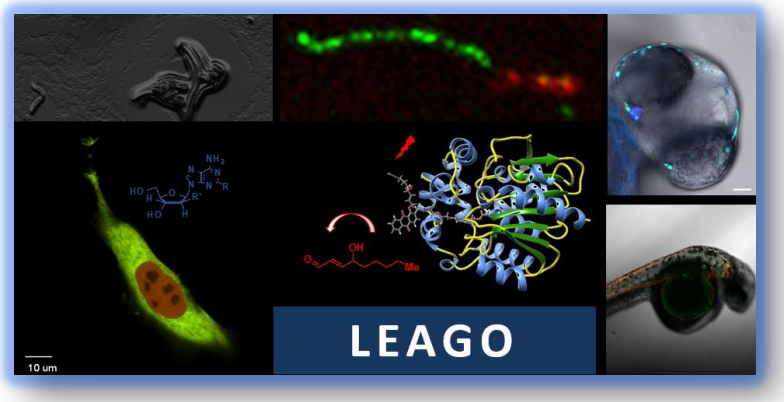
Welcome to CH-313: Chemical Biology

Prof. Yimon Aye https://leago.epfl.ch/



Laboratory of Electrophiles And Genome Operation

Lecture Week 13:

ETC-OXPHOS (continued)

& tools to monitor

cell energy status

2022 Dec 13th (Room: CH B3 31): 8:15 am – 10:00 am

https://moodle.epfl.ch/course/view.php?id=14462

Part II: Targeting metabolic regulation

3 stages of cellular respiration

Week	Date	Topic	Notes
9	15th Nov	**Glycolysis**	
10	22nd Nov	…. and isotopic tracing / pathway flux analysis	**PSet 5**
11	29th Nov	**Tricarboxylic acid (TCA) cycle**	
12	6th Dec	…. and Warburg effect	**PSet 6**
13	13th Dec	**Oxidative phosphorylation (Oxphos)**	
14	20th Dec	Methods to monitor cell energy stat	**PSet 7**

Glycolytic Signaling Switches in Cancer (the Warburg Effect aka aerobic glycolysis)

Anaerobic vs. Aerobic Metabolism (lactate production vs. TCA cycle)

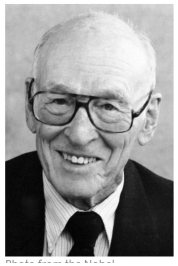
Electron transport chain (ETC) and Oxidative phosphorylation (Oxphos)

Methods to monitor cell energy status
ECAR and OCR

ATP – the universal energy carrier in the living cell

The Nobel Prize in Chemistry 1997

"for their elucidation of the enzymatic mechanism underlying the synthesis of adenosine triphosphate (ATP)"



Paul D. Boyer
The Nobel Prize in Chemistry 1997

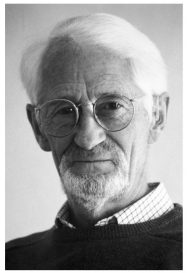
Born: 31 July 1918, Provo, UT, USA

Died: 2 June 2018, Los Angeles, CA, USA

Affiliation at the time of the award: University of California, Los Angeles, CA, USA

Prize motivation: "for their elucidation of the enzymatic mechanism underlying the synthesis of adenosine triphosphate (ATP)."

Prize share: 1/4



Jens C. Skou
The Nobel Prize in Chemistry 1997

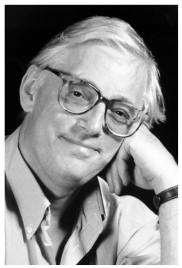
Born: 8 October 1918, Lemvig, Denmark

Died: 28 May 2018, Aarhus, Denmark

Affiliation at the time of the award: Aarhus University, Aarhus, Denmark

Prize motivation: "for the first discovery of an ion-transporting enzyme, Na+, K+ -ATPase."

Prize share: 1/2



John E. Walker
The Nobel Prize in Chemistry 1997

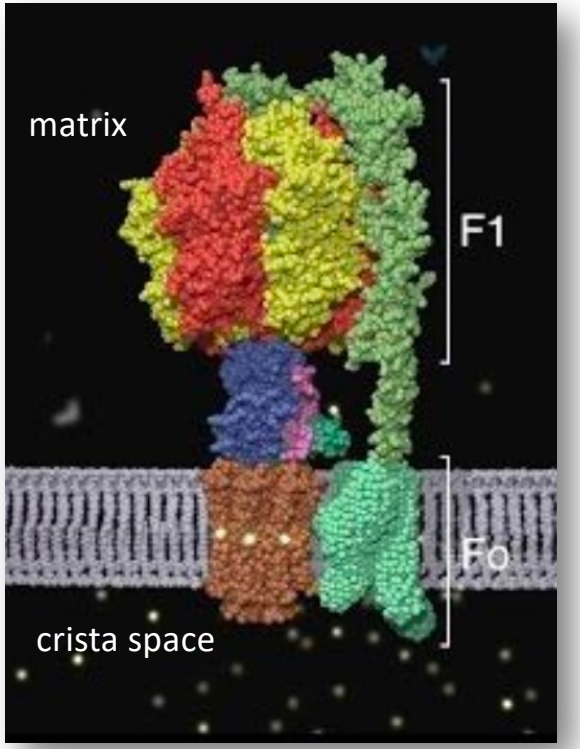
Born: 7 January 1941, Halifax, United Kingdom

Affiliation at the time of the award: MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Prize motivation: "for their elucidation of the enzymatic mechanism underlying the synthesis of adenosine triphosphate (ATP)."

