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Agency

Respirometric Screening and Characterization of Mitochondrial Toxicities Induced by ToxCast Chemicals

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Office of Research and Development
National Center for Computational Toxicology

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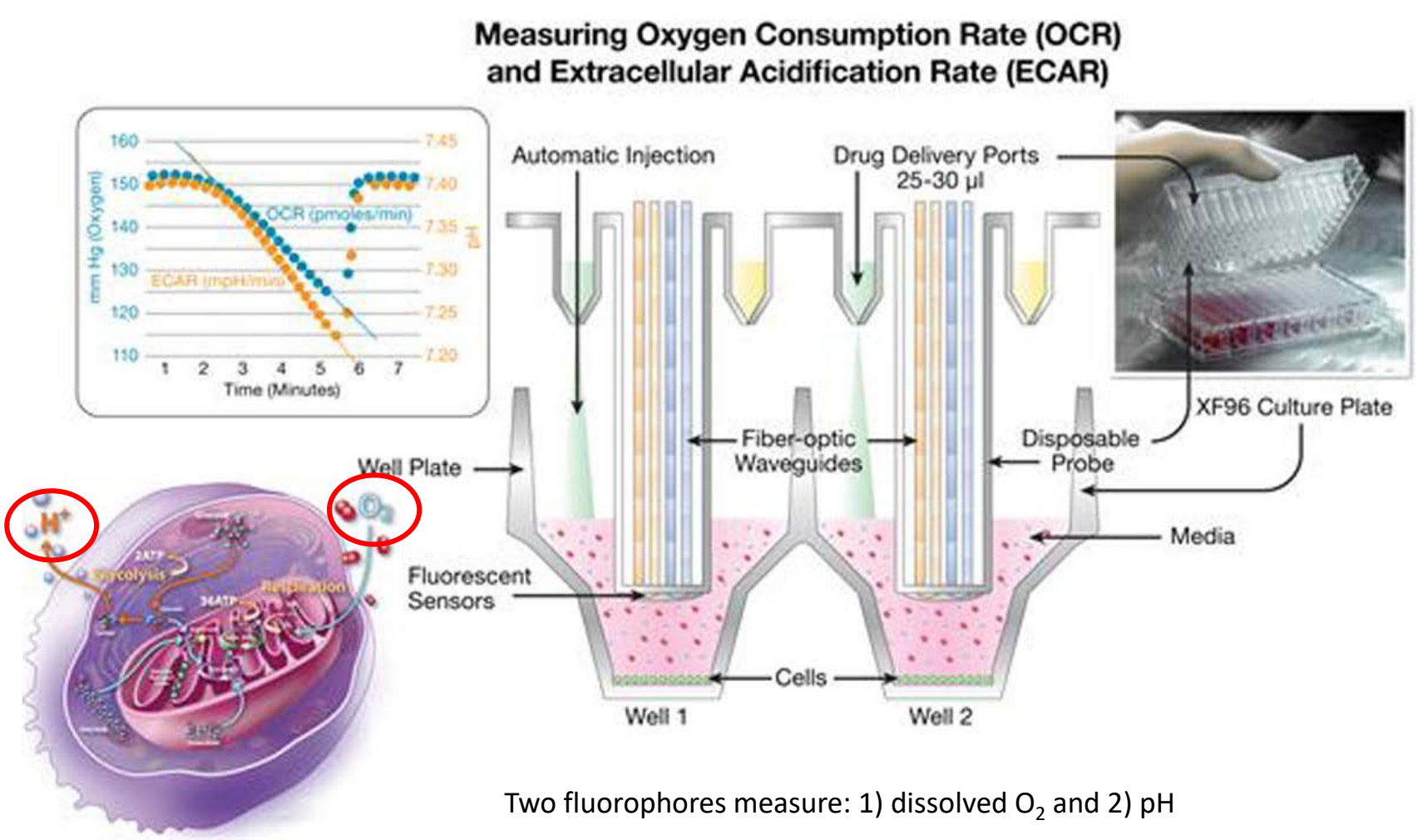
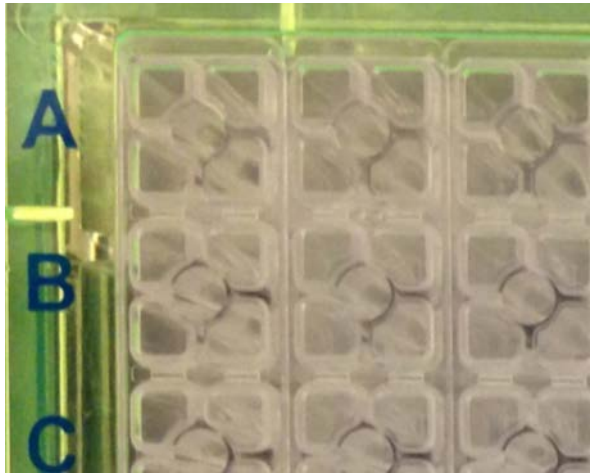
Mitochondria as Targets of Toxicity

- Mitochondria are critical in eukaryotic cells because they generate >90% of the cellular supply of ATP
- Also key to regulating cell cycle/growth, differentiation and apoptosis
- Many chemicals are known to impair mitochondrial function through various mechanisms:
  - Electron transport chain (ETC; Complexes I-IV) inhibition
  - Uncoupling and Ionophores
  - Phosphorylation (Complex V) inhibition
  - Transport inhibition (ATP)
  - Kreb cycle inhibitors
- Disease states associated with genetic mitochondrial disorders provide insights about possible adverse outcomes
- In many of these cases, mitochondria have normal morphology- the impact is functional, not structural
- Current ToxCast/Tox21 high-throughput test methods typically use immortalized/tumor cells (Warburg Effect) cultured in high-glucose medium (Crabtree Effect), and thus are impervious to mitochondrial insult
- ToxCast/Tox21 mitochondrial assays have focused on two endpoints: mitochondrial mass (swelling) and mitochondrial membrane potential (MMP)
- These assay use dye probes to measure structural mitochondrial defects due primarily to membrane changes and are not sensitive to chemicals that impair mitochondrial function through other mechanisms (i.e. ETCi)
- The Seahorse XF Analyzer platform measures mitochondrial function, so it is sensitive to *most* mechanisms of disruption

