# Journal clubs

# Format

Four teams

(3 persons per team)

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Four papers to discuss

(One each day; dates in following slide)

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One paper = two teams

Team 1: present and defend the manuscript

Team 2: find weaknesses, point deficiencies, contest results

(Besides teams involved, everybody will be evaluated)

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(One each day; dates in following slide)

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Team 1: present and defend the manuscript

Team 2: find weaknesses, point deficiencies, contest results

(Besides teams involved, everybody will be evaluated)

1/3 of exam evaluation

(10 points out of 30)

# Dates

## April 4th and April 9th

AC will lecture about most relevant technologies in metabolism

## April 10th and April 29th

Team building, paper selection, slide preparation, discussion

May 2nd: paper X

May 7th: paper Y

May 9th: paper W

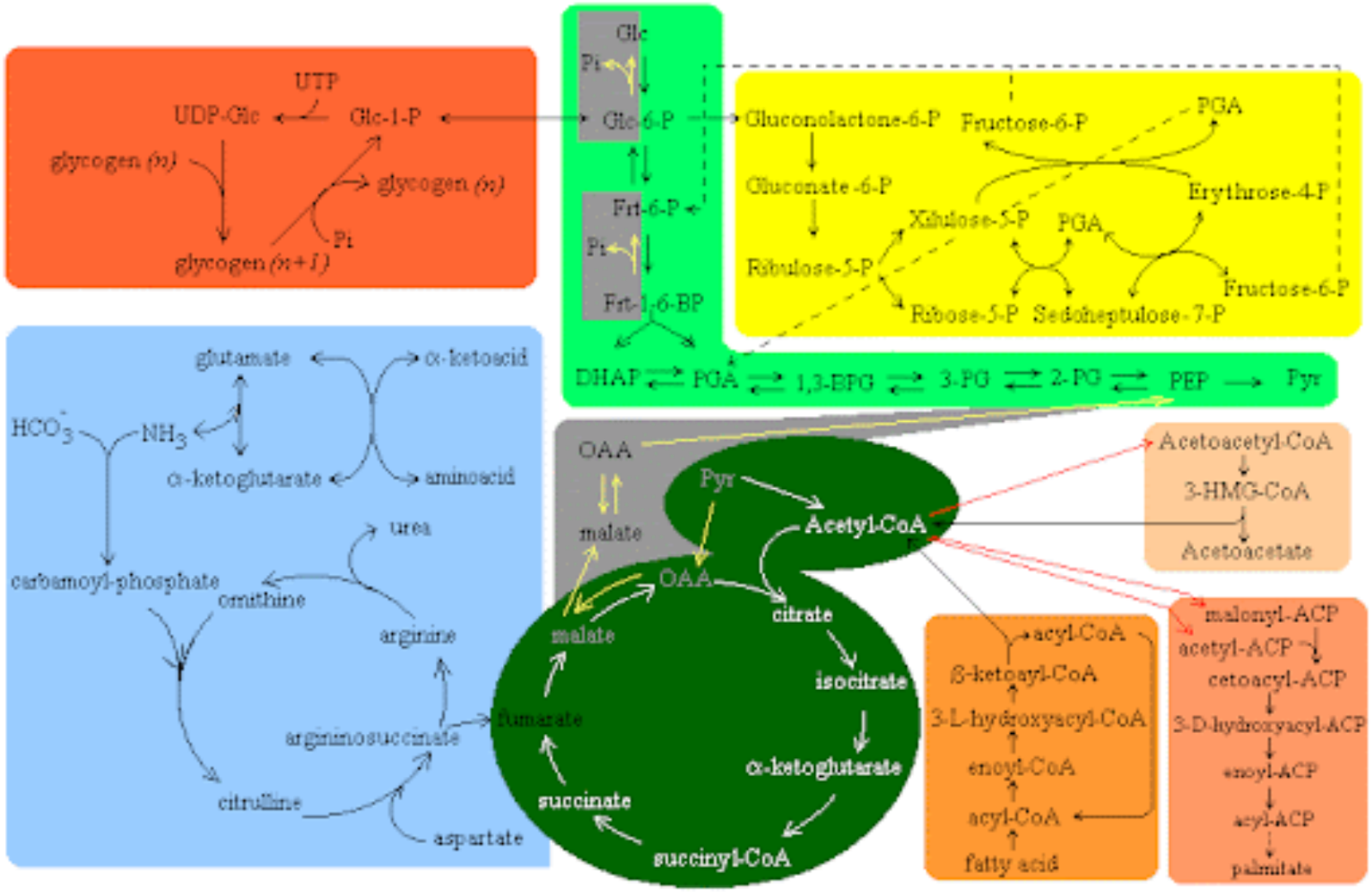
May 14th: paper Z

# Papers


1. Banh et al, Cell, 2020 - PMID:33142117
2. Diaz-Cuadros et al, Nature, 2023 - PMID:36599986
3. Ravichandran et al, Cancer Discov, 2022 - PMID:35771494
4. Baixauli et al, Nature, 2022 - PMID:36171294

# Methods in Metabolism

# What is the difference with Biochemistry I, II, III, ….?



# What is the difference with Biochemistry I, II, III, ….?

# Metabolic Metro Map

## Carbohydrate Metabolism

Ascorbate (Vitamin C)
Sugar Acids
Double/Multiple Sugars & Glycans
Glyco-genolysis
Simple Sugars
Inositol-P
Amino Sugars & Sialic Acids
Glyco-genesis
Nucleotide Sugars
Hexose-P
Gluconeo-genesis
Glycolysis
Triose-P
Glycerol
Pentose Phosphate Pathway
Pentose-P
Tetrose-P
Glyoxylate
Carbon Fixation
← $CO_2$
Photo-respiration
$O_2$ → $H_2O_2$
P-glycerate
Direct / C4 / CAM Carbon Intake
$CO_2$
$O_2$
$H_2O$ → $O_2$
Light
NADPH, ATP
Light Reactions
Photosystems
Chloroplast
Peroxisome
Cytosol

## Photosynthesis

## Cellular Respiration

Homoserine Group & Lysine
Aspartate Group
P-glycerates
Serine Group
Malate
Oxalo-acetate
Citrate Shuttle
Pyruvate
Alanine
Lactate
Fermentation
Polyketides
Pyruvate Decarb-oxylation
Acetyl-CoA
Branched Amino Acids
Citrate
Citric Acid Cycle
$CO_2$ ←
Succinyl-CoA
α-Keto-glutarate
Ketogenic & Glucogenic Amino Acids
Ketolysis
Ketogenesis
Propionyl-CoA
NADH, $FADH_2$
Ketone Bodies
Mitochondrion
$O_2$
$O_2$ → $H_2O$
ATP & Heat
NADH, $FADH_2$
Oxidative Phosphorylation
Respiratory Chain
$CO_2$
Mitochondrion
Urea Cycle
Amino Acid Deamination
$NH_3$ → Urea
Creatine & Polyamines
Arginine
Urea
feeders to Gluconeogenesis
Glutamate Group & Proline
δ-ALA
Succinate
Bile Pigments
Hemes
Chlorophylls
Cobalamins (Vitamin $B_{12}$)
Chloroplast
Glyoxylate Cycle
Acetyl-CoA
Peroxisomal Beta Oxidation
Peroxisome

## Amino Acid Metabolism

## Vitamin & Cofactor Metabolism

## Nucleotide & Protein Metabolism

Ribosome
Lysosome/Proteasome
Various Vitamin B's
Neurotransmitters & Thyroid hormones
Proteolysis
Glycosyl-ation
Golgi Body
Amino Acids
Translation
Proteins
Glycoproteins & Proteoglycans
Transciption & Replication
Nucleus
Pentose-P
PRPP
Nucleotides
Nucleic Acids
Cofactors
Vitamins & Minerals
Shikimate Pathway
Aromatic Amino Acids & Histidine
Shikimate
Antioxidants
Quinones (Vitamin K) & Tocopherols (Vitamin E)
Plastid
MEP Pathway
MEP
MVA Pathway
MVA
NADPH
Terpenoid Backbones
Terpenoids & Carotenoids (Vitamin A)
Retinoids (Vitamin A)
Steroidogenesis
Cholesterol
Bile Acids
Calciferols (Vitamin D)
Steroids
Endoplasmic Reticulum
Fatty Acid Synthesis
Fatty Acid Elongation
Endo-cannabinoids
Glycero-phospholipids
Glyco-sphingolipids
Golgi Body
Glycerolipids
Sphingolipids
Lipogenesis
Acyl-CoA
Waxes
Beta Oxidation
Lipolysis
Eicosanoids
Fatty Acids
Polyunsaturated Fatty Acids