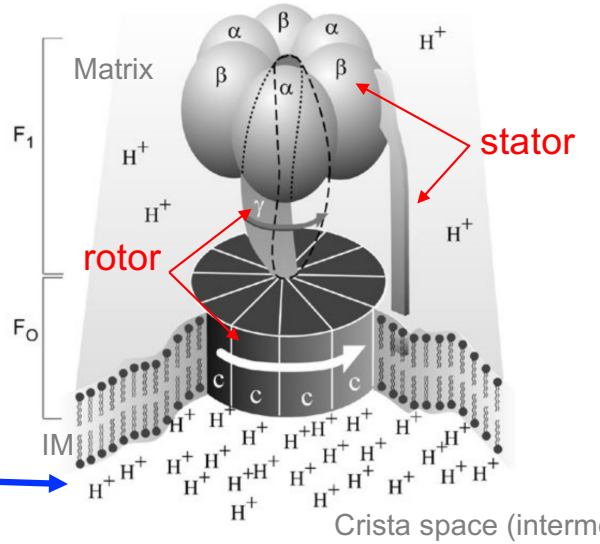
Prize share: 1/4

The human body on average contains only 250 grams (8.8 oz) of ATP, and each day we turn over this amount of ATP in a day, at rest. But if you're at hard work, more amount surely..



Q: show in the figure where is matrix **vs.** intermembrane space (crista space, more specifically).

Figure 1:



Q: Why are there more H+ on this side of IM?

Figure 1. Simplified picture of ATP syntase The Fo part through which hydrogen ions (H+) stream is located in the membrane. The F1 part which synthesises ATP is outside the membrane. When the hydrogen ions flow through the membrane via the disc of c subunits in the Fo part, the disc is forced to twist around. The gamma subunit in the F1 part is attached to the disc and therefore rotates with it. The three alpha and three beta subunits in the F1 part cannot rotate, however. They are locked in a fixed position by the b subunit. This in turn is anchored in the membrane. Thus the gamma subunit rotates inside the cylinder formed by the six alpha and beta subunits. Since the gamma subunit is asymmetrical it compels the beta subunits to undergo structural changes. This leads to the beta subunits binding ATP and ADP with differing strengths (see Figure 2).

Figure 2. Boyer's "Binding Change Mechanism" The picture shows the cylinder with alternating alpha and beta subunits at four different stages of ATP synthesis. The asymmetrical gamma subunit that causes changes in the structure of the beta subunits can be seen in the centre. The structures are termed open betaO (light grey sector), loose betaL (grey sector) and tight betaT (black sector). At stage A we see an already-fully-formed ATP molecule bound to betaT. In the step to stage B betaL binds ADP and inorganic phosphate (Pi). At the next stage, C, we see how the gamma subunit has twisted due to the flow of hydrogen ions (see Figure 1). This brings about changes in the structure of the three beta subunits. The tight beta subunit now becomes open and the bound ATP molecule is released. The loose beta subunit becomes tight and the open becomes loose. In the last stage the chemical reaction takes place in which phosphate ions react with the ADP molecule to form a new ATP molecule. We are back at the first stage.

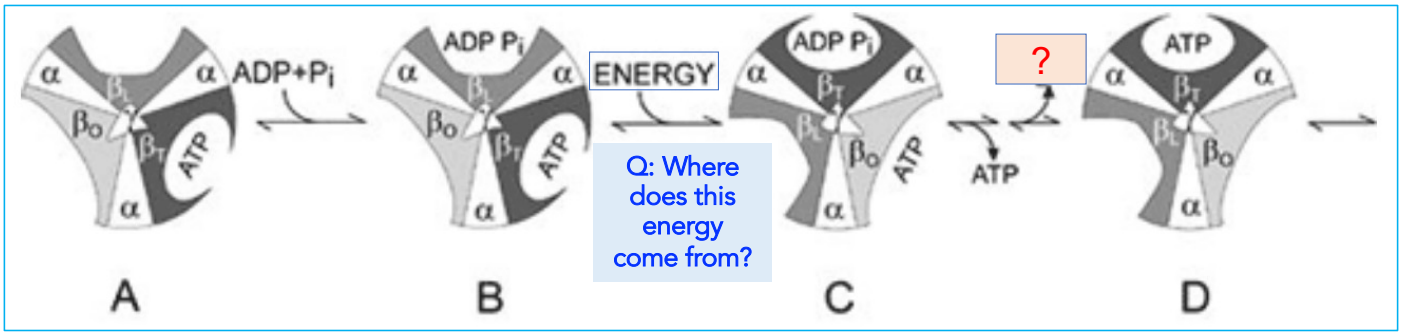
[Link to ATP synthase in action](https://www.youtube.com/watch?v=kXpzp4RDGJI):

https://www.youtube.com/watch?v=kXpzp4RDGJI

Q: How does ATP synthases differ from the majority of enzymes (in terms of substrate binding/product release)?

Figure 2:

View from top

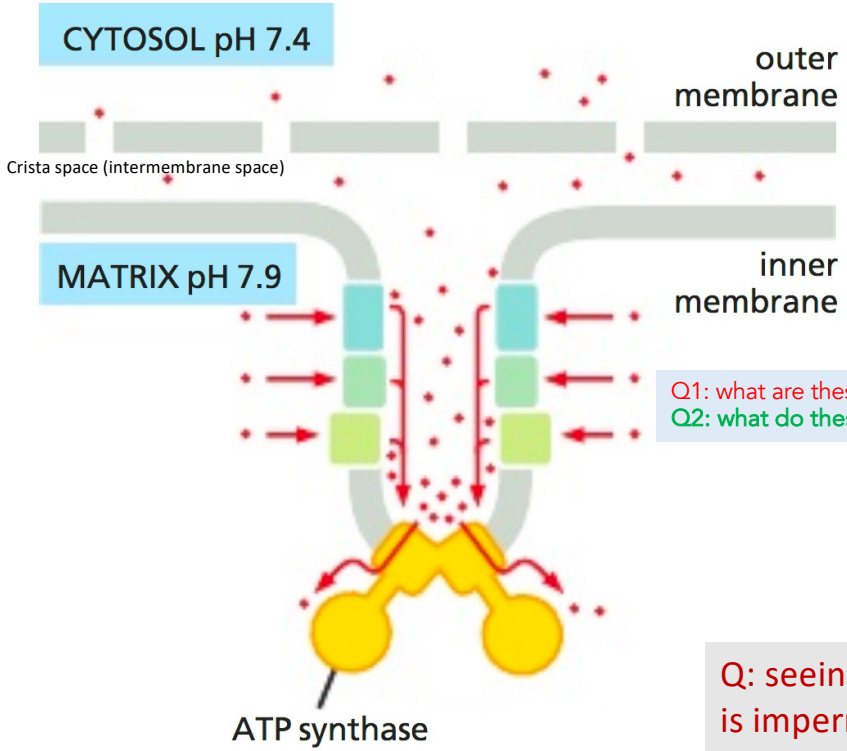


Q: Where does this energy come from?