Seahorse XF Platform

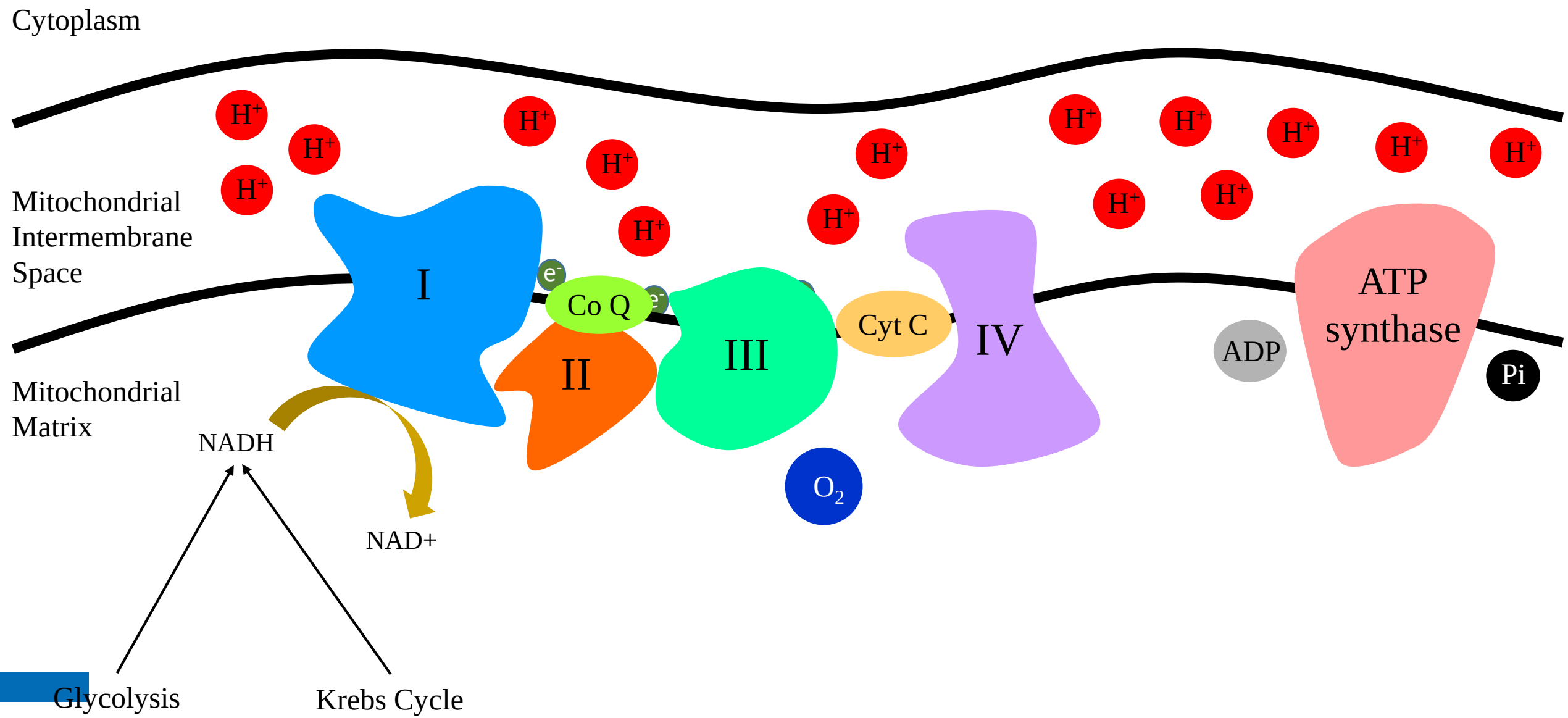




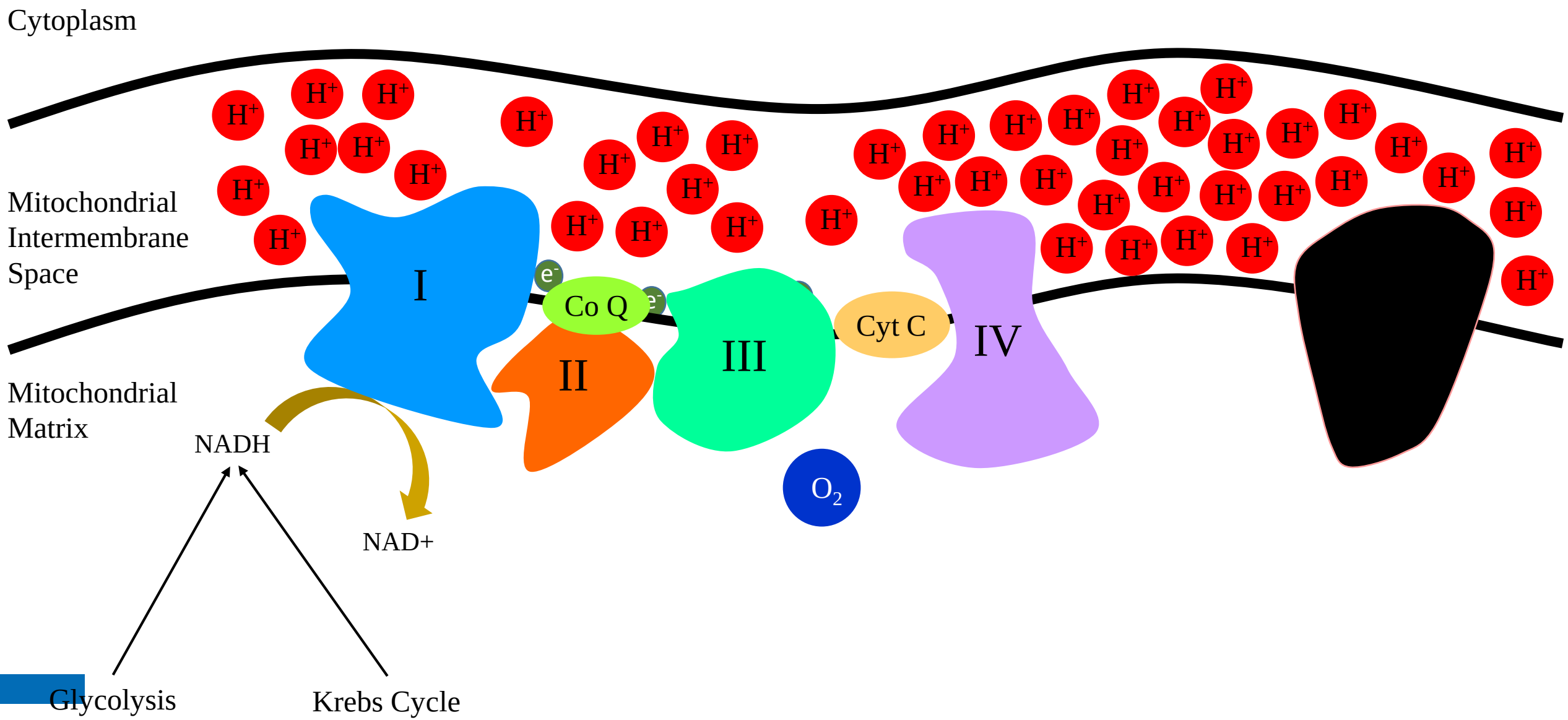
Two fluorophores measure: 1) dissolved O_2 and 2) pH

Direct, non-invasive analyte measurement of oxidative phosphorylation and glycolysis in real time

