Seahorse Analysis

Mitochondrial Stress Test

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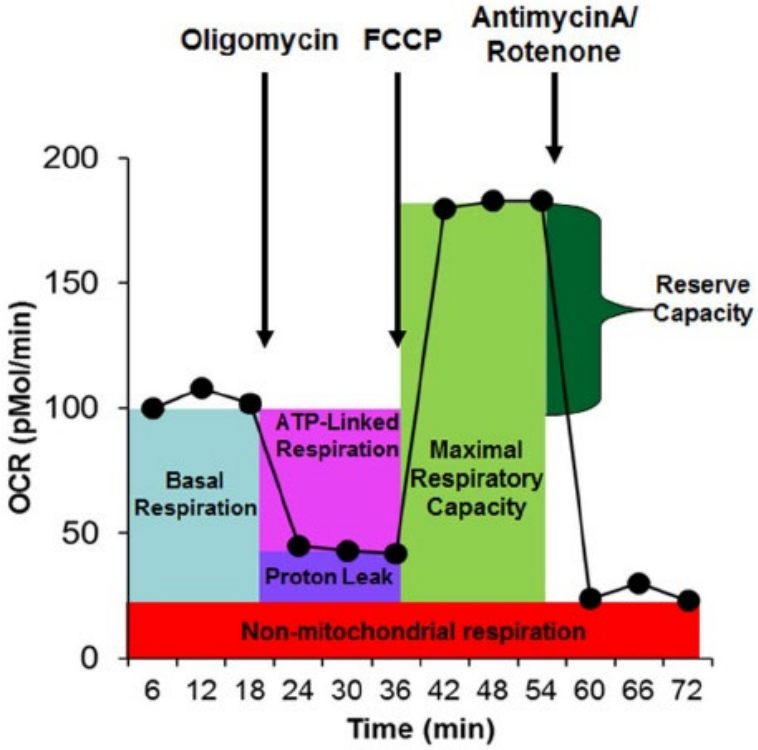
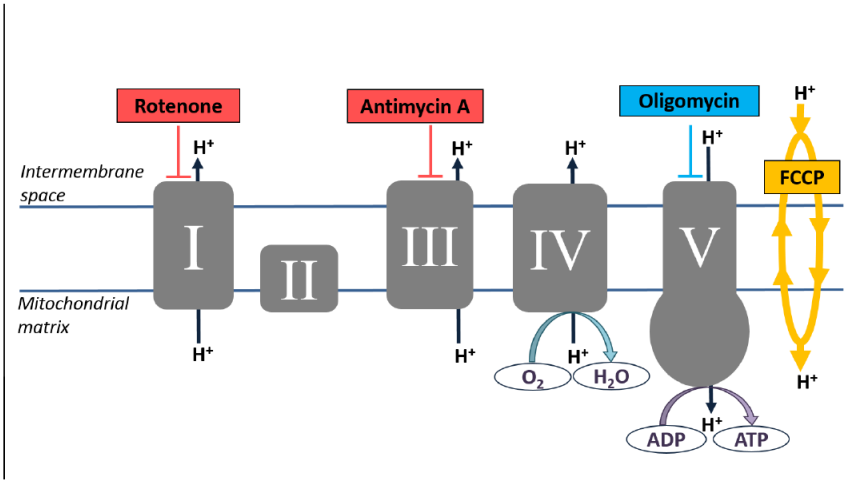
I. Introduction

a. The Seahorse Mitochondrial Stress Test is a method to measure mitochondrial function in any cell type. The expected curve and defined terminology are below:

Seahorse (gives you a read out of which cells are more metabolically active):

- **OCR** – oxygen consumption rate → measures mitochondrial respiration
- **ECAR** – extracellular acidification rate → measures proton excretion (more for glycolysis)
    - If the cells are more glycolytic, they will excrete more lactate which will make the media more acidic (acids release H^+).





- **Oligomycin** → inhibits ATP synthase
- **FCCP** → proton uncoupler (permeabilizes the cell membrane to allow protons to leak through)
- **Antimycin A** (inhibits complex III) and **Rotenone** (inhibits complex I).

b. Parameter Definitions

• **Basal respiration:** Oxygen consumption used to meet cellular ATP demand and resulting from mitochondrial proton leak. Shows energetic demand of the cell under baseline conditions.

• **ATP production:** The decrease in oxygen consumption rate upon injection of the ATP synthase inhibitor oligomycin represents the portion of basal respiration that was being used to drive ATP production. Shows ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell.

• **H+ (Proton) leak:** Remaining basal respiration not coupled to ATP production. Proton leak can be a sign of mitochondrial damage or can be used as a mechanism to regulate the mitochondrial ATP production.

- Proton leak tells you about the structural integrity of the mitochondria because when ATP synthase is blocked by oligomycin the protons (hydrogens) cannot be pumped from the intermembrane space into the matrix of the mitochondria (as the ETC passes electrons hydrogens move to the intermembrane space). So you have a big pool of protons in the intermembrane space, but since the cell wants to achieve equilibrium and concentrations always move from high to low, if the mitochondrial membrane is damaged the protons will diffuse ("leak") into the mitochondrial matrix and you will have a high proton leak.