Also 1979 and 1988 Nobel Prizes in Chemistry mark the contributions toward understanding of ETC enzymes and photosynthesis/cellular respiration processes.

ATP production by ATP synthase dimers **at cristae ridges**

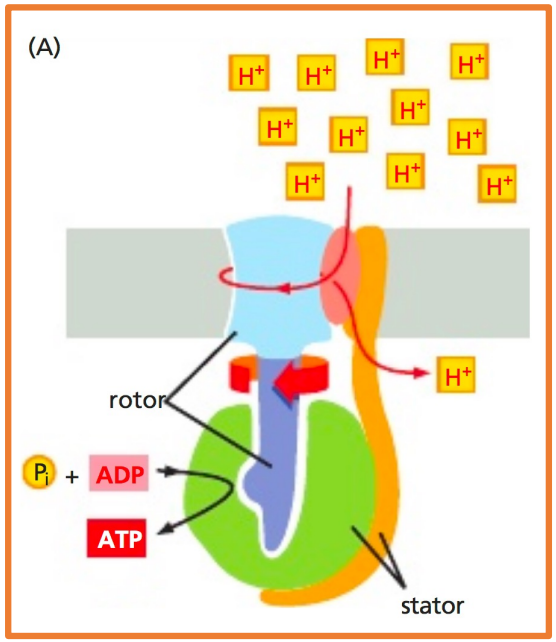
Question: Electron micrographs show that mitochondria in heart muscle have a much higher density cristae than mitochondria in skin cells - why do you suppose this should be?



Q1: what are these Red arrows?
Q2: what do these 3 "rectangles" represent?

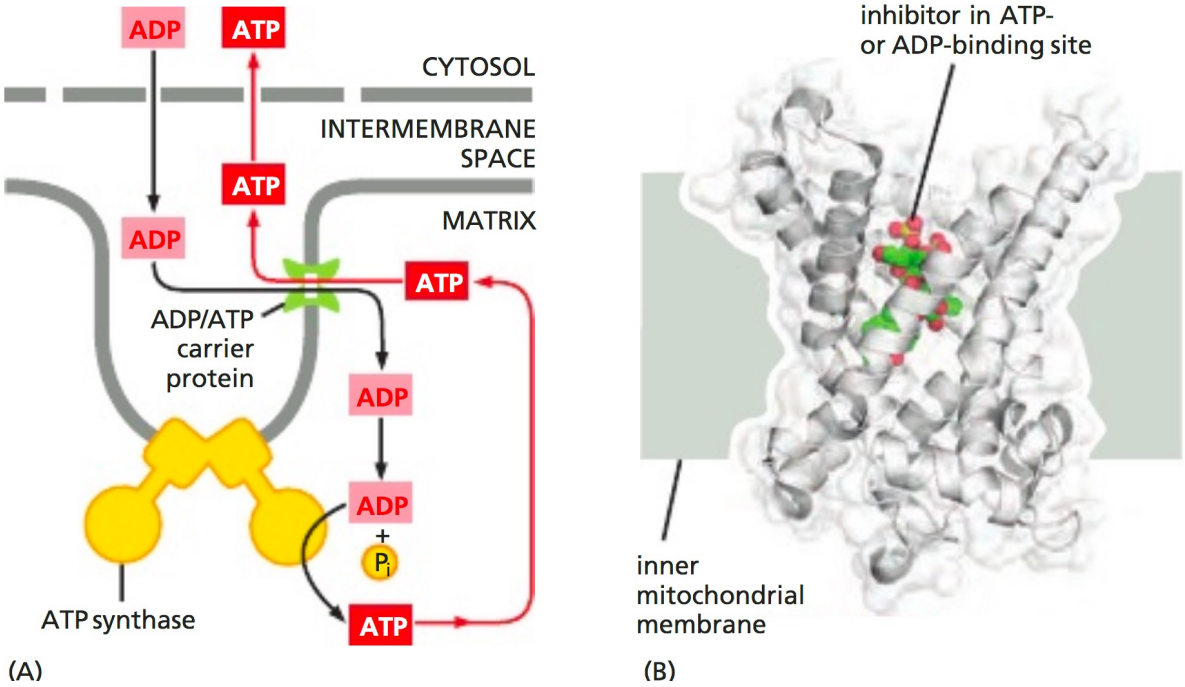
(proton sink created by ATP synthase)

ATP synthase



Q: seeing that ATP is made inside matrix, and since we know inner-membrane is impermeable to charged entities, how do newly-synthesized ATP molecules get back out to cytoplasm and elsewhere in the cell? And further, how do ADP substrate molecules even get inside matrix in the first place?

How does ATP get back out to cytosol? (by ADP/ATP carrier protein)



The ADP/ATP carrier protein is a small membrane protein that carries the ATP produced on the matrix side of the inner membrane to the intermembrane space, and the ADP that is needed for ATP synthesis into the matrix.

Fig. B. x-ray structure of the ADP/ATP carrier shows six transmembrane alpha helices that define a cavity that binds either ADP or ATP. The substrate is replaced by a tightly bound inhibitor (colored). When ADP binds from outside the inner membrane, it triggers a conformational change and is released into the matrix. In exchange, a molecule of ATP quickly binds to the matrix side of the carrier and is transported to the intermembrane space. From there the ATP diffuses through the outer mitochondrial membrane to the cytoplasm, where it powers the energy-requiring processes in the cell.