Basal Respiration



ATP Synthase Inhibition



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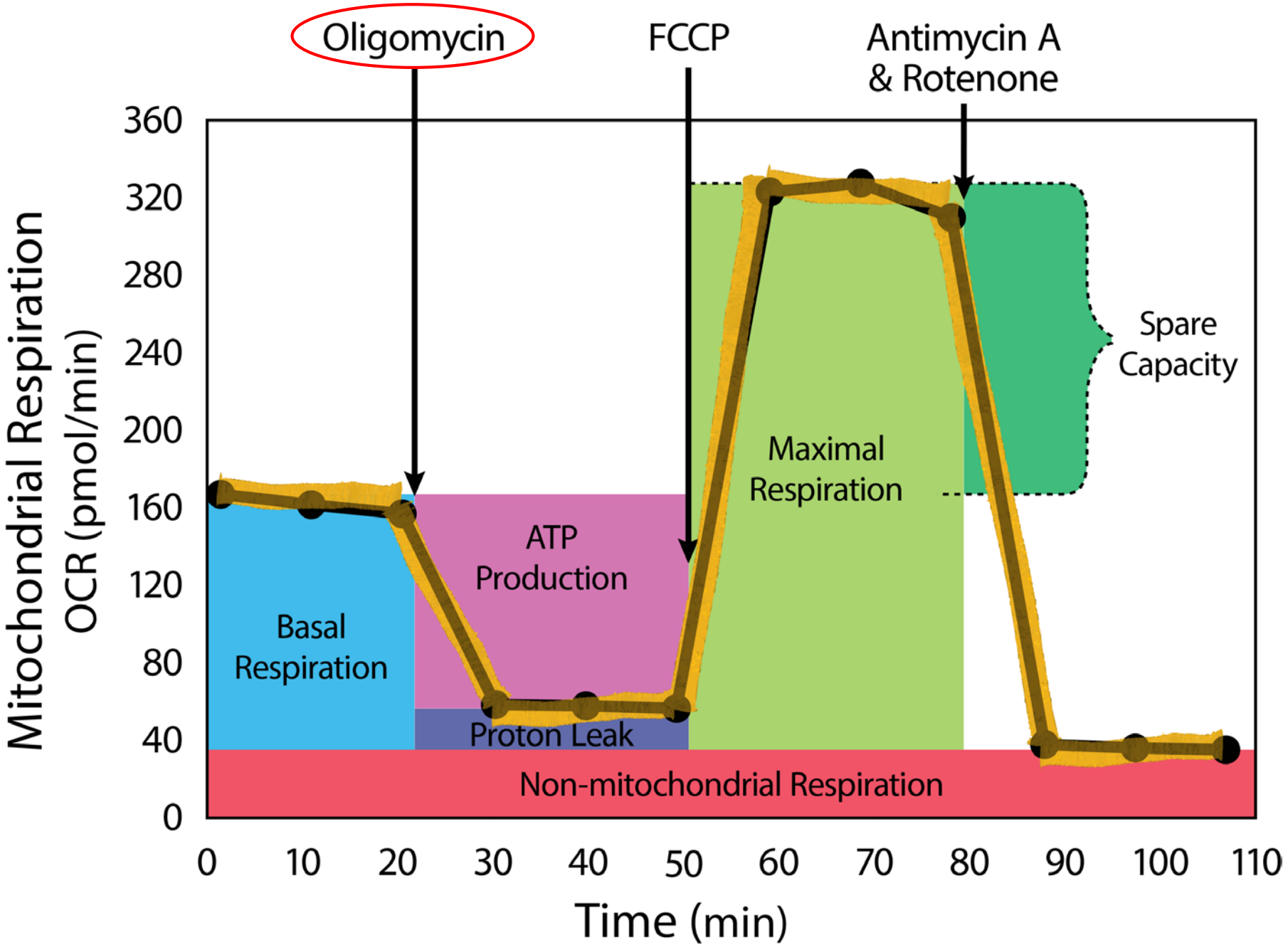
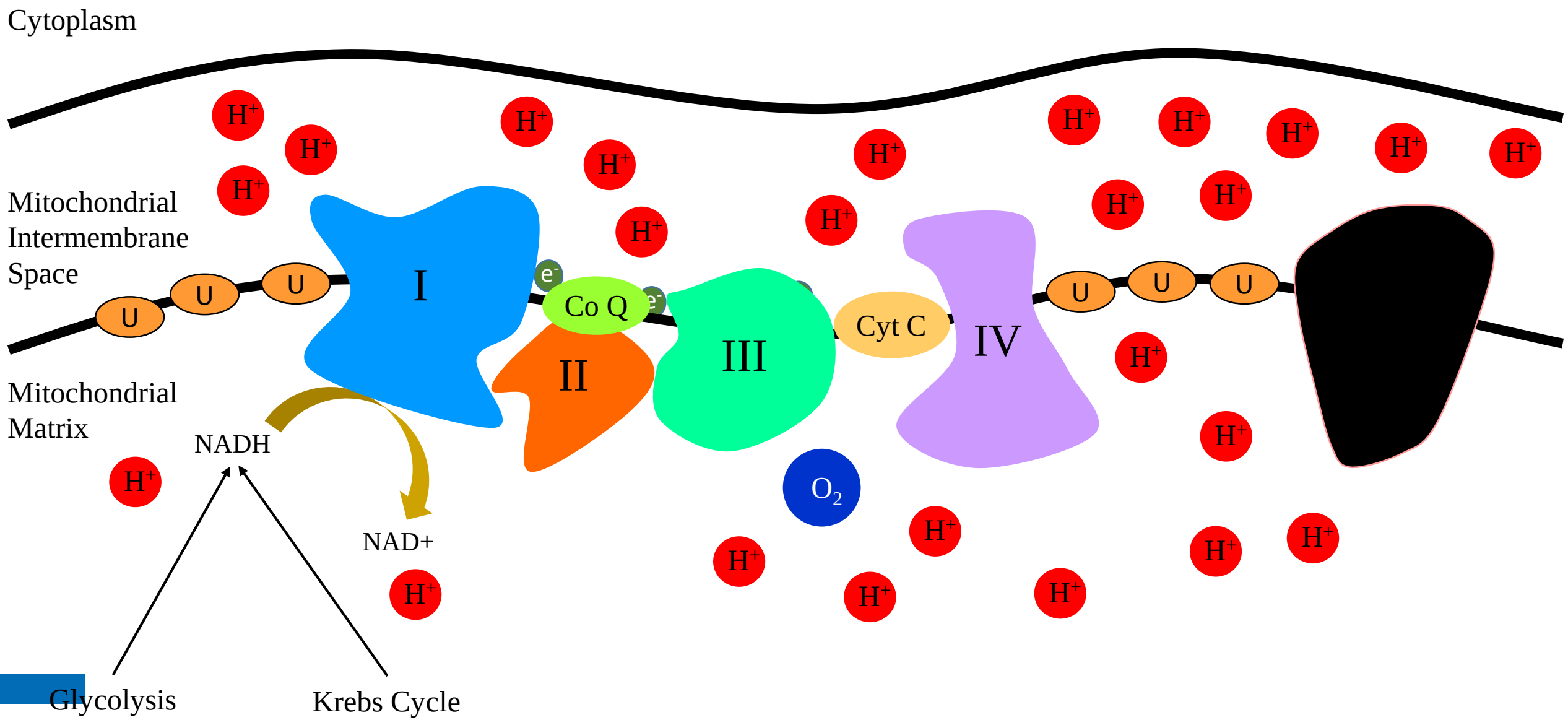
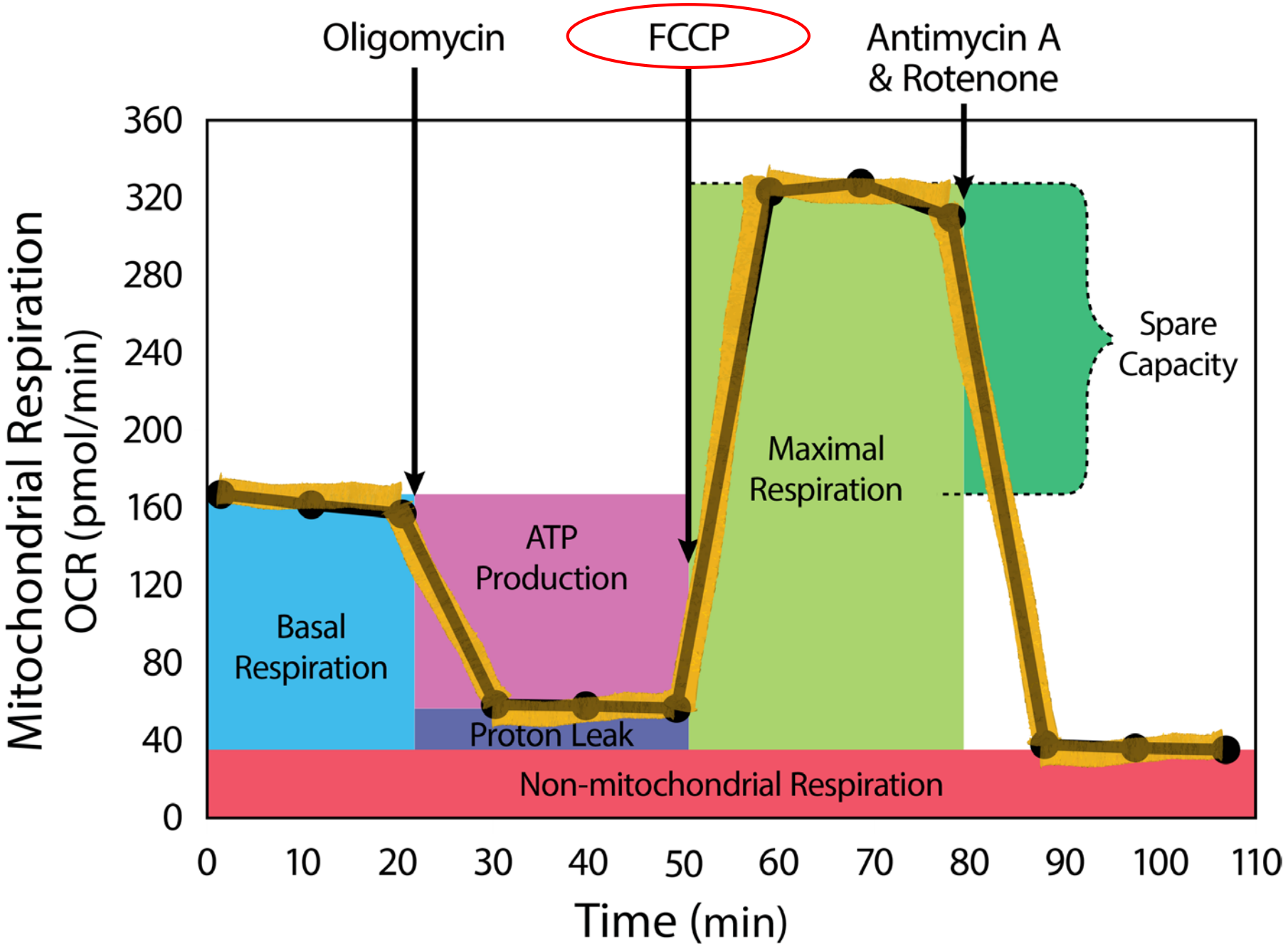
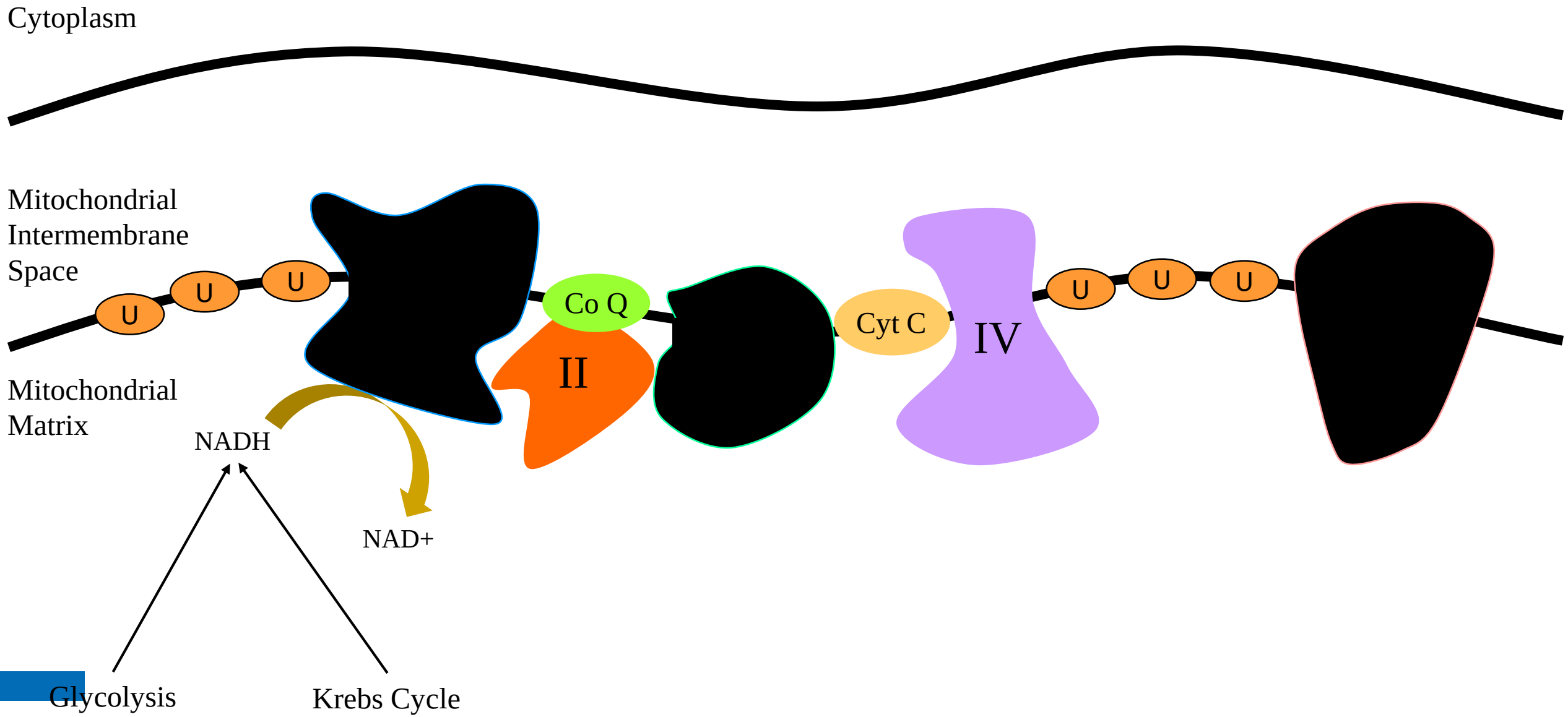