## Lipid Metabolism

## Messengers

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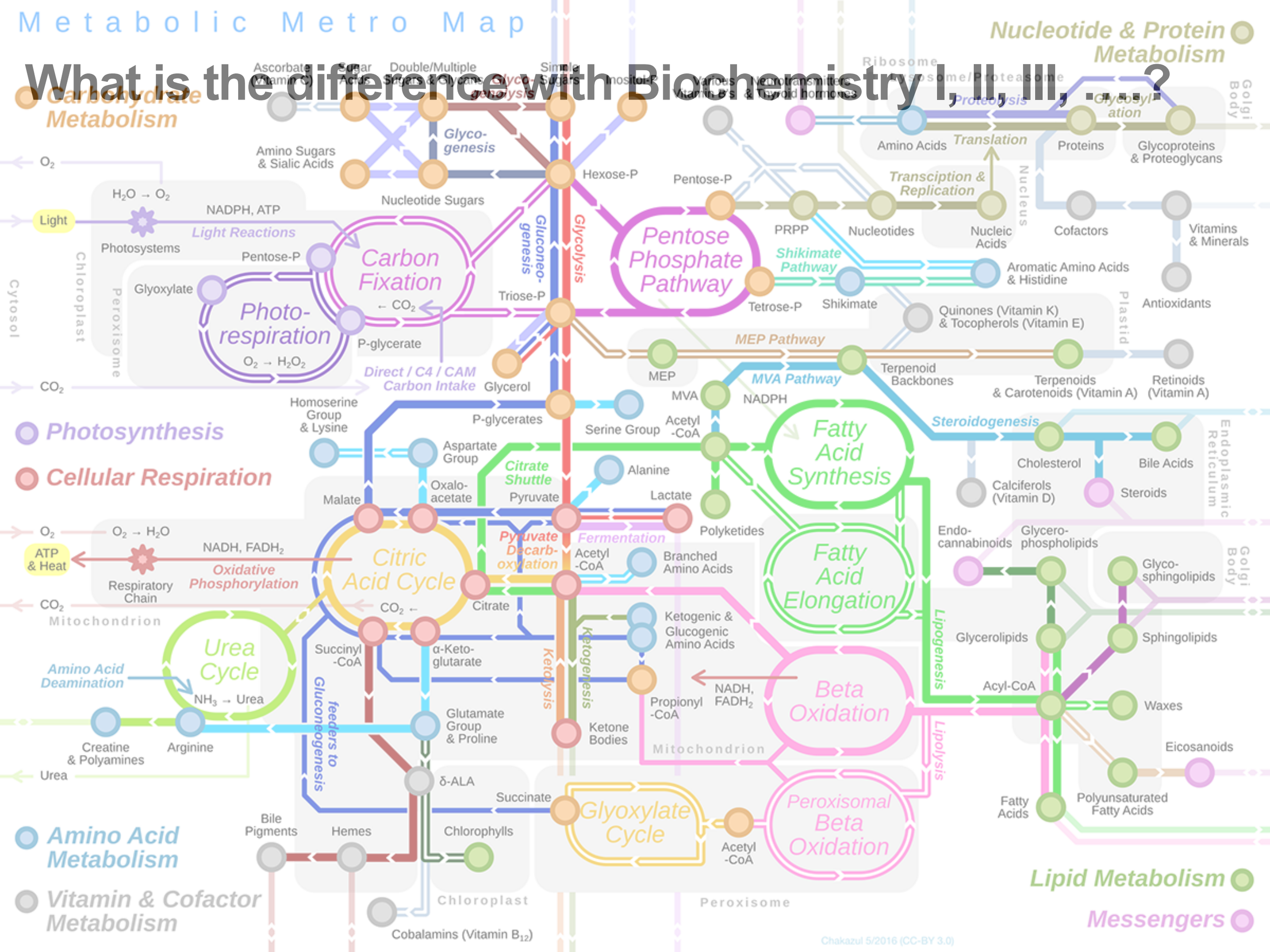


# Metabolic assays seek to:

Measure metabolites and macromolecules

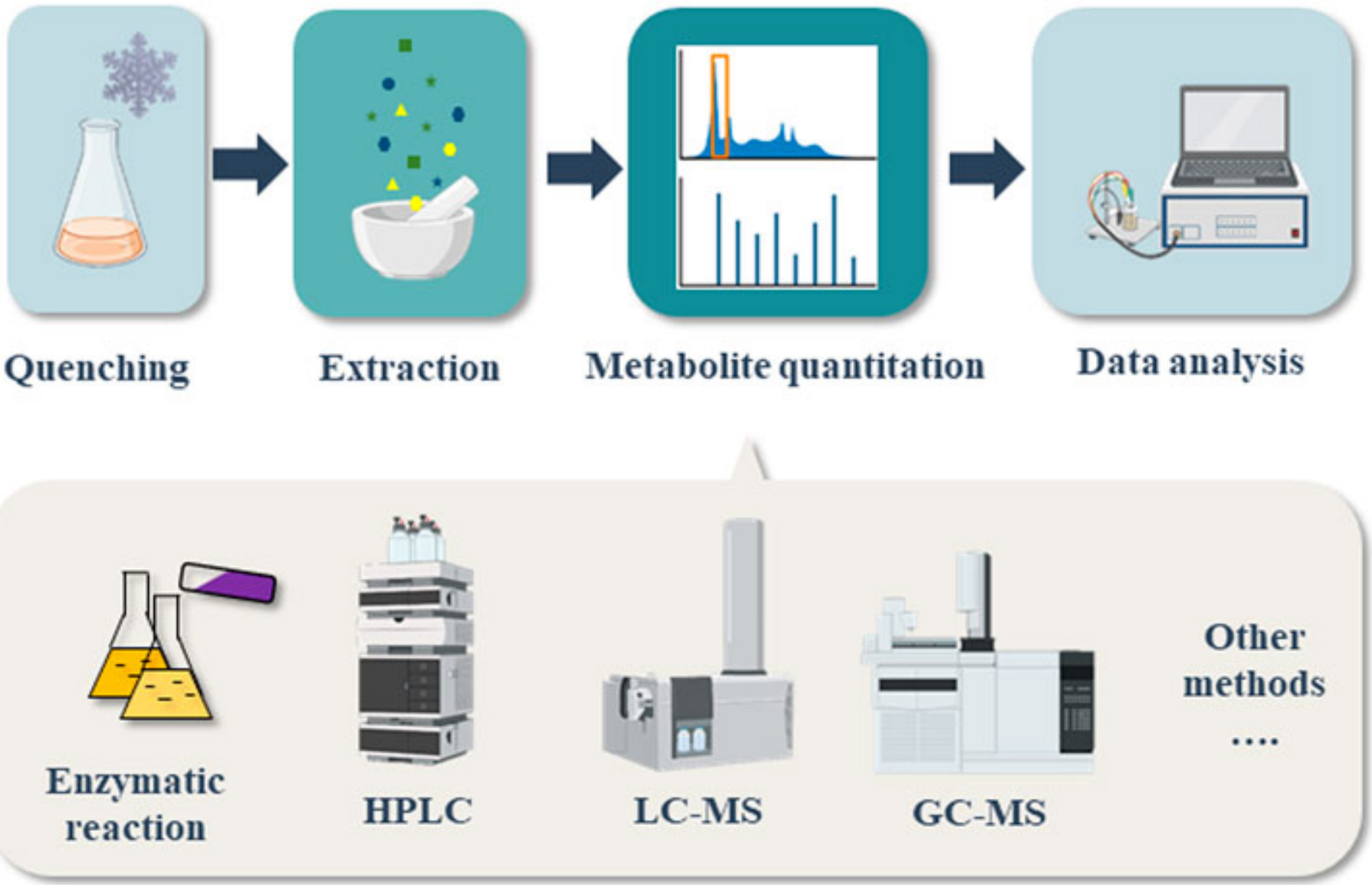
Measure energy equivalents and co-facors