In-Class Exercise: Concepts on ADP/ATP transport

Although mitochondria can transport both ATP and ADP, there is *a strong bias in favour of exchange of external ADP for internal ATP in actively-respiring mitochondria*. You hypothesize that this bias is due to *concentration gradient* for ADP import and ATP export (due to ATP synthesis inside mitochondrion). To test this hypothesis, you conduct 4 experiments (see Table) using isolated mitochondria, to **measure the initial rates of entry of ATP and ADP**. [Note: substrate (electron-donor metabolites such as succinate) addition makes mitochondria begin to respire. Recall our previous discussions on the effect of DNP (dinitrophenol) which is an uncoupler reagent as it collapses the pH gradient, and oligomycin, which is an ATP synthase inhibitor. In all experiments, note that enough time has been allowed to elapse for equilibration to occur].

Q1. Which one of the four experiments disproves your hypothesis?

Q2. Propose another chemically-well-reasoned and biologically-sound hypothesis that would correctly explains the observed biased exchange under some conditions and unbiased exchange under others.

experiment	Substrate presence	Inhibitor	Relative rates of entry into matrix
1	no	none	ADP = ATP
2	yes	none	ADP > ATP
3	yes	dinitrophenol	ADP = ATP
4	yes	oligomycin	ADP > ATP

The key to understanding these results is to recognize that an exchange of ADP for ATP is *not* electrically neutral. ATP carries 4 (--)ve charges, and ADP carries only 3. As a result, each exchange of ADP for ATP increases the negative charge on the side of the membrane that receives the ATP. In a respiring mitochondrion there is an electrochemical gradient across the mitochondrial inner membrane (IM) such that the outside of the membrane is positive. The resulting proton-motive force drives the exchange of an external ADP for an internal ATP, thereby reducing the positive charge on the outside of the membrane.

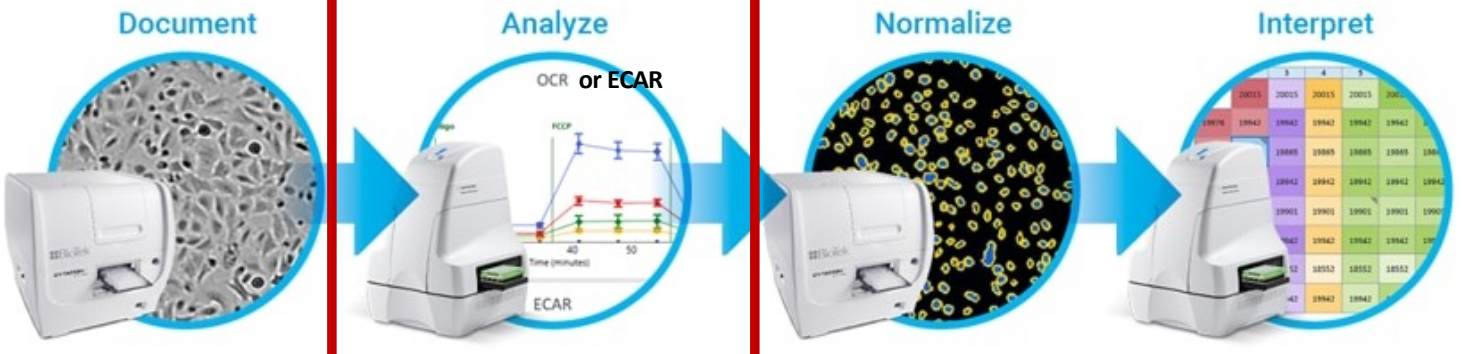
In the results shown in the table, the exchange of external ADP was favored over that of external ATP under conditions in which the membrane was charged: when substrate was present without an inhibitor, and when substrate was present with an inhibitor of ATP synthase (oligomycin). When the electrochemical gradient was absent (i.e., when there was no substrate, or when the membrane was made permeable to protons (DNP treatment)), ADP and ATP were exchanged at equal rates.

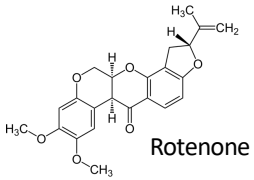
Note: The electrogenic nature of the ADP/ATP transfporter has important physiological consequences. In essence, the exchange harnesses the energy of the electrochemical gradient to drive transport so that the cytosolic ratio of ATP/ADP remains high (up to 50). This concentration difference provides up to 1/3rd of the free-energy change (ΔG) for ATP hydrolysis in the cytoplasm.

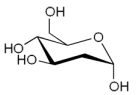
Methods to monitor cell energy status: ECAR and OCR

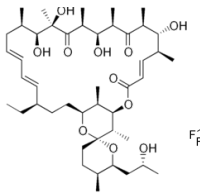
Extracellular acidification rate (ECAR) and OCR (oxygen consumption rate)

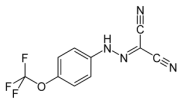
Typical workflow of XF (extracellular flux) assay:

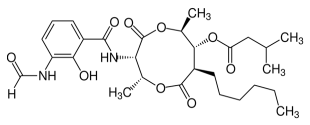



Rotenone

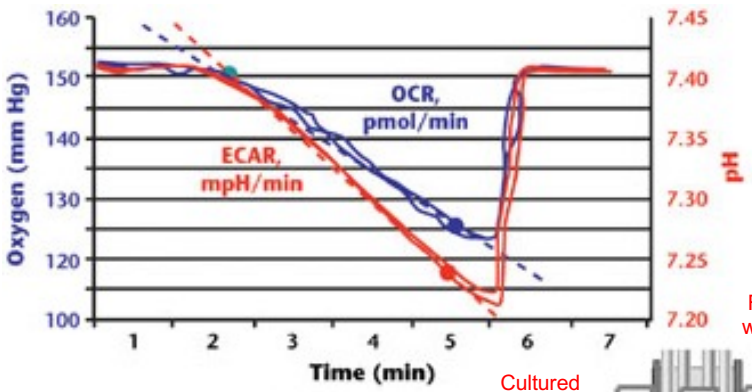

2-DG

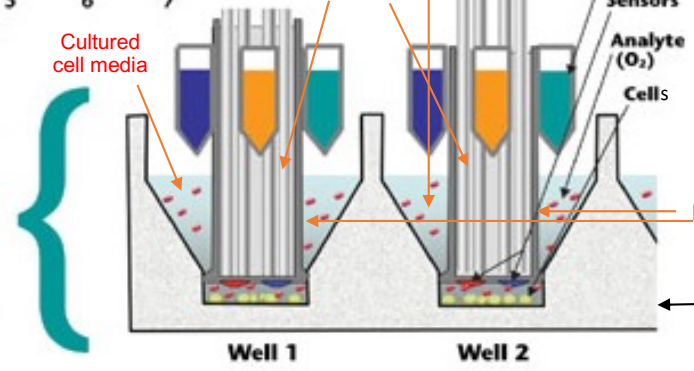

oligomycin

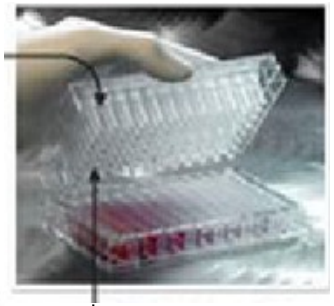

FCCP


Antimycin A

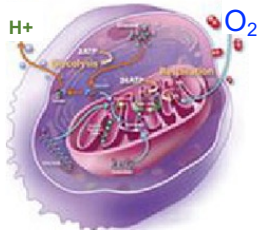
Aside: just for your interest: instrument analyzing extracellular flux (XF) (aka Seahorse analyzer)







Multi-well XF-plate

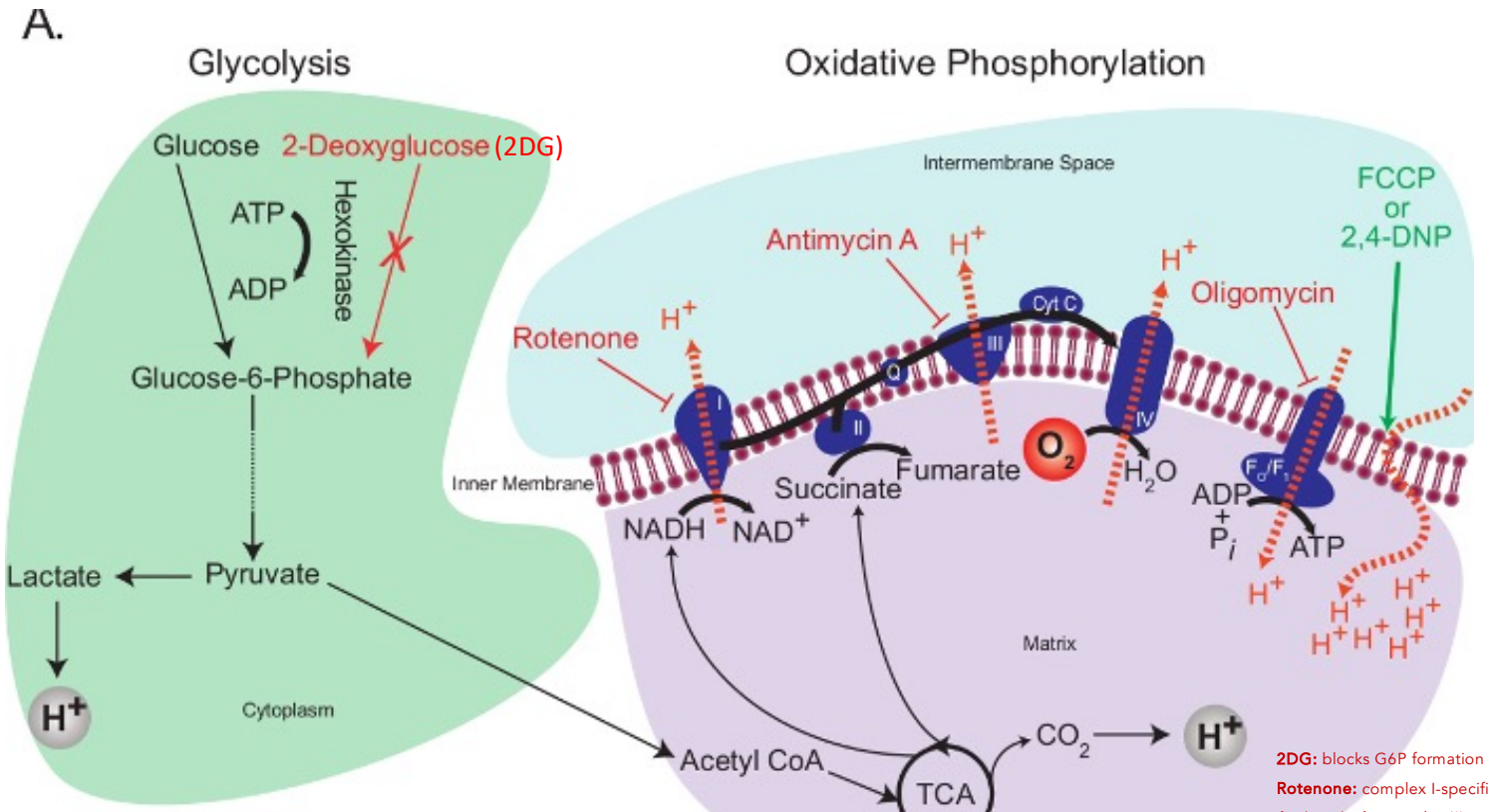


Measuring Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR)

https://www.jove.com/v/54918/an-optimized-protocol-to-analyze-glycolysis-mitochondrial-respiration

Methods to monitor cell energy status:
Extracellular acidification rate (ECAR) and OCR (oxygen consumption rate)