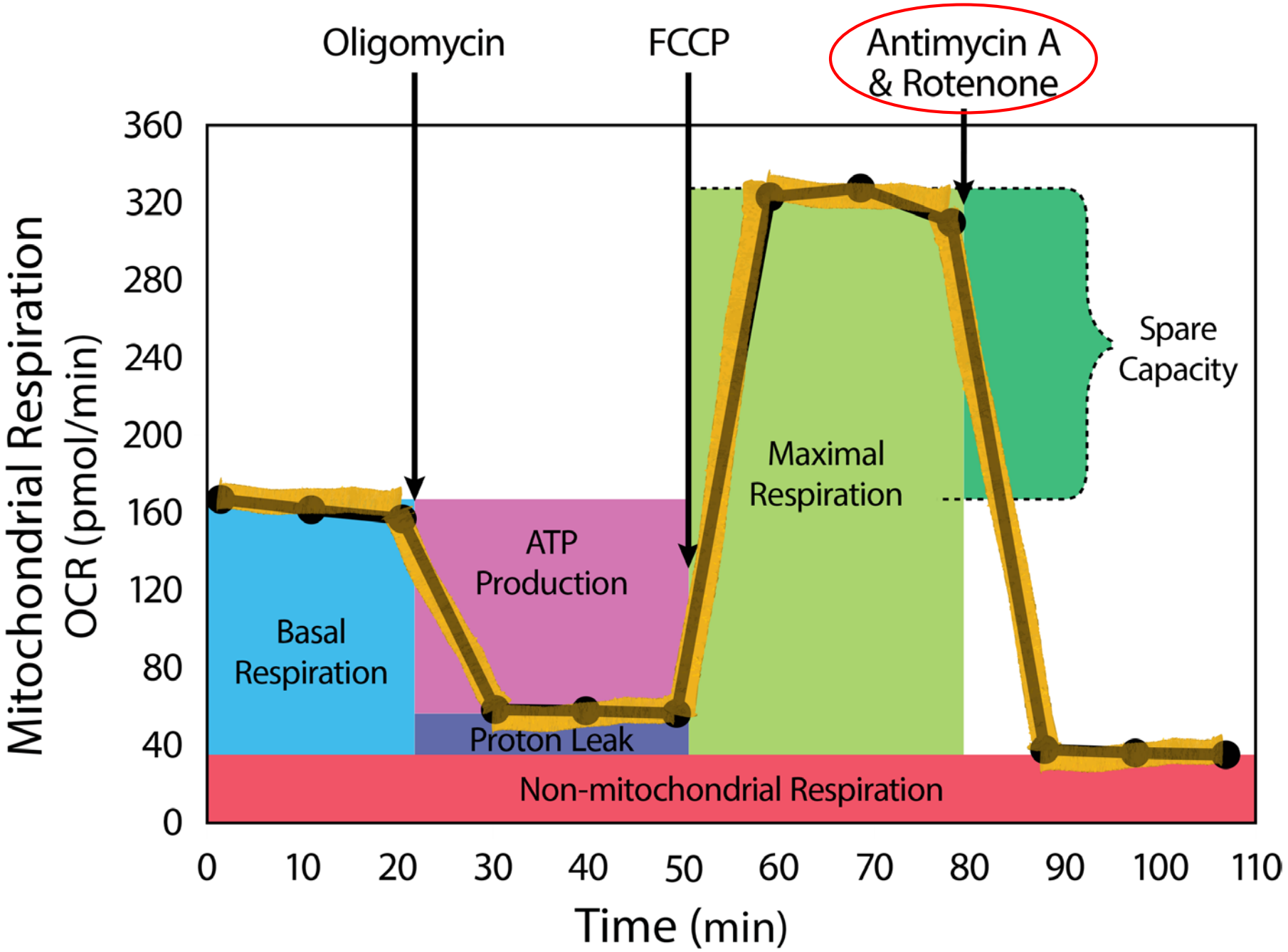
Uncoupling Respiration from ATP Synthesis




Oligomycin
FCCP
Antimycin A
& Rotenone
Mitochondrial Respiration
OCR (pmol/min)
360
320
280
240
200
160
120
80
40
0
Basal
Respiration
ATP
Production
Proton Leak
Maximal
Respiration
Spare
Capacity
Non-mitochondrial Respiration
0
10
20
30
40
50
60
70
80
90
100
110
Time (min)

ETC Inhibition

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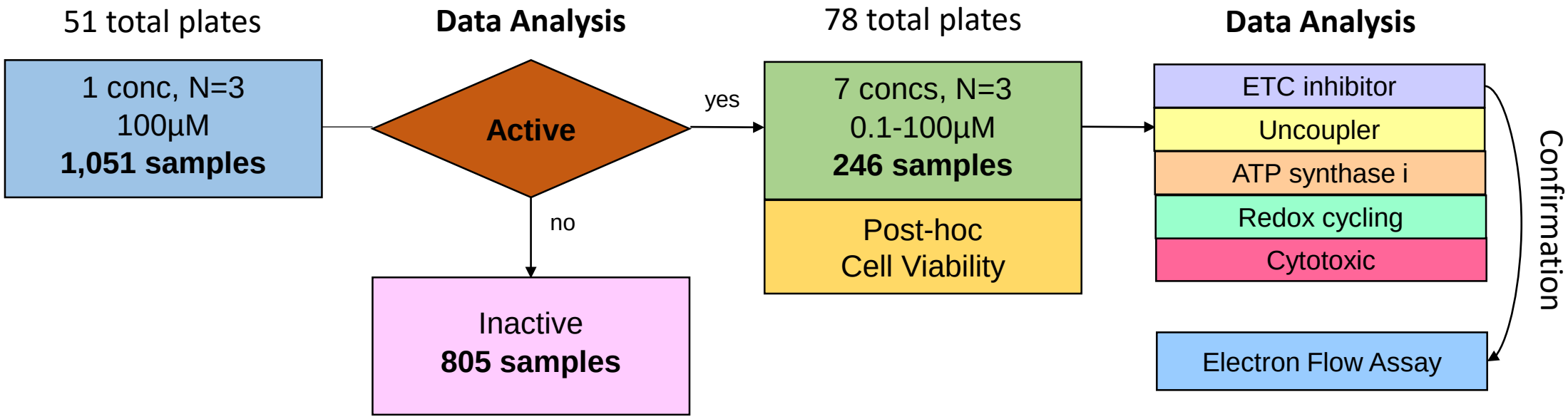
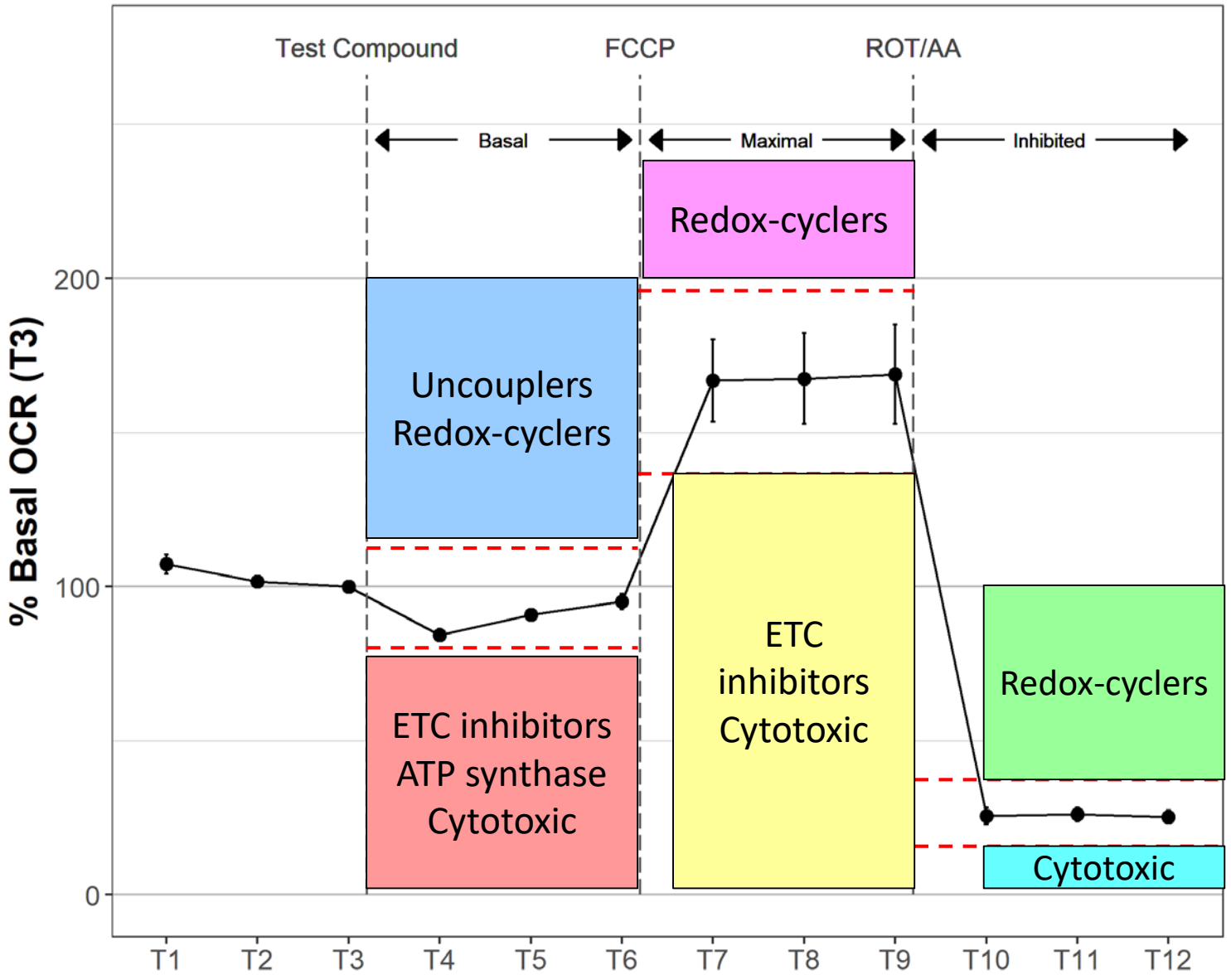
Seahorse Screening Project- Tiered Overview