Measure enzymatic activities

Measure activity of metabolic organelles

# Measure metabolites and macromolecules

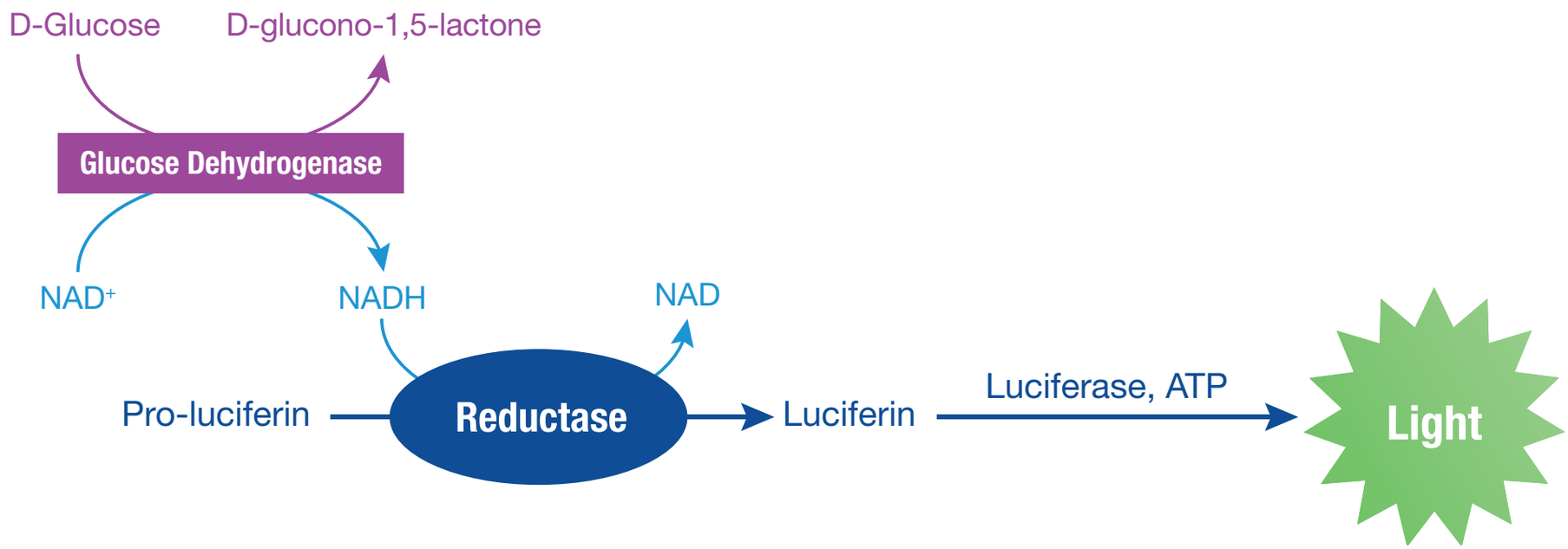
Quantitative measures rely on biochemical analytical approaches:




*Wang & Luo,* ***Front Bioeng Biotech****, 2022*

# Enzymatic assays

Couples the oxidation/reduction of a specific metabolite with the generation of a co-factor required for the production of a quantifiable output (luminescence, fluorescence, color).



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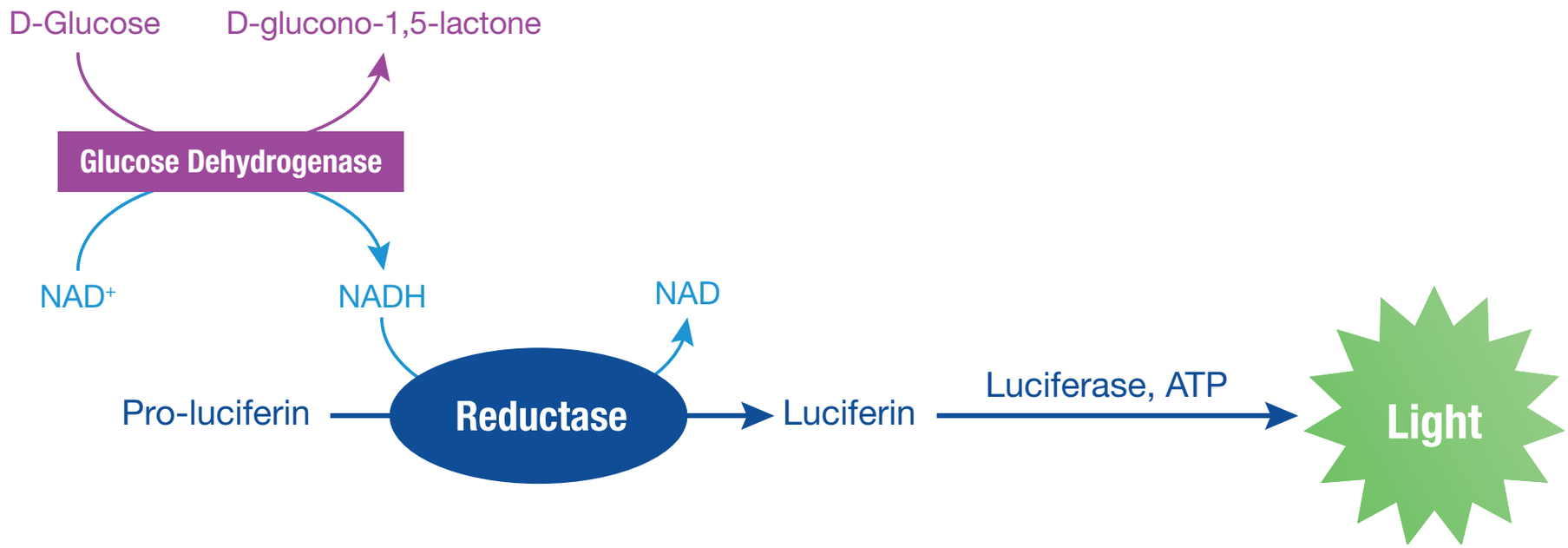


**Requires:**

- De-proteinization of samples
- Specific enzyme (recombinant)
- NAD(P)/H degradation
- Proportionality

# Enzymatic assays

Couples the oxidation/reduction of a specific metabolite with the generation of a co-factor required for the production of a quantifiable output (luminescence, fluorescence, color).

**Pros:**

- Easy and quick
- Accessible

**Cons:**

- Poorly sensitive
- Poor linearity

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- **ONE METABOLITE AT THE TIME**

# Enzymatic activity

Switching on/off the genes that encode specific enzymes or regulating their activity contributes to control/regulate cell metabolism

- Allosteric regulation
- Availability of co-factors
- Endogenous activators/inhibitors
- Feedback mechanisms
- **Compartmentalization**

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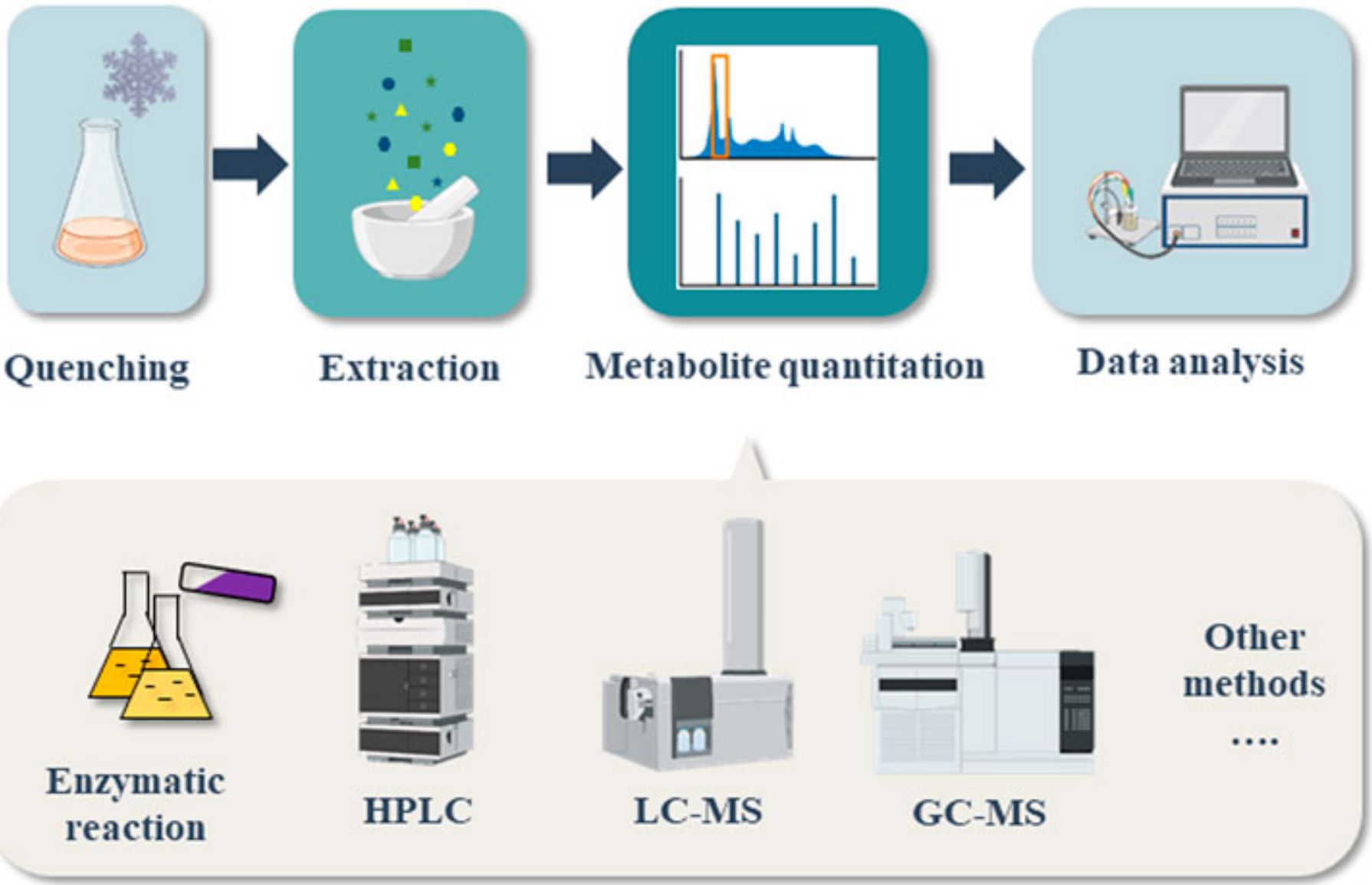
# Measure metabolites and macromolecules