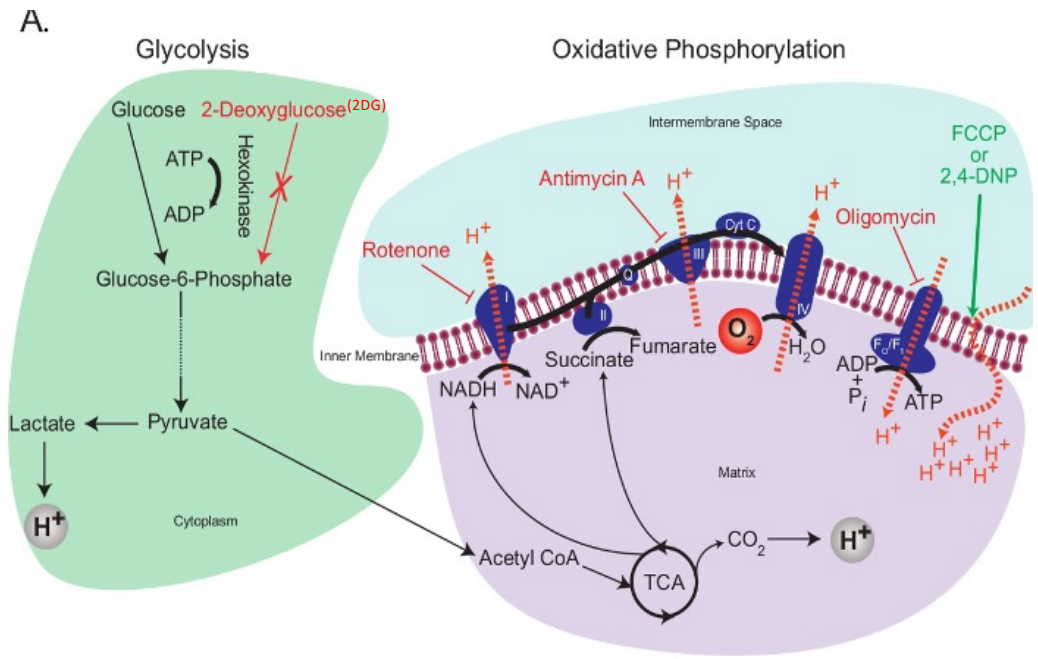
.....featuring small-molecule metabolic modulators used in the extracellular flux assays (which interfere these pathways at specific points as shown)



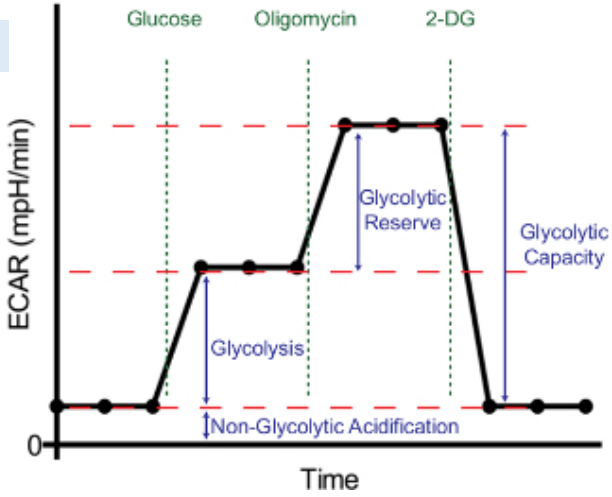
First, consider the specific impact of each molecule, (2DG, rotenone, antimycin A, oligomycin, and FCCP/2,4-DNP). Then, consider the data on the following slide

2DG: blocks G6P formation so blocks glycolysis.
Rotenone: complex I-specific inhibitor of ETC
Antimycin A: complex III-specific inhibitor of the ETC
Oligomycin: inhibitor of ATP synthase
DNP or FCCP: uncoupling agent (intervene specific events related to ETC, H+ gradient, and ATP synthesis)

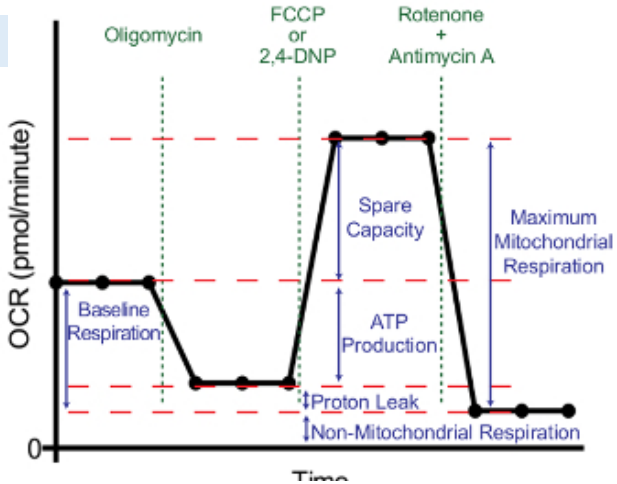
In-class discussion – on Methods to monitor cell energy status: Extracellular acidification rate (ECAR) and OCR (oxygen consumption rate)



In starved cells:



In non-starved cells:



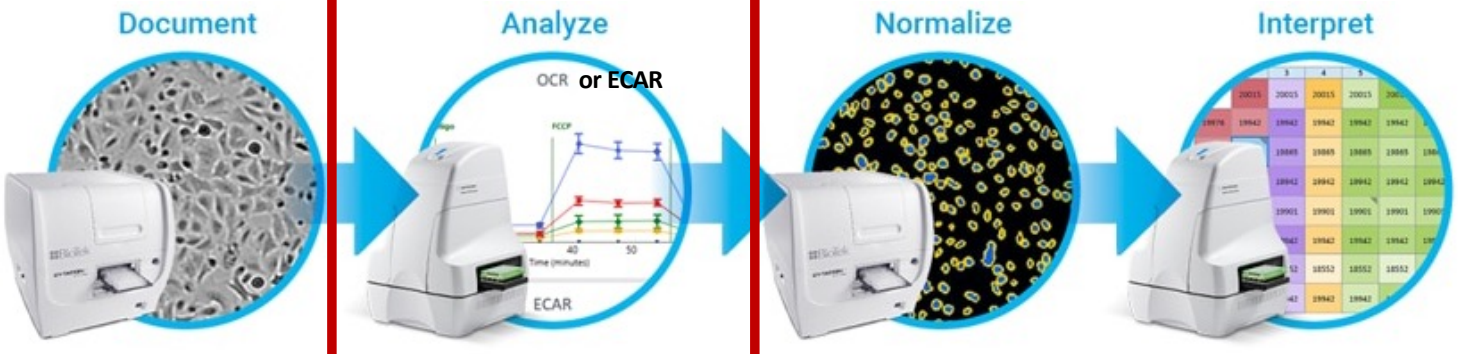
Q: Explain the data in glycolysis stress test (upper plot)