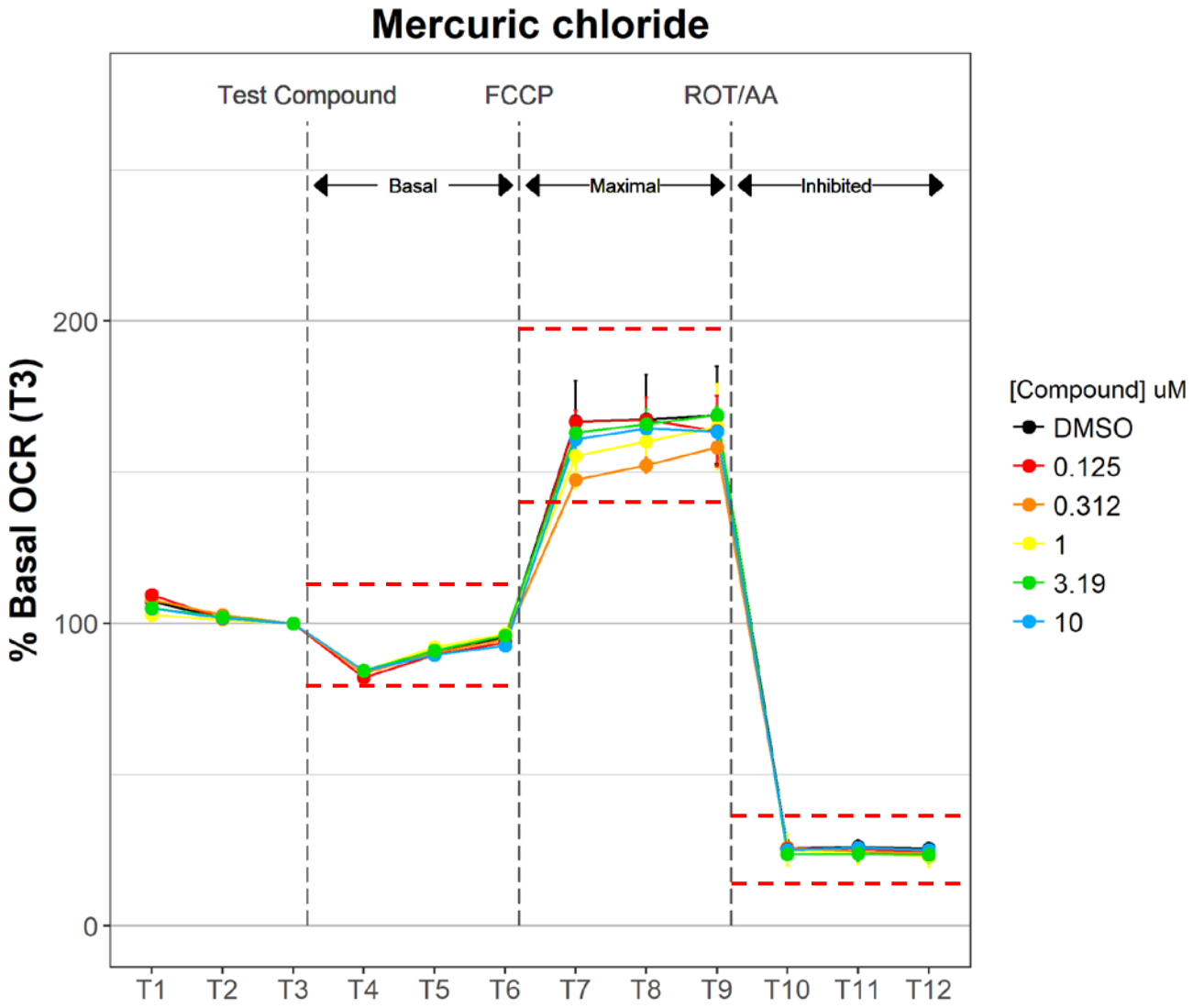
- Human **HepG2** hepatocellular carcinoma cells (50% glycolytic)
- Screening assay comprised of 4 temporal windows separated by 3 sequential injections (reagent additions):
  - Port A: **DMSO** (vehicle), **Fenpyroximate** (ETCi), **2,4-Dinitrophenol** (Uncoupler), **Blinded Test Samples**- (Basal Respiration)
  - Port B: 250nM **FCCP**- (Maximal Respiration)
  - Port C: 1uM **Rotenone** + 1uM **Antimycin A**- (Inhibited Respiration)
- Total assay time is > 75 minutes. Cell viability was measured on cells at conclusion of Seahorse run (mutli-conc plates only)
- Each assay plate accepted/rejected on 5 QC criteria:
  - %CV (DMSO)
  - $rZ'_{OCR\downarrow}$ (DMSO/FENP)
  - $AC50_{FENP}$
  - $rZ'_{OCR\uparrow}$ (DMSO/DNP)
  - $AC50_{DNP}$

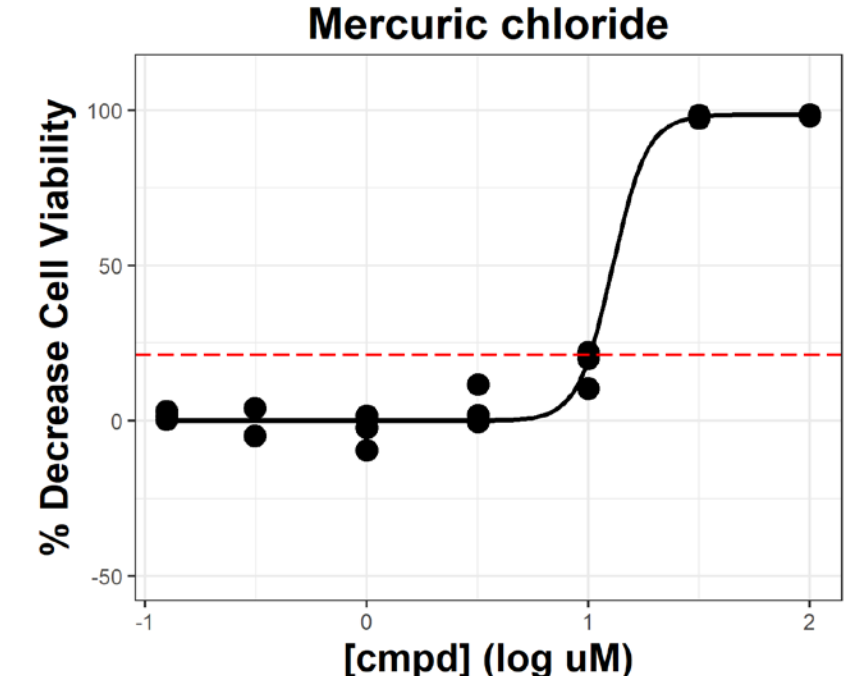
ToxCast Screening Protocol

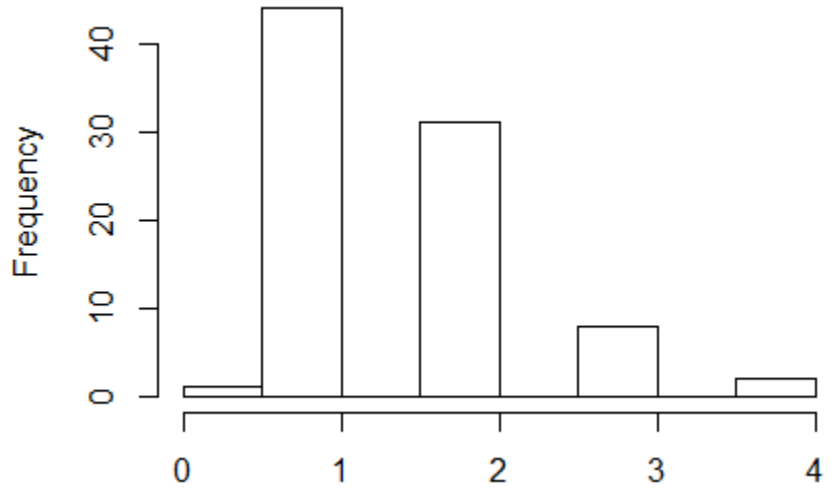


- Replaced Oligomycin injection with test compounds/controls
- Used variation in vehicle (DMSO) response to establish activity thresholds (cut-offs)
- Tracked activity throughout time course of assay to identify potential mitochondrial toxicants (single concentration) and then to confirm activity and define mechanism (concentration-response)
- Anticipated that most actives would decrease OCR

Cytotoxicity Filtering







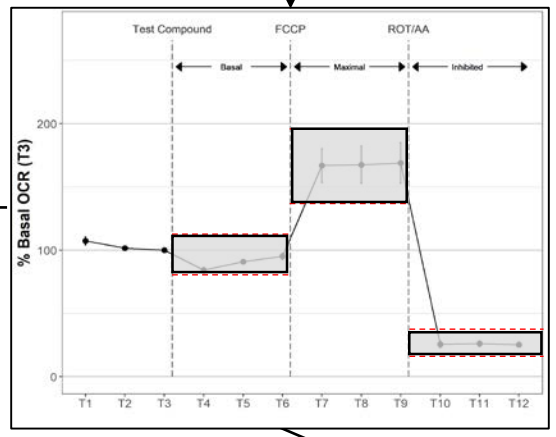
Number of concs filtered by spid

Binning Actives by Mechanism

Conc x sample is cytotoxic?