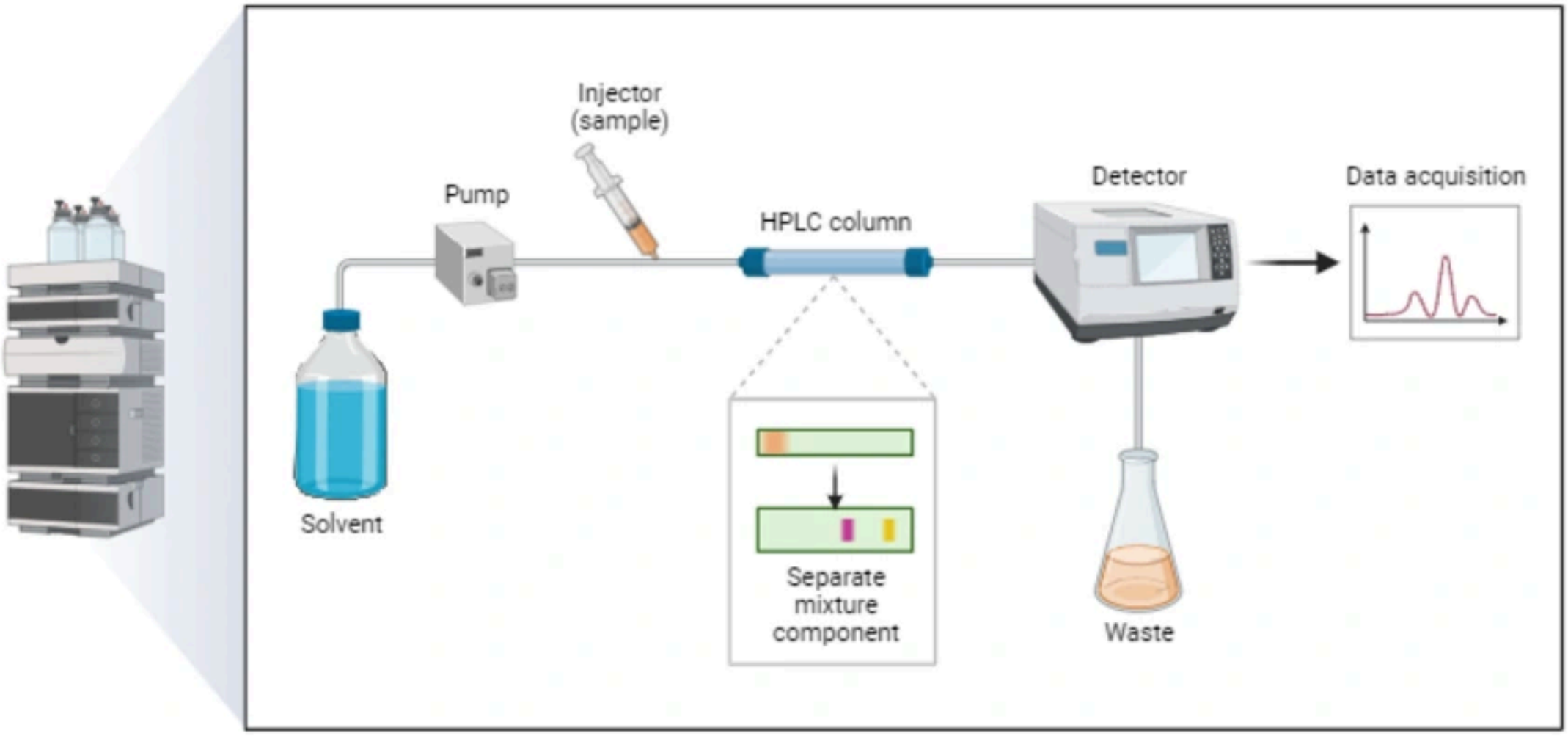
Quantitative measures rely on biochemical analytical approaches:




*Wang & Luo,* ***Front Bioeng Biotech****, 2022*

# High-performance liquid chromatography (HPLC)

It allows the separation, identification, and quantification of each component in a mixture. The fundamental concept behind HPLC originates from column chromatography, a process in which a mixture is passed through a stationary phase, and the various components separate out due to their multiple interactions with the stationary phase.



# High-performance liquid chromatography (HPLC)

The sample is carried by the mobile phase and driven through the column after being inserted into the HPLC system via the injector. The different components experience varying degrees of retention as the mixture interacts with the stationary phase, leading to their separation down the column.
Depending on the type of analytes, the eluted components are next determined using a suitable detector, such as ultraviolet (UV) or visible light detectors, detectors of refractive index, or mass spectrometers.

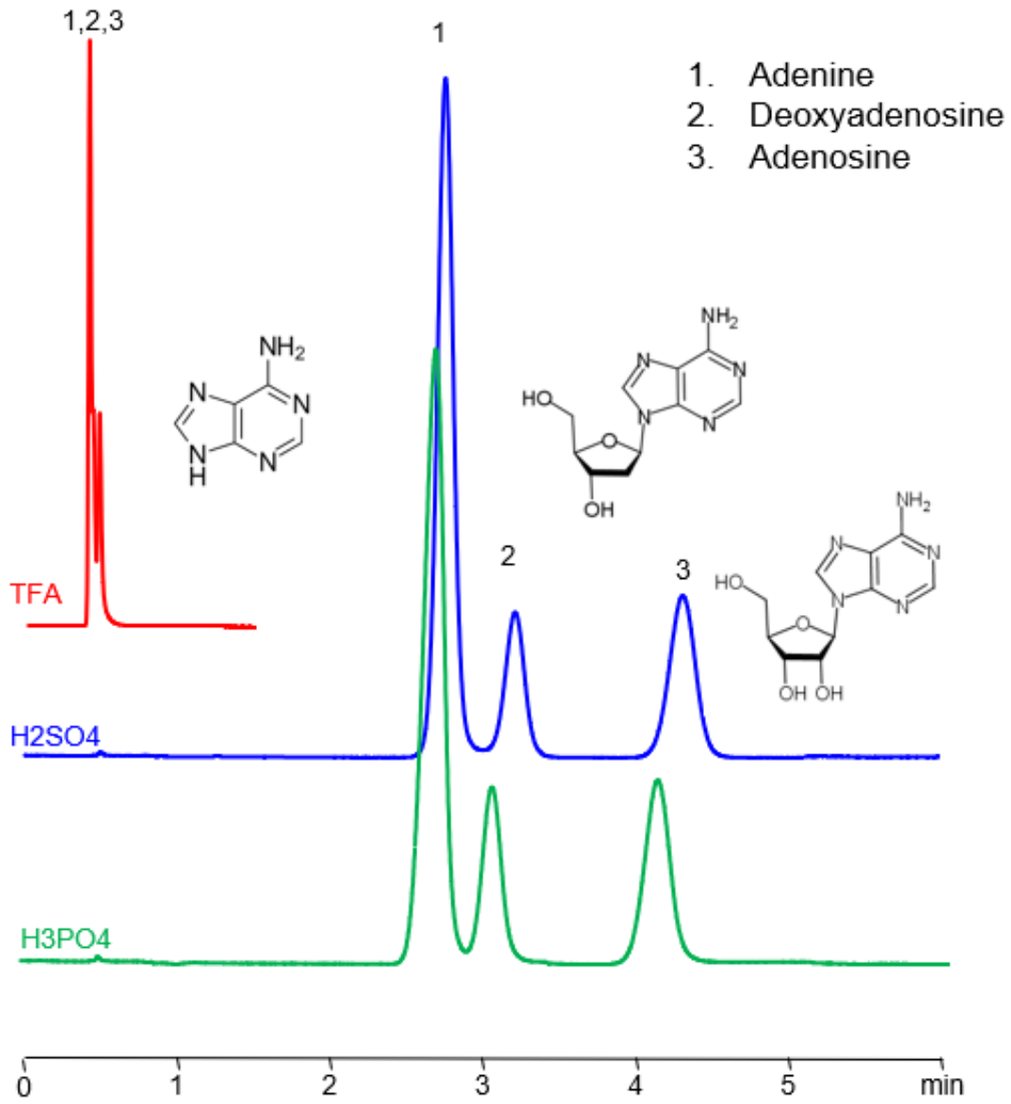
**Pros:**

- Rapid
- Cheap

**Cons:**

- Metabolite identification
- Poor adaptability

# High-performance liquid chromatography (HPLC)




| | |
|---|---|
| **Column**: | BIST B+ |
| **Column size:** | 4.6 × 50 mm, 5 µm |
| **Column part number:** | TBP-46.50.0510 |
| **Mobile phase:** | MeCN – 85% |
| **Buffer:** | 0.2% |
| **Flow rate:** | 1,0 mL/min |
| **Detection:** | UV 260 nm |

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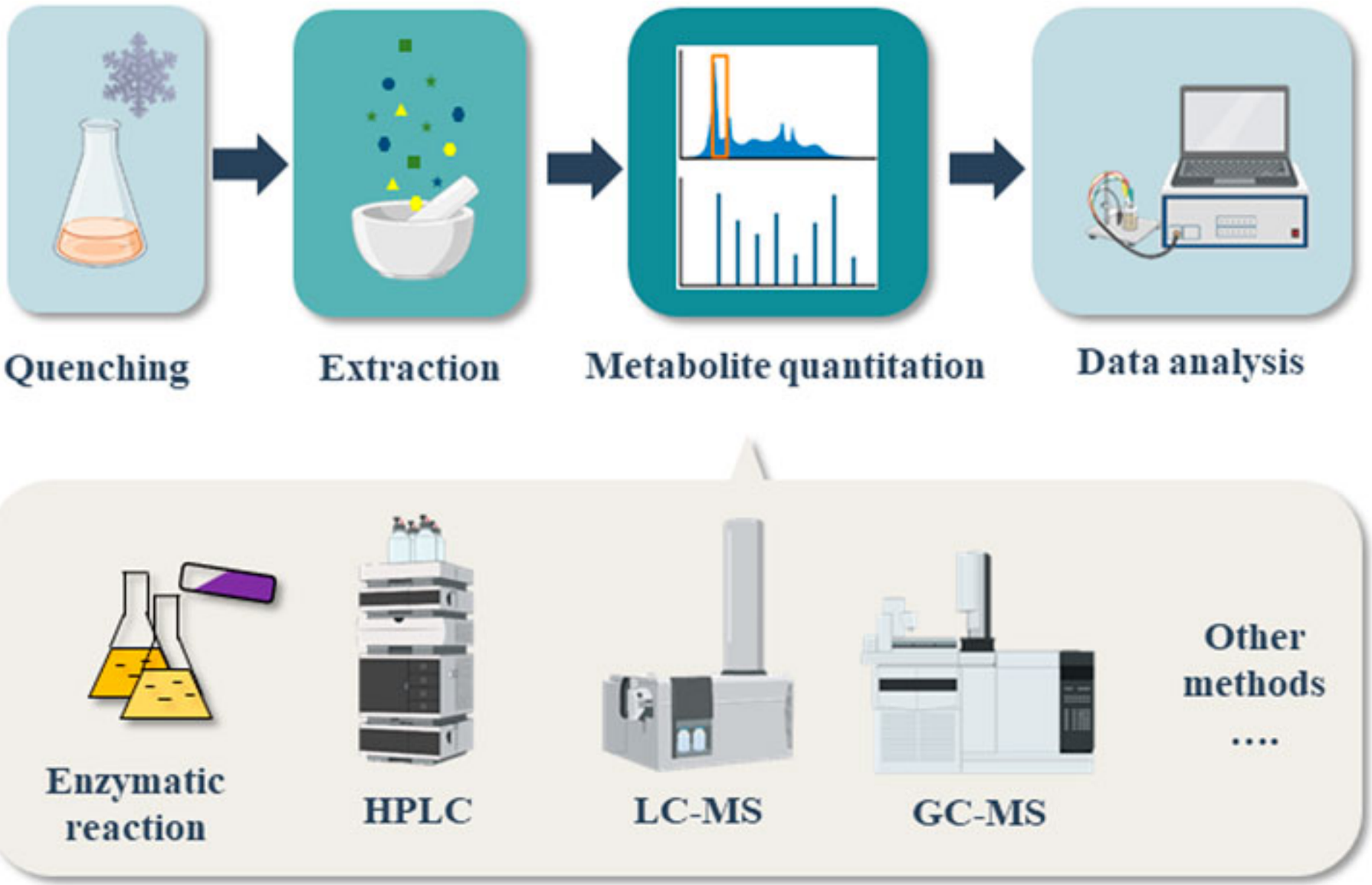
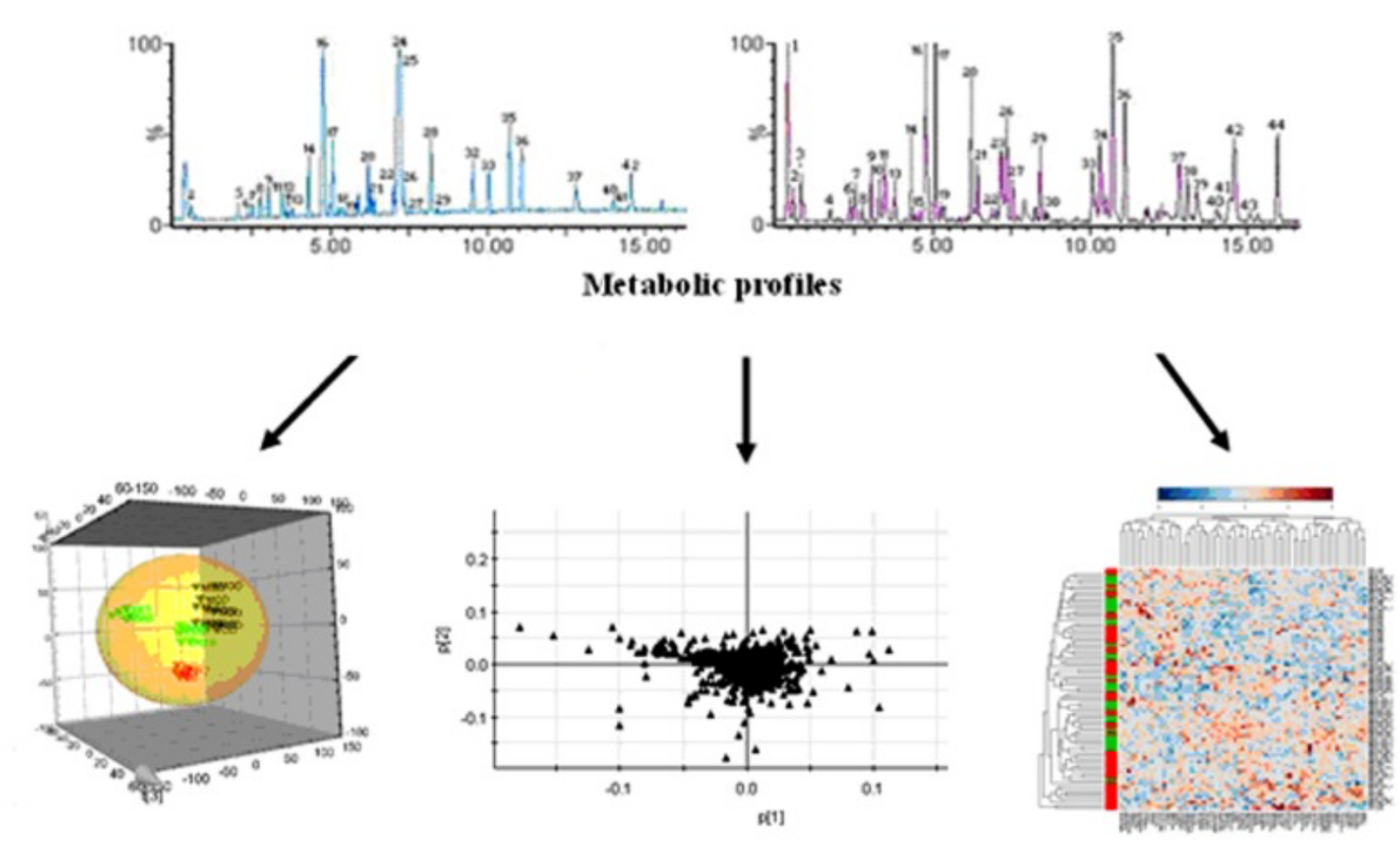



*Wang & Luo,* ***Front Bioeng Biotech****, 2022*

# Spectrometry (NMR or MS)

Analytical approaches that resolve a mixture of components into spectra defined by multiple peaks (where each peak correspond to a different molecule).
These peaks are separated according to different physical properties (mass, charge, solubility, etc).
You obtain a “fingerprint” that is a snapshot of cell metabolism at a given time.




Common types of analysis include those that quantify principal components, associate hierarchical clusters, create partial least squares, discriminant function, or even form artificial neural networks. Collectively, this analysis helps identify and discriminate the function of the metabolites in the sample, where databases can secondarily be used to validate specific pathway activity.

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**Pros:**

- Sensible
- Robust
- Large-scale approach (METABOLOMICS)

**Cons:**

- Difficult (requires extensive training)

# Spectrometry (NMR or MS)

Metabolomic studies often rely on nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) techniques for the identification and quantification of metabolites.

NMR is a quantitative, robust, reproducible, and high-throughput analytical technique associated with a straightforward and simple sample preparation procedure. MS-based techniques are more sensitive, in particular when using liquid chromatography (LC) connected to a tandem MS/MS for quantitative analysis in the multiple reaction monitoring mode.

In NMR, a wide variety of samples may also be used, including plasma, serum, saliva, etc., and these must be deproteinized prior to analysis through precipitation or extraction to weaken the intensity of interfering resonances. After derivatization and buffering the samples undergo analysis to produce a specific NMR profile, constitutive of many peaks that represent various molecules within the sample. For NMR, various nuclei spectras may be used, including 1H, 13C, 15N and 31P, and the profile can be interpreted using multivariate statistical analysis with pattern recognition software.

# Mass Spectrometry (GC-MS or LC-MS)

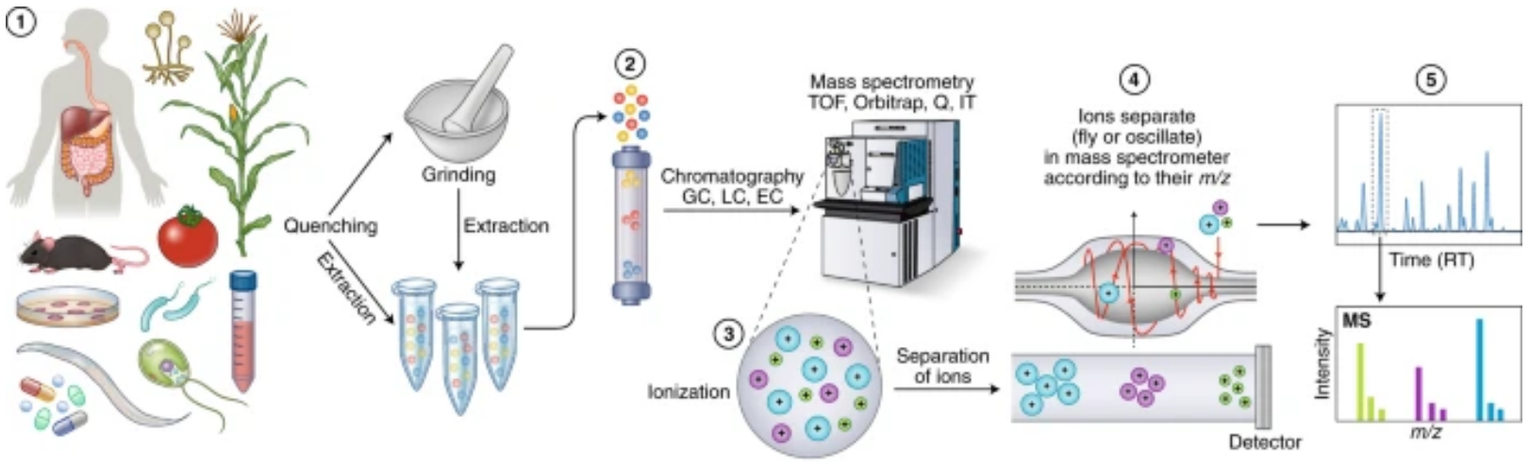
Similar to NMR, mass spectrometry (MS) easily distinguishes the masses of molecular components, or more specifically, ionized molecules and fragments.

1. Samples are prepared accordingly then undergo a rapid quenching step to quickly stop any metabolic activity within the cell. If required, samples must undergo a purification step to separate the cellular phase from the extracellular medium.
2. The sample will undergo an extraction phase that aims to remove and dissolve the maximum amount of original sample metabolites as possible. There is no one-size-fits-all for extraction, as these steps may be variable and must be empirically determined for each sample and reagent combination.
3. Samples are concentrated to partially, if not totally, remove leftover solvents.

Usually, samples will undergo another technique for better resolution prior to MS analysis.

Liquid chromatography (LC) is most commonly used to separate unneeded metabolites according to a column and eluent, though gas chromatography (GC) can be used to separate volatile and non-volatile metabolites as well.

# Mass Spectrometry (GC-MS or LC-MS)




**Sample preparation and extraction**
- Avoid environmental perturbation during harvesting
- Control environment: harvesting at the same time and under the same conditions
- Snap-freezing in liquid nitrogen
- Enzyme quenching: completely terminate all enzyme activities
- Standards spiked into the quenching solvent
- Grinding, isolation of cells, fast-filtration or aspiration

**Sample replication and randomization**
- At least four biological replicates, preferably more
- Technical and analytic replicates are worthy of consideration
- Randomization of samples throughout workflows is essential
- In large-scale studies, quality-control samples and batch correction are essential

**Chromatography–mass spectrometry**
- Separation methods, composition of the mobile phase, column properties and injection volume
- Metabolites are within their range of detection
- Avoid ion suppression: dilution of extracts, sonication, filtration or centrifugation, recovery test
- Choosing ionization source and type of detection mode, MS method, scan number and speed, MS/MS and energy for fragmentation

Metabolites are identification with computational approaches, including ion annotation, spectral interpretation and spectral matching.

*Targeted* metabolomics: spectra matches known analytical standards

*Alseekh et al,* ***Nat Methods****, 2021*

**Biological samples**

- Experimental design
- Growth conditions, treatments, tissue type
- Replication ($n = 4$), quality control
- Care before and during sampling
- Freeze and then extract
- Storage conditions

↓

**Quenching and extraction**

- Tissue samples: quick freeze, liquid $N_2$ grinding, liquid $N_2$ clamping, pulverization, lyophilization and cell lysis
- Biofluid/cell culture extraction: cold organic solvent, aspiration or filtration
- Concentration/drying under $N_2$ or cold speed-vac for short time; SPE column for clean-up or enrichment of extract
- Recovery test, spike internal standard

↓

**Chromatography separation**

Liquid chromatography:
- Wide range of compounds, reverse-phase column (silica C18), mobile phase, injection volume

Gas chromatography:
- Low-molecular-weight compounds, volatile, derivatization (MSTFA, BSTFA), packed column, carrier gas, injection mode

- Sample separation based on chemical properties
- Reduce ion suppression
- Improve detection of low-abundance compounds
- Separation of isomers
- Reduce the interfering compounds

↓

**Mass spectrometry**

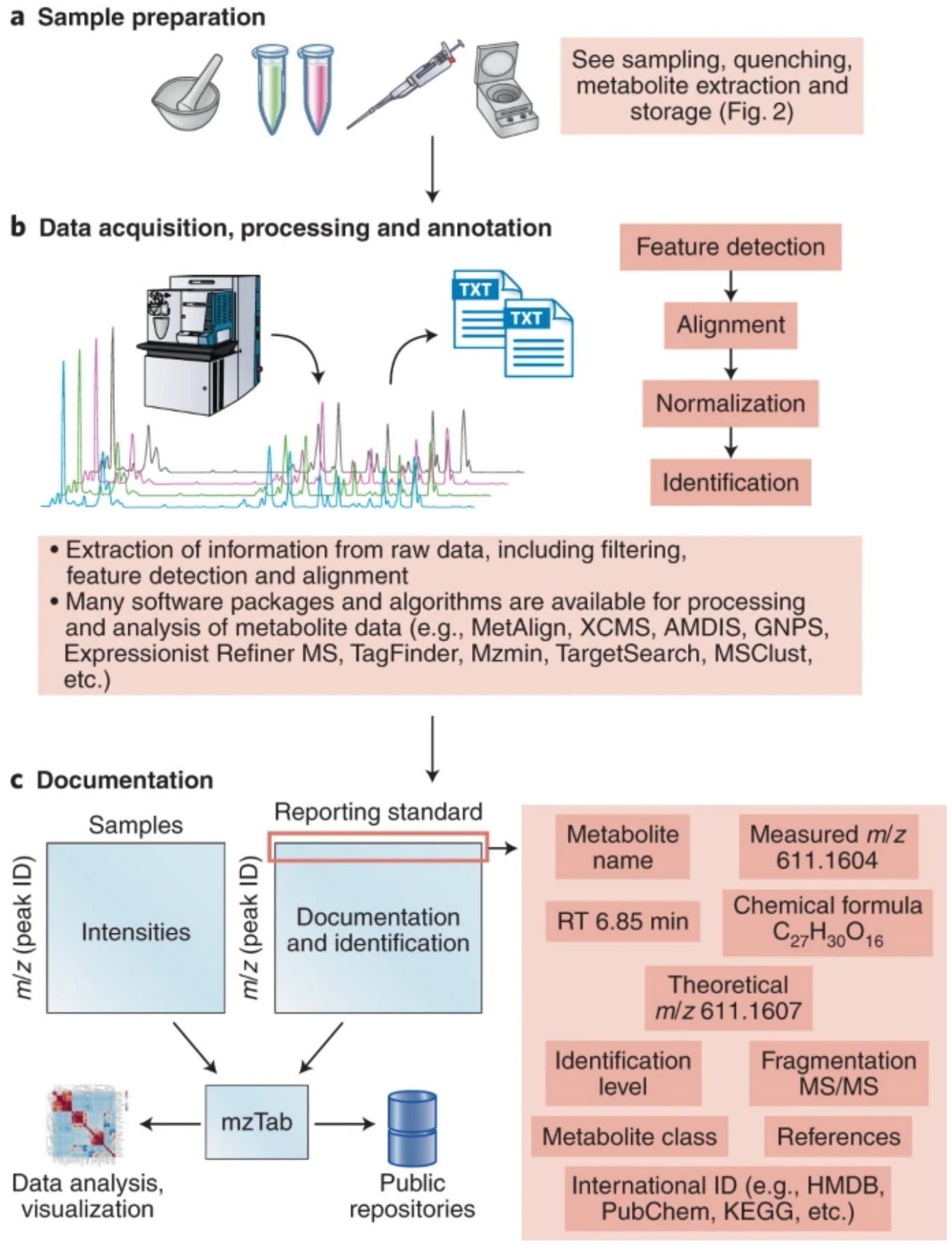
- Ionization source, ionization type (ESI, EI, APCI, etc.), polarity, voltage, temperature, vacuum
- Mass analyzer (TOF, Orbitrap, ion trap, FT–ICR, etc.)
- Resolution, sensitivity, mass accuracy, scan rates, acquisition mode (full scan, MS/MS, SIM, MRM, ddMS, etc.)

↓

**Data processing, metabolite identification and data analysis**

- Convert raw MS data ($m/z$) to intensities table
- Requires filtering, detection and normalization
- Identification and documentation
- Data analysis and representation

# Mass Spectrometry (GC-MS or LC-MS)




*Alseekh et al,* ***Nat Methods****, 2021*

# Mass Spectrometry (GC-MS or LC-MS)

More than 1 million different metabolites occur across the tree of life, with between 1,000 and 40,000 estimated to occur in a single species.

Even the most comprehensive methods cannot provide firm upper limits for metabolite number. Current capabilities for detection and quantification of metabolites fall a long way short of being comprehensive. Currently, combinations of the most comprehensive methods are able to quantify 700 of the 3,700 metabolites predicted to be present in *Escherichia coli*, 500 of the 2,680 metabolites predicted to be present in yeast, 8,000 of the 114,100 metabolites predicted to be present in humans and only 14,000 of the over 400,000 metabolites predicted to be present in the plant kingdom. Chemical diversity, rapid turnover times and broad dynamic range in cellular abundance currently prohibit the possibility of using single-extraction and single-analysis procedures to measure all metabolites.

To tackle these challenges:

- Different extraction techniques / matrices
- Combinations of analytical methods
- Rigorous standards for normalization

# Enzymatic activity

Switching on/off the genes that encode specific enzymes or regulating their activity contributes to control/regulate cell metabolism

- Allosteric regulation
- Availability of co-factors
- Endogenous activators/inhibitors
- Feedback mechanisms
- **Compartmentalization**

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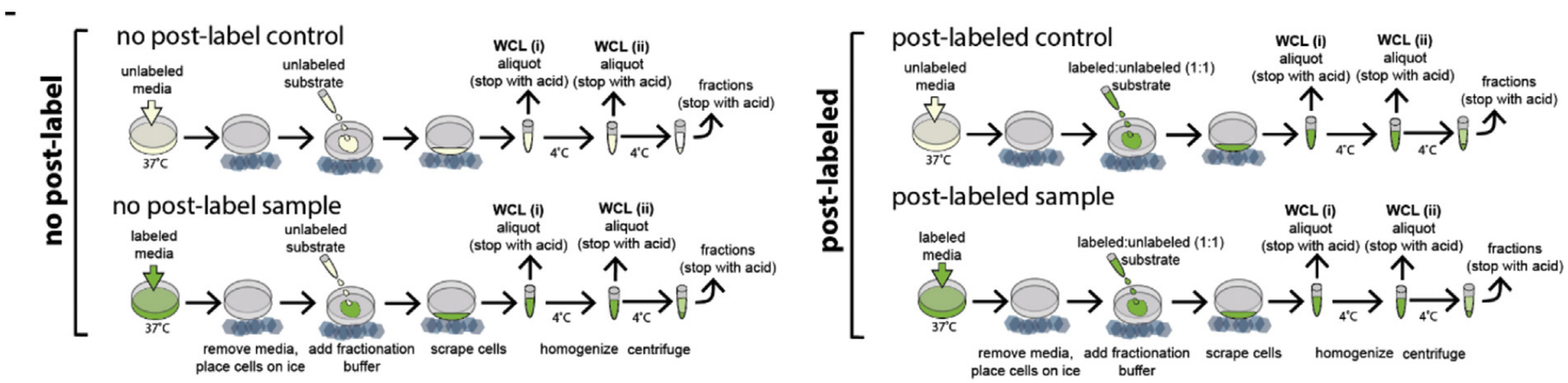
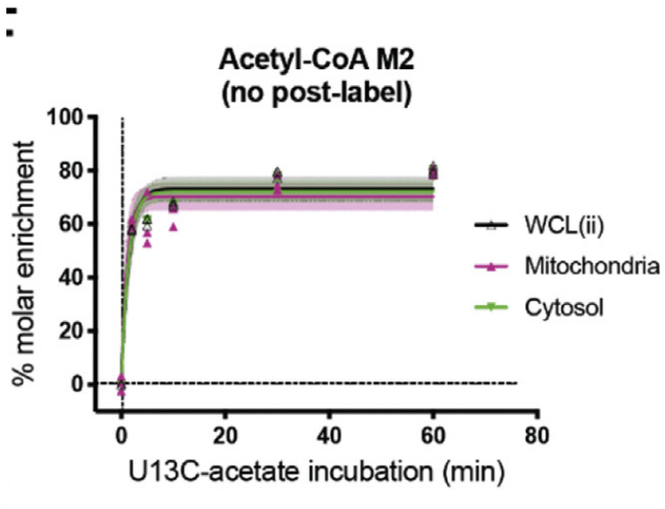
Yet, they hardly capture the complexity metabolic regulation *in vivo*.

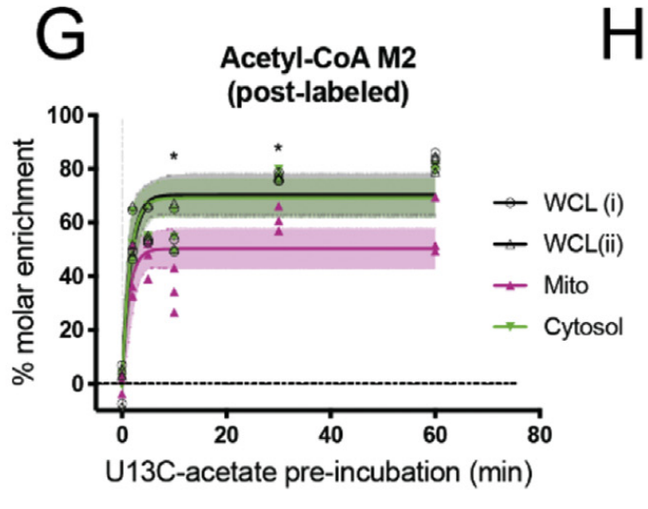
# Subcellular fractionation introduces MS bias

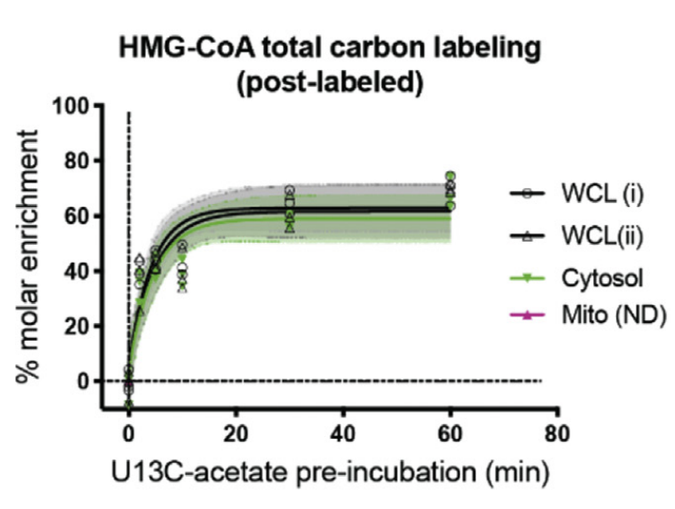
Metabolic reactions are extremely dynamic and occur post harvesting.

Long fractionation protocols affect readouts.




*Trefely et al,* ***Mol Metab****, 2019*

# Metabolic assays seek to:

Measure metabolites and macromolecules

Measure energy equivalents and co-facors

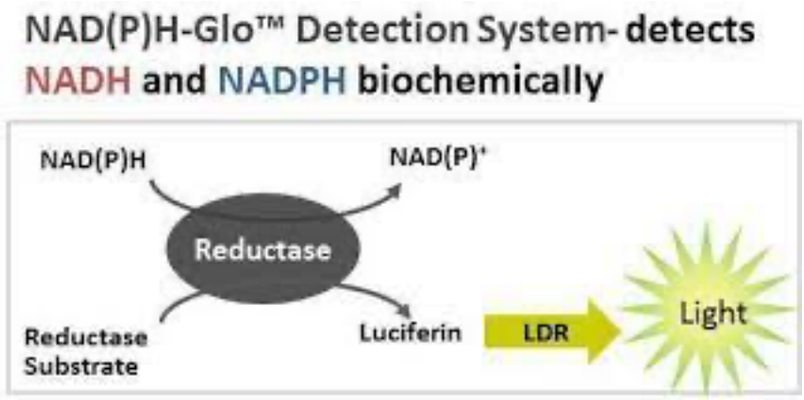
Measure enzymatic activities

Measure activity of metabolic organelles

# Measure energy equivalents and co-factors

ATP and NAD(P)/H levels can be easily measured in vitro using enzymatic assays (commercially available).

However, they do not capture an essential level of regulation: availability at different compartments



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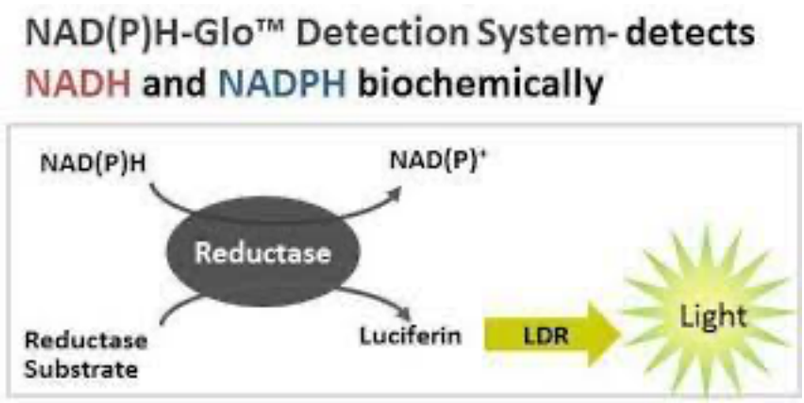
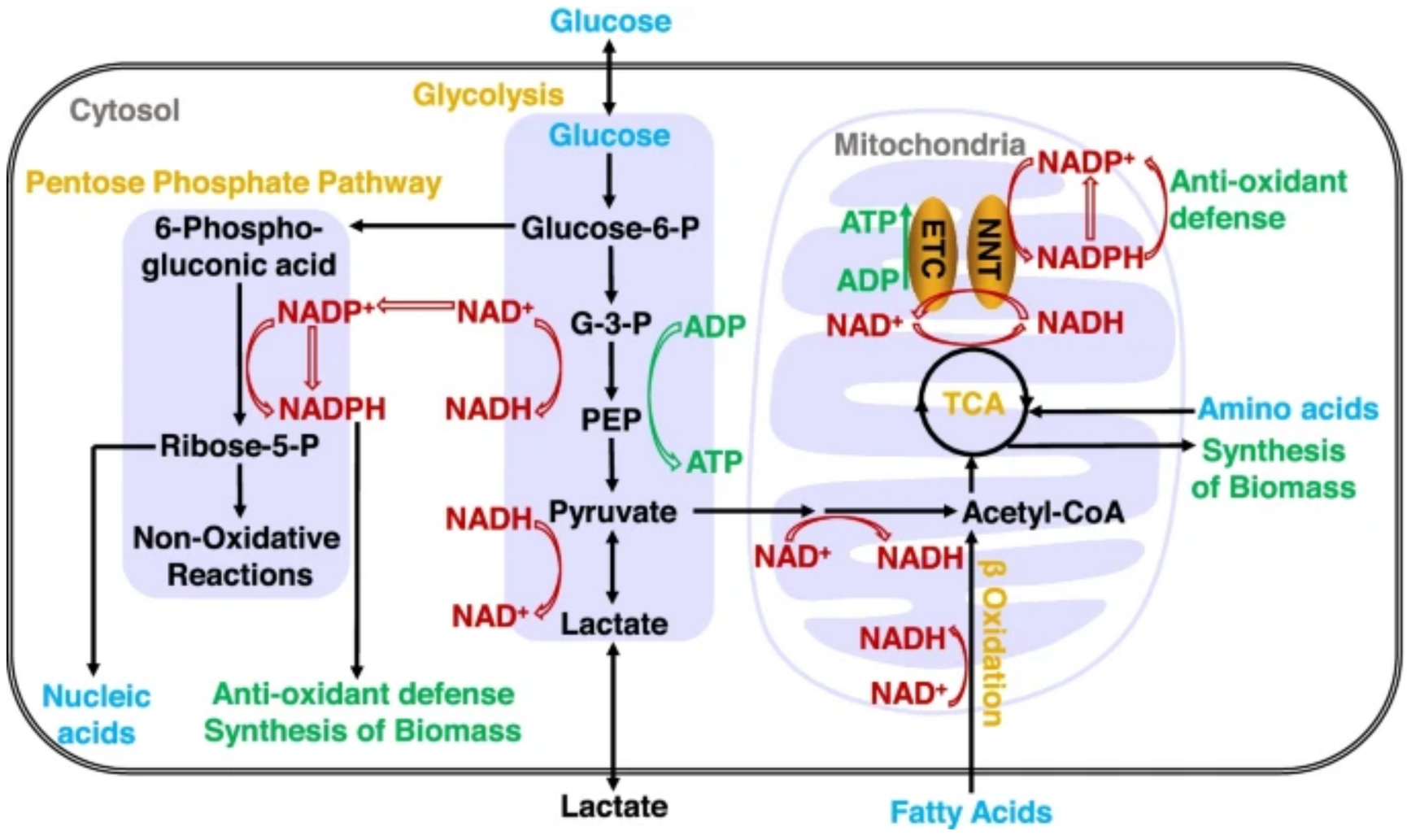