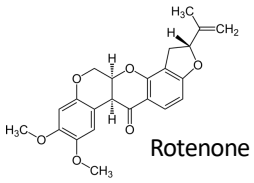
Q: Explain the data in mitochondrial stress test (lower plot)

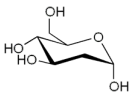
Revisiting Slide 8 above…

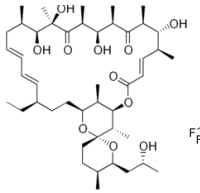
Typical workflow of XF (extracellular flux) assay:

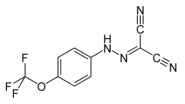
Q: what is the goal of this step? Why is this necessary? Is normalization of cell number alone sufficiently rigorous?

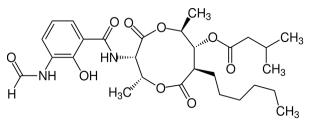



Rotenone

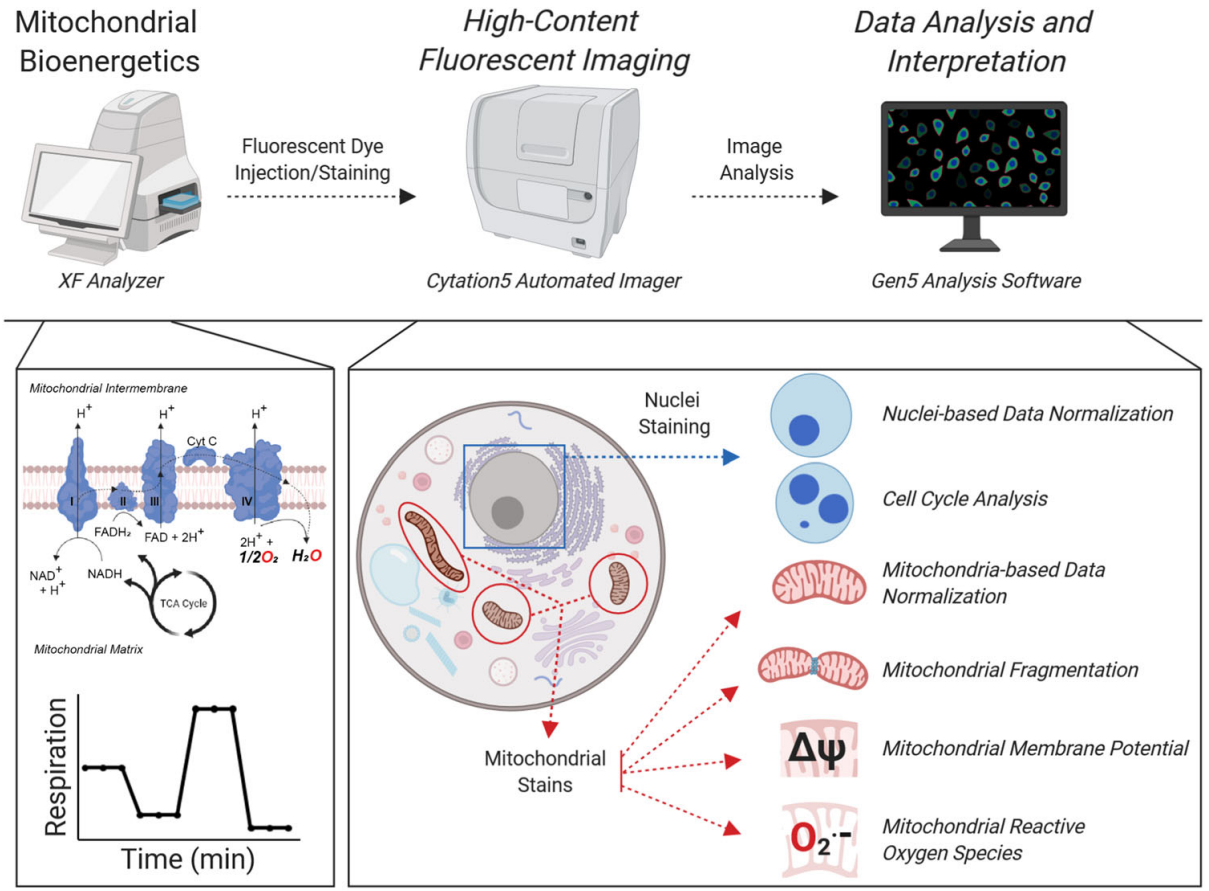

2-DG


oligomycin


FCCP


Antimycin A

Platform to integrate the metabolic flux assay with high-content imaging:



COMMUNICATIONS BIOLOGY | (2020)3:271 | https://doi.org/10.1038/s42003-020-0988-z

At the instrument level, cells are processed using the Seahorse metabolic flux assay and immediately stained with a variety of nuclear and mitochondrial dyes, which is completely integrated in the Seahorse bioanalyzer assay. The plates are then abstracted and imaged on a Cytation5 Automated Imager for downstream image analysis and interpretation. At the biochemical level, the metabolic flux assay provides OCR and ECAR data and information on other mitochondrial bioenergetic properties
(by Mito Stress Test). The cells are then stained with nuclear and mitochondrial dyes that provide information on the cellular properties noted.