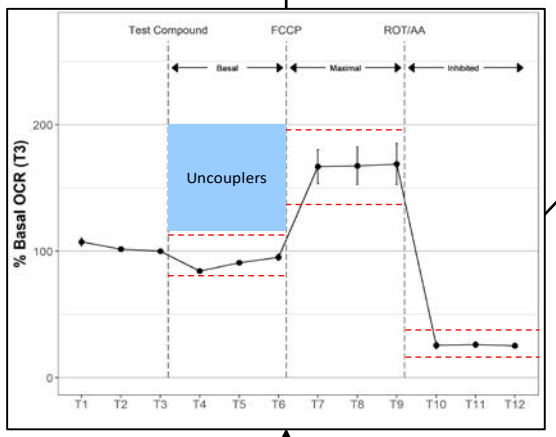
yes → Filter Out

no → Fit each window x direction to TCPL models



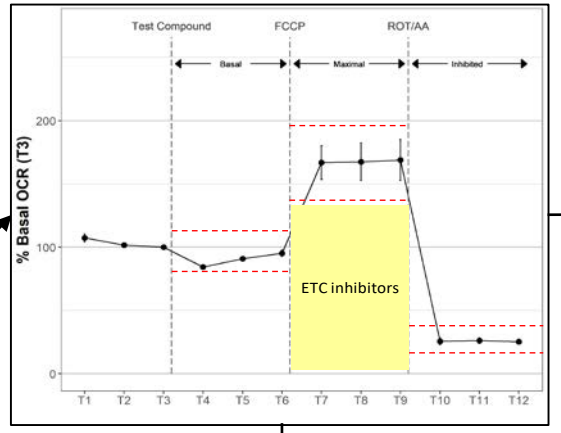
yes → Inactive (57)

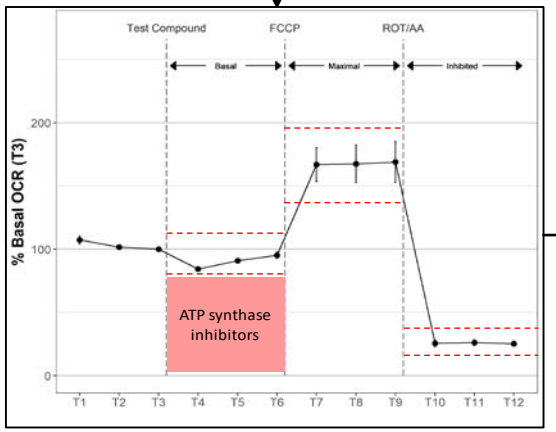
no → Uncoupler (16)



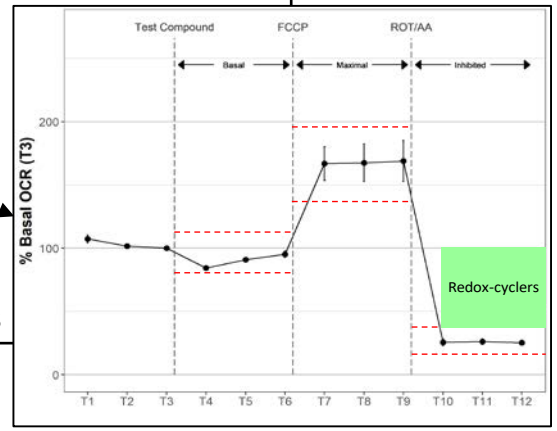
yes → ETC inhibitor (125) → confirm → Electron Flow Assay

no → ATP synthase Inhibitor (40)



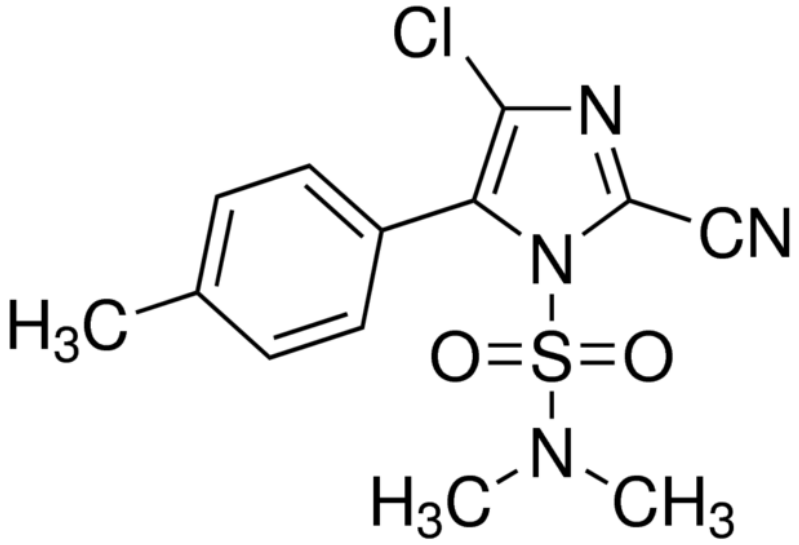


no → Redox Cycler (4)

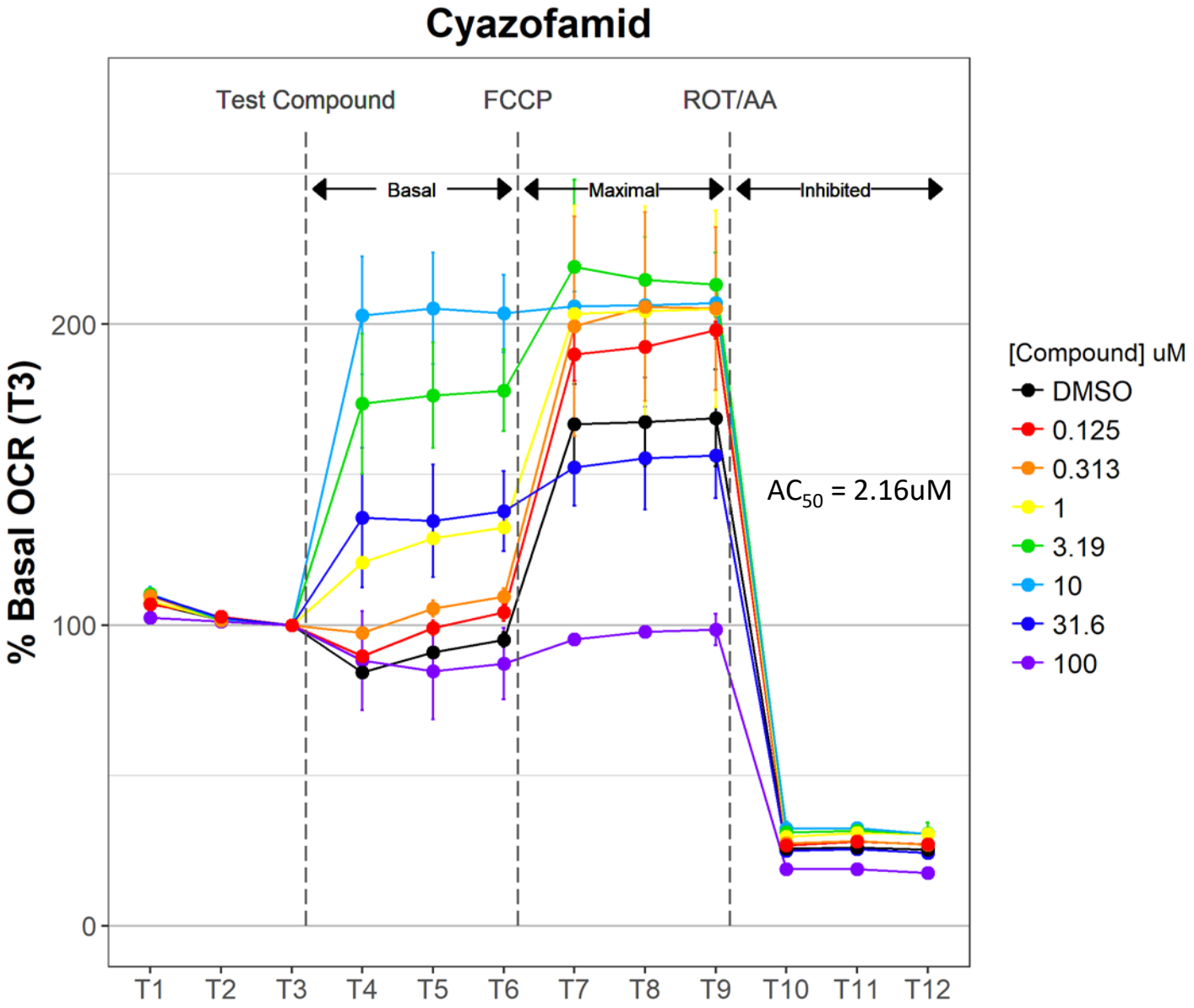


*probe interference (2)

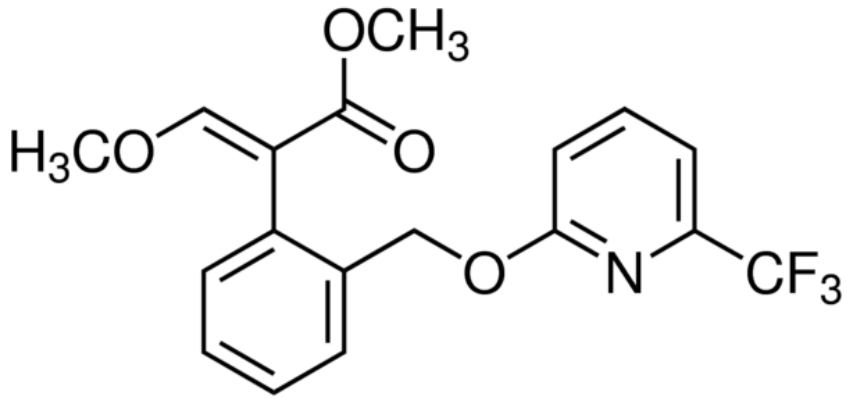
Example: Uncoupler



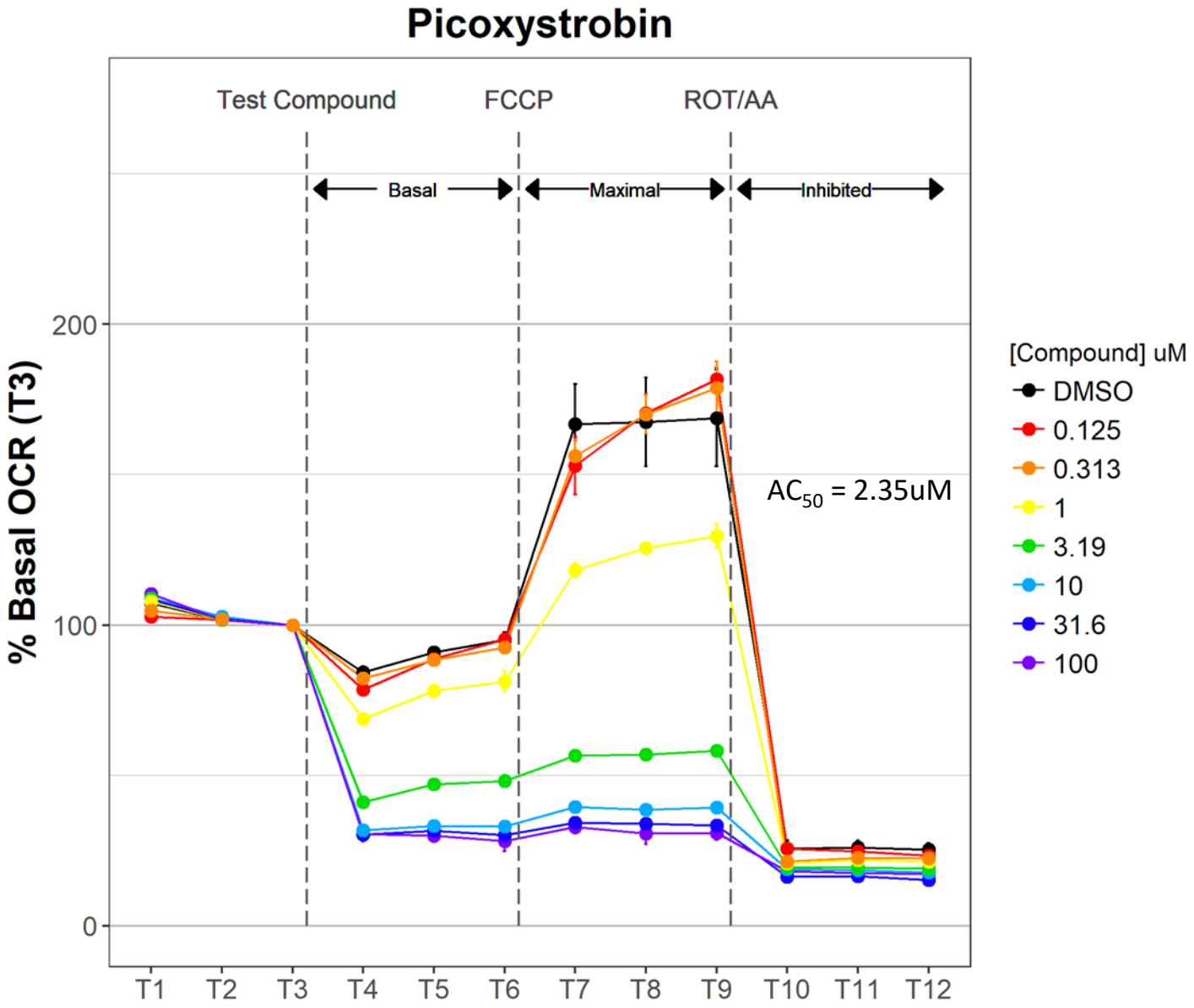
- Used as a systemic fungicide in crop protection products
- Inhibits all developmental stages of fungi by influencing respiration in the mitochondrial cytochrome bc 1 complex (III)



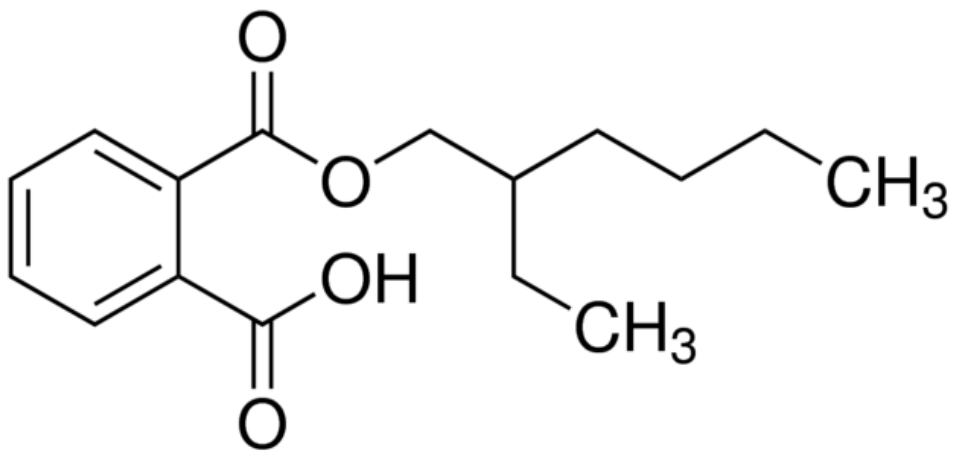
Example: Electron Transport Chain Inhibitor



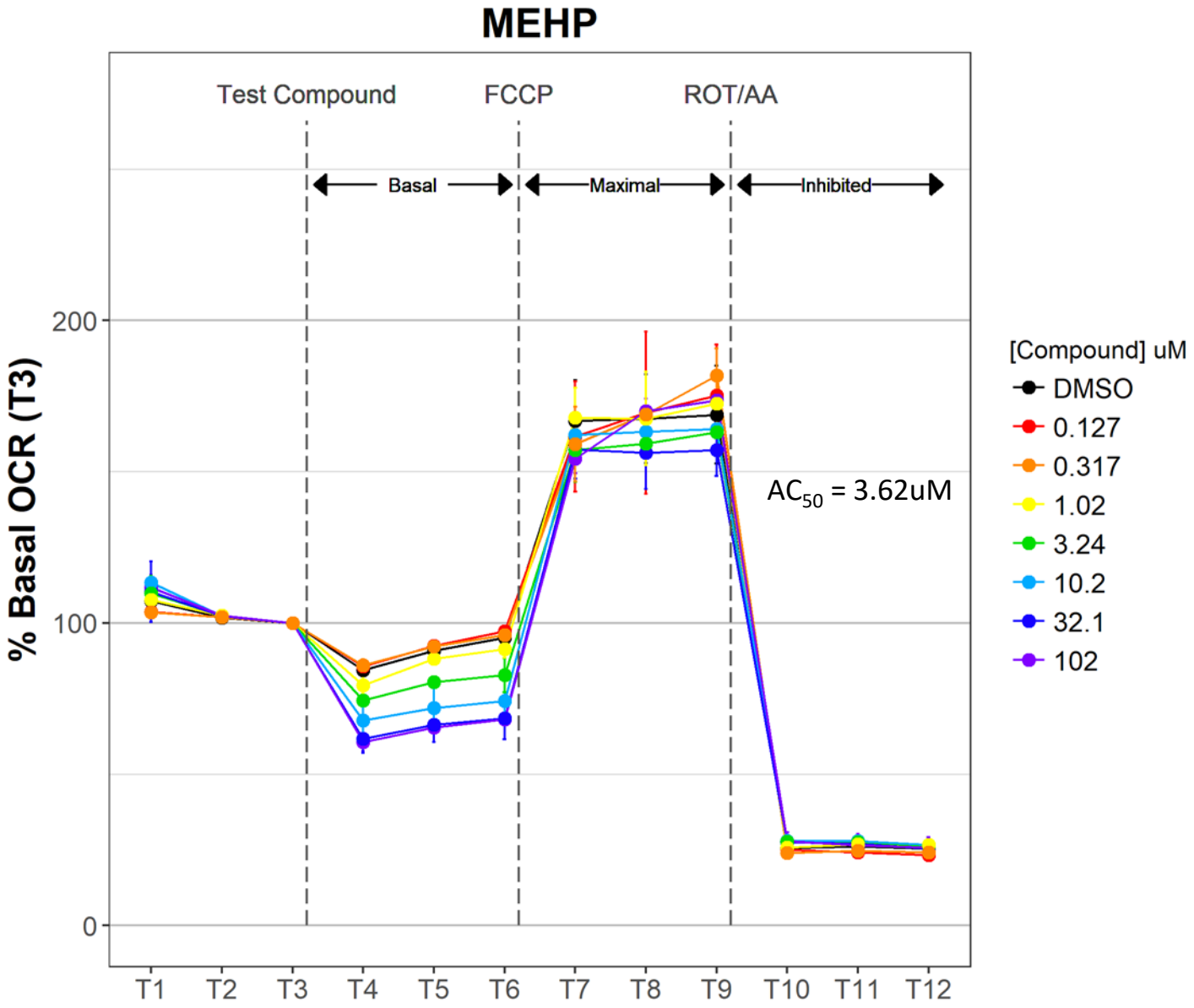
- Used as a fungicide against certain fungal diseases in wheat and barley
- Blocks the electron transport between cytochrome b and cytochrome c1 (complex III)