• **Maximal respiration:** The maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity, which causes rapid oxidation of substrates (sugars, fats, amino acids) to meet this metabolic challenge. Shows the maximum rate of respiration that the cell can achieve.

• **Spare respiratory capacity:** This measurement indicates the capability of the cell to respond to an energetic demand as well as how closely the cell is respiring to its theoretical maximum. The cell's ability to respond to demand can be an indicator of cell fitness or flexibility.

• **Spare respiratory capacity percentage:** This shows spare respiratory capacity as a fold change difference from basal OCR instead of a relative subtracted difference. *This shows a relative difference (like a fold change) rather than an absolute difference. This gives you the scale of difference between treatments and allows us to understand the comparative ratio of the numbers. This way everything is compared as a fold change on the same scale and relative difference, rather than absolute change.*

• **Nonmitochondrial respiration:** Oxygen consumption that persists due to a subset of cellular enzymes that continue to consume oxygen after rotenone and antimycin A addition. This is important for getting an accurate measure of mitochondrial respiration.

• **OCR/ECAR Ratio:** If the ratio is higher you have more reliance on mitochondrial respiration. If it's low, you are relying more on glycolysis.

II. Goal (Expected Result)

You will have an outputted OCR and ECAR file with the measurements at multiple timepoint during the assay. Running calculations from these results will give you information on the mitochondria's functional capacity.

III. Methodology & Tools

a. Installing Packages/Software

The following tools used for this analysis are:

- **Wave Seahorse Software**
- **GraphPad Prism**
- **R & R Studio**
- **Python 2.7 & Python Pandas**

b. Obtaining & Formatting Data Files

i. Open the seahorse file outputted from the machine in the Wave software and click on Add → Overview to see the OCR & ECAR curves.

ii. While you are looking at the OCR curve, click on wells in the plate layout to see if any of the wells failed (meaning the curve doesn't exist for that well and the line plateaus).

iii. Click on all the failed wells you wish to exclude from the analysis (they will be greyed out).

iv. Next, to export the data click on Export, then GraphPad Prism.

v. Copy and paste the OCR & ECAR data from GraphPad into Excel as a transposed matrix, add a "Group" column to indicate which group your sample belongs in, then save the file as a CSV.

1. We are exporting the data to GraphPad then copying it to excel instead of exporting to Excel directly because the output to GraphPad provides you with averages of each well for each timepoint – the Excel output contains raw data we do not need.

vi. The excel CSV file should now look like this:

Time (minutes)	Group	1.413248	8.029409	14.62033	21.30337	27.87647	34.46636	41.10752	47.69904	54.27648	60.92578	67.50982	74.08912
B cell HF 010	B cell HF	5.874303	5.604285	5.342845	4.357883	5.541929	6.165468	10.76959	10.69469	10.8715	6.677042	7.542431	7.201583
B cell HF 010	B cell HF	6.969056	6.442794	6.081051	5.136133	6.546033	6.916194	10.5377	10.74928	10.45432	6.831753	7.315611	7.168182
B cell HF 010	B cell HF	7.676725	7.150002	6.680417	5.034397	6.799732	7.437819	12.13844	11.69943	12.15838	7.700104	8.005525	7.769876
B cell HF 010	B cell HF	8.638697	7.994267	7.629323	6.171677	7.4487	7.735954	13.3405	12.45309	12.28603	8.200596	8.426214	8.181559
B cell HF 010	B cell HF	8.051233	7.568512	7.190429	5.310756	6.906499	7.34701	12.94774	12.63619	12.63284	8.031172	8.027576	8.057201
B cell HF 011	B cell HF	8.140874	7.611454	7.287746	5.306836	7.25761	7.662084	12.87557	12.36901	12.49022	8.065959	8.570187	8.125981
B cell HF 011	B cell HF	7.698856	7.158086	6.758947	5.538206	6.800909	7.386692	12.47194	11.96613	11.66525	7.721564	8.336981	8.032861
B cell HF 011	B cell HF	8.33131	7.221127	6.812399	5.602988	7.032689	7.333315	13.58102	13.13564	13.03242	7.709621	8.334929	8.312592
B cell HF 011	B cell HF	8.692059	7.640514	7.38334	5.417712	7.103824	7.839146	13.62558	13.1377	13.19218	8.284839	8.800062	8.604657
B cell HF 011	B cell HF	7.297591	7.033671	6.803768	4.759404	6.572941	7.132878	12.84611	12.53799	12.35089	7.518092	7.996888	8.027003

vii. Data Pre-processing steps for R & python scripts:

1. If you wish to exclude entire rows (wells) from the analysis be sure to remove them. DO NOT remove individual values yet (reference step 2 below).
2. If you wish to exclude any individual row value from the analysis, replace the value with "NaN". The Python/R scripts will skip NaN values and exclude them from analysis.
    a. If each column does not contain same number of rows, the program will fail.
3. You **MUST** replace all negative OCR & ECAR values with NaN in the CSV file!
4. Make sure to label each column with the corresponding biological replicate (ex: B cell Mouse 1)
5. **ALL BIOLOGICAL REPLICATES MUST HAVE THE SAME UNIQUE PREFIX** (ex: B cell Mouse 1, B cell Mouse 2, CD4 Mouse 1, CD4 Mouse 2, etc...) & prefixes must **NOT** overlap between biological groups.
    a. The biological group names must be unique from each other, from example, you cannot have B cell HF and B cell HF_SPM because both contain the same prefix 'B cell HF' and they are different biological groups.
    b. That is why you should change it to B cell HF and B cell SPM_HF to make sure all prefixes are unique. Or else the program will assign that sample to the wrong group. ***Make sure the ECAR & OCR Group names match***
    c. Also, the OCR & ECAR files must contain the sample samples, meaning sample 1 cannot be in the OCR file but completely missing from the ECAR file

(it's ok if the number of technical replicates differs). If you wish to remove an entire sample for one file but not the other place NaNs for that sample's values.

c. **Calculating Outliers**

i. To analyze whether a technical replicate is an outlier, run the **seahorse_outlier_analysis.R** program, or calculate outliers with the 1.5*IQR method.

ii. In the seahorse_outlier_analysis.R program, the outliers will be outputted into a matrix datafile where you will have the biological group, timepoint, and outlier OCR or ECAR value outputted to a file (example below).

c("B cell Lean 2"	"X60.91076"	"27.012544")
c("B cell Lean 2"	"X67.498093"	"29.071465")
c("B cell Lean 2"	"X74.097237"	"29.478494")
c("B cell Lean 3"	"X21.279686"	"22.35429")

iii. Using the output above, the R program will replace all outliers with NaN. This segment of the code is under the comment: "**#replacing outliers with NaN**".

d. Calculating Parameters

i. The python program runs the equations to calculate different parameter outputs for the Mito Stress Test. The equations used for the calculations are below:

Parameter Value	Equation
Non-mitochondrial Oxygen Consumption	Median rate measurement after Rotenone/antimycin A injection
Basal Respiration	(Last rate measurement before first injection) – (Non-Mitochondrial Respiration Rate)
Maximal Respiration	(Maximum rate measurement after FCCP injection) – (Non-Mitochondrial Respiration)
H+ (Proton) Leak	(Minimum rate measurement after Oligomycin injection) – (Non-Mitochondrial Respiration
ATP Production	(Last rate measurement before Oligomycin injection) – (Minimum rate measurement after Oligomycin injection)
Spare Respiratory Capacity	(Maximal Respiration) – (Basal Respiration)
Spare Respiratory Capacity as a %	(Maximal Respiration) / (Basal Respiration) × 100
Acute Response	(Last rate measurement before oligomycin Injection) – (Last rate measurement before acute injection)
Coupling Efficiency	ATP Production Rate) / (Basal Respiration Rate) × 100

	Experimental Group #1						
	Measurement	Assay Well - A1 OCR (pmol/min)	Assay Well - A2 OCR (pmol/min)	Assay Well - A3 OCR (pmol/min)	Assay Well - A4 OCR (pmol/min)	Average OCR	
Baseline OCR	1	170.02	172.80	169.96	175.99	172.19	
	2	165.36	163.62	167.00	166.42	165.60	
	3	160.50	158.25	159.44	161.75	159.98	<< Last rate measurement before 1st injection
Injection 1 - Oligomycin	4	65.44	53.05	61.53	59.22	59.81	
	5	61.80	49.44	59.62	56.94	56.95	
	6	61.93	49.55	59.06	56.04	56.65	<< Minimum rate measurement after Oligo injection
Injection 2 - FCCP	7	319.58	310.94	312.59	315.98	314.77	
	8	327.95	320.32	325.77	330.73	326.19	<< Maximum rate measurement after FCCP Injection
	9	297.17	292.30	301.35	299.68	297.63	
Injection 3 - Rot/AA	10	51.77	34.62	44.99	39.45	42.71	
	11	49.28	33.24	44.13	39.05	41.42	
	12	47.03	31.80	42.66	39.20	40.17	<< Minimum after Rot/AA Injection (Non-Mitochondrial Oxygen Consumption)

We use median rate

ii. When running the python program (**seahorse_analysis.py**), make sure to correctly list the biological group names as described in step ***vii***. Make sure to also change the input and output file names. The output file names are located on lines 121 and 133-135.

1. Open bash & run: **python seahorse_analysis.py**. You will get 4 output files, we will use the output file from line 121 (first csv output) as input for our R graphing script.

e. Statistical Analysis & Graphing

i. Use the **seahorse_comboPlots.R** script to generate statistics & graph the results.
    1. If you have many biological groups and would like to run a particular set at a time, then run the command on the line under the comment (**#filter by group**). There you can exclude particular groups from the dataframe and only analyze a few at a time.

ii. You will get a graph output like the one below:
*The assumption of normality is checked in the code with the Shapiro-Wilks test to decide whether to use a T-test or Wilcoxon test for statistical comparisons.