An in-class exercise / problem to think about (on OCR concepts/measurements):

Q1: During the handling of seahorse analyzer plates, labels in the two sets of wells got mixed up. One set originates from starvation-resistant cell line and the other set from starvation-sensitive cell line. Note: each set contained data obtained in the presence and absence of glucose.
Could you help assign the right set of wells based on the OCR data shown?
(NOTE: Starvation-resistant cells are cells that can use their 'glucose stores' and continue functioning their metabolic activity, even when starved…)

Hint: FCCP (uncoupler reagent) makes IM permeable to H^+

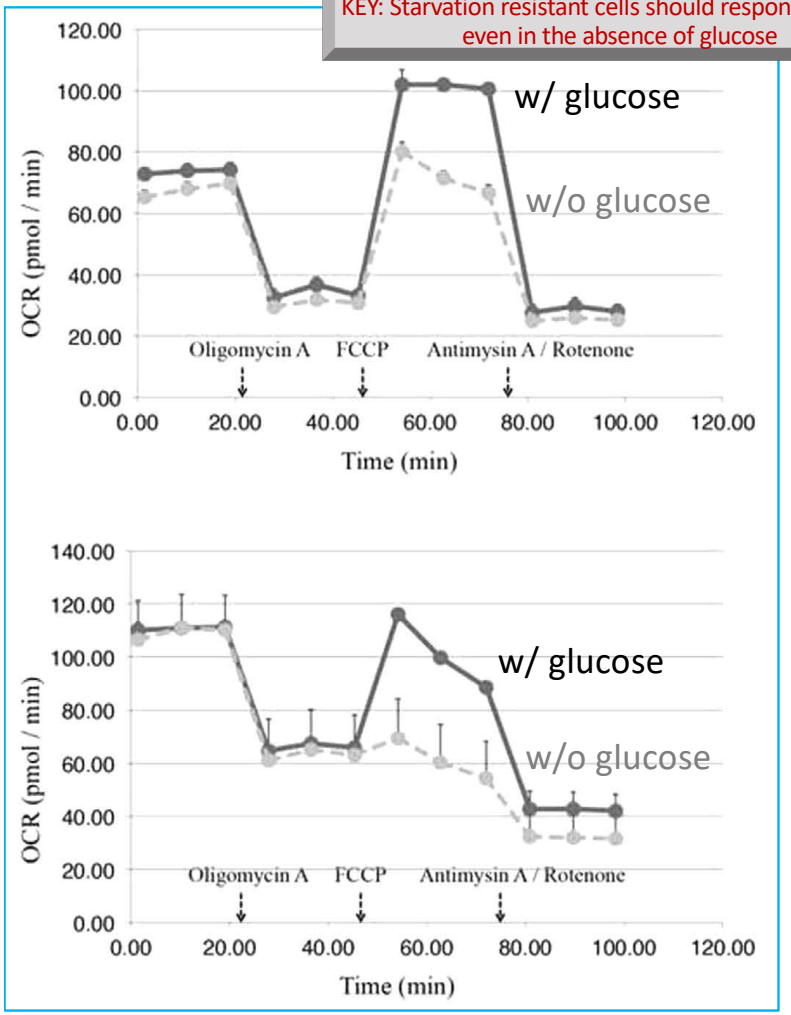
Q2: What could be potential target(s) of antimycin A and rotenone. Choose one or more correct answer(s):

a) Complex I
b) Complex III
c) Inhibits ATP synthase
d) Inhibits ADP/ATP carrier

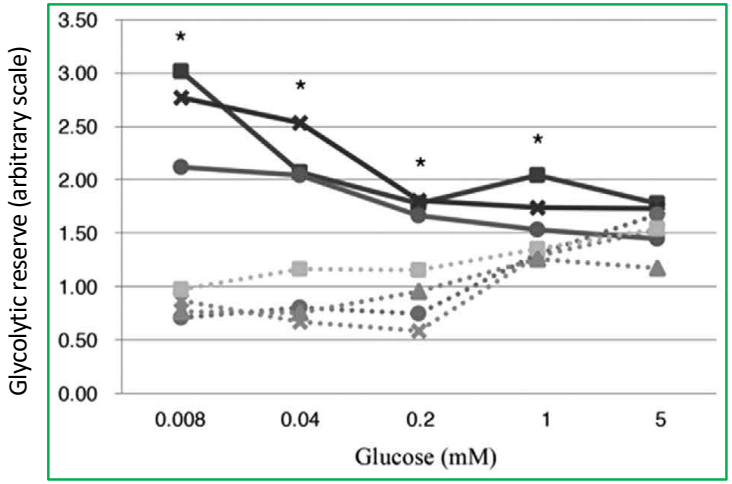
Note: you're only expected to interpret the data given in the problem, w/o being able to recall/remember what Rotenone or antimycin A does…

KEY:: based on the data shown in the question, all we can deduce is that both Antimysin A and Rotenone decrease OCR to the lowest level, meaning that the shutting off the first stage of Ox Phos occurs. Blocking either complex I or III or both should result in this observation.
Whereas inhibiting ATP synthase and/or ATP/ADP carrier should not affect OCR

KEY: Starvation resistant cells should respond to FCCP, even in the absence of glucose



A problem on OCR: continued…



Q3: additional analysis that measures how much energy is stored in these cell lines (so-called glycolytic reserve/glucose stores) was undertaken to yield the data shown above:

Designate the correct labels for dotted vs. solid lines as:

starvation-resistant cell lines vs. starvation–sensitive cell lines

KEY: Starvation-resistant lines possess high glycolytic reserves under low glucose conditions (0.008 – 1 mM), and similarly at high glucose concentrations (5 mM). However, there are no glycolytic reserves in the starvation-sensitive cell lines.

Learning outcomes (Week 13: CH-313 Chemical Biology - Synopsis)

- ETC-OXPHOS : underlying redox chemistry, biochemistry, and biology with focus on ATP synthesis and ADP → ATP conversion

- OCR, ECAR, and experimental design, data interpretations, and limitations underlying these state-of-the-art technologies