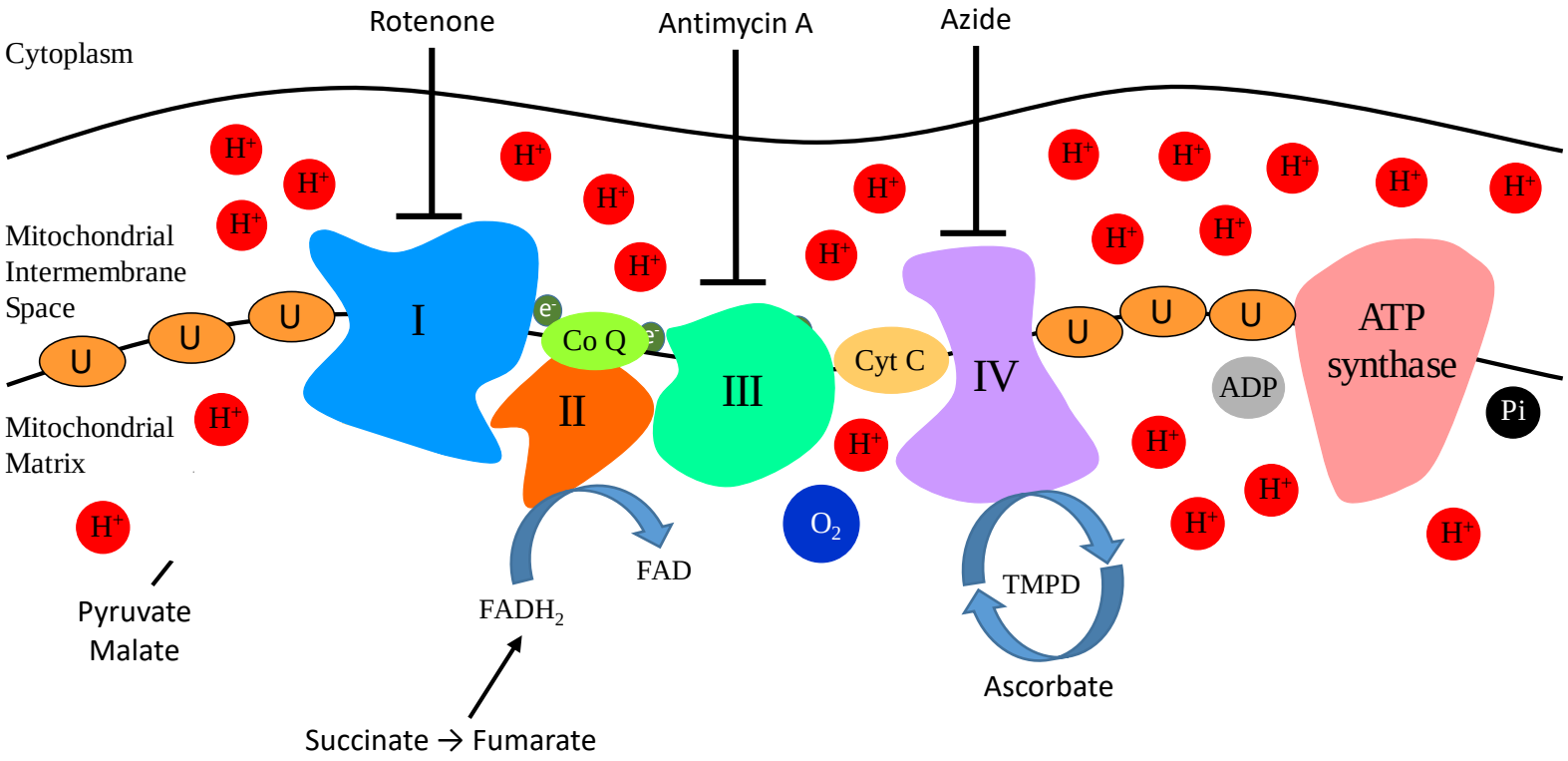
Example: ATP Synthase Inhibitor

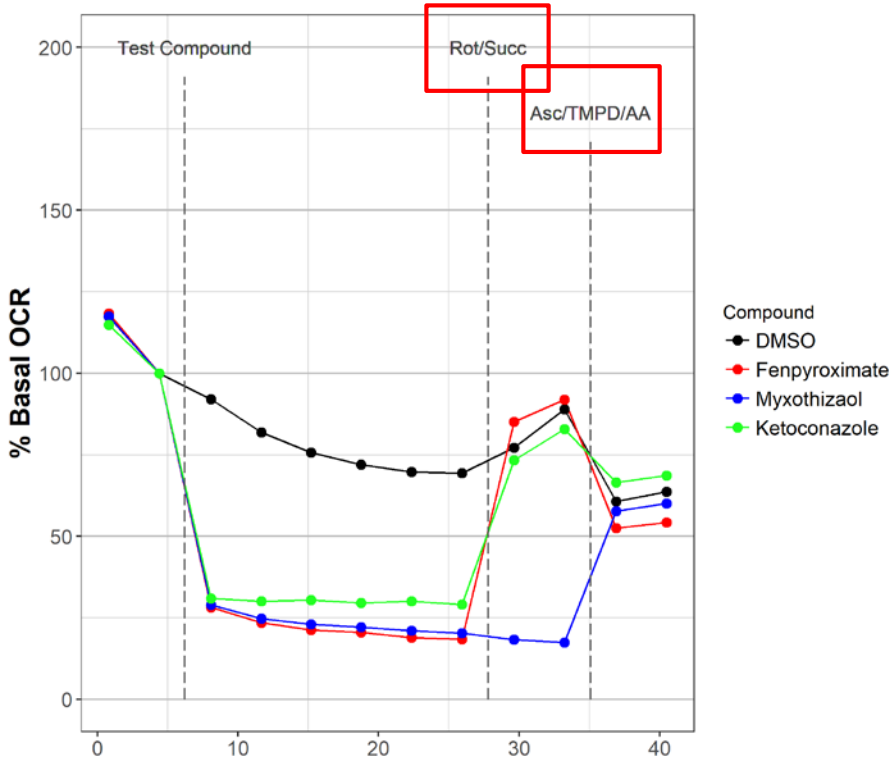


- Metabolite (hydrolysis) of plasticizer di-2-ethylhexyl phthalate (DEHP)
- Suspected androgen disruptor
- No known mitochondrial action



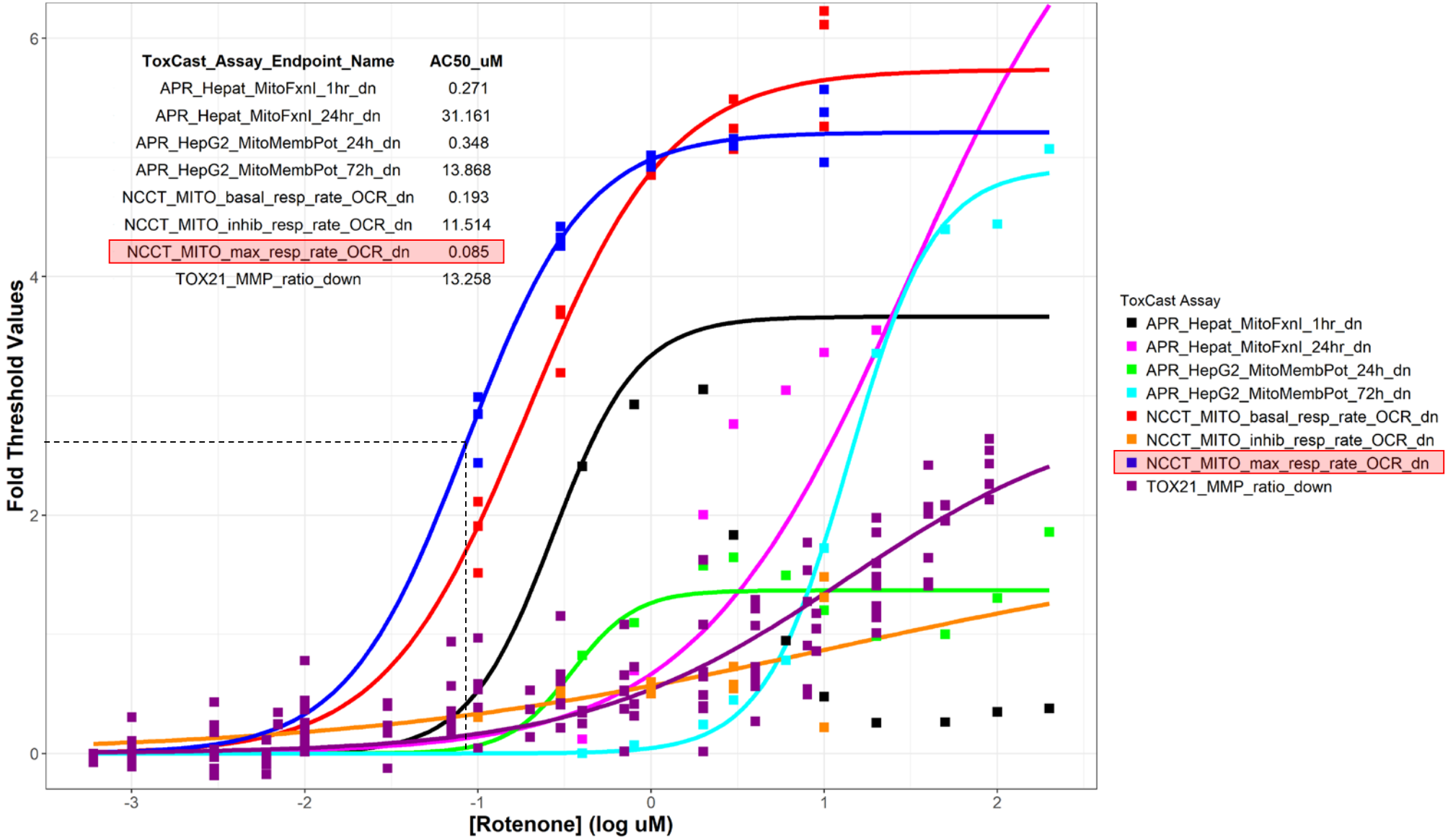
Confirmation: Electron Flow Assay





- Permeabilized HepG2 cells
- Fully uncoupled with FCCP

Enhanced ETC Inhibitor Detection: Rotenone



Acknowledgements

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NHEERL

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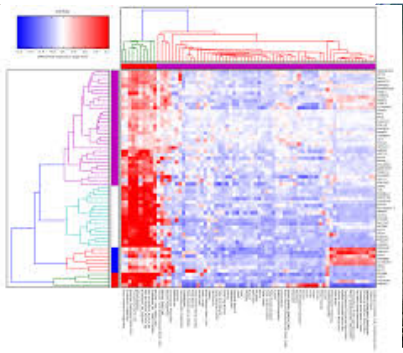

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