glucose is depressed in the diabetic islets as compared to the normal islets.



OCR vs. time for Normal Human Islets
Shirihai Lab unpublished data



OCR vs. time for Diabetic Human Islets
Shirihai Lab unpublished data

Comparisons of normal human islets versus diabetic human islets run in the Islet Capture Microplate. Red lines - Blank injection at A; oligomycin at B. Blue lines - 20mM glucose injection at A; oligomycin at B.

VII. Notes, Suggestions and Comments

The methods described above have been used successfully with whole pancreatic islets isolated from both mouse and humans. We believe that whole islets from other species can be used by following this protocol, however, the tissue, species (including age and sex), and method of isolation will contribute to the overall activity and other variables associated with the whole islets.

Starting values, ranges, and optimization: it is recommended that the following parameters be explored and optimized depending on the overall goal(s) of the experiment and research topic.

- Amount of whole islets per well
- The concentration of substrates and compounds injected
- Mix, Wait and Measure times

VIII. Contact information for questions:

Brian Benoit, Seahorse Bioscience BBenoit@seahorsebio.com
www.seahorsebio.com

Orian Shirihai, Boston University Shirihai@bu.edu
www.Shirihai-lab.org

Jakob Wikstrom, Boston University Jakobwikstrom@gmail.com
www.Shirihai-lab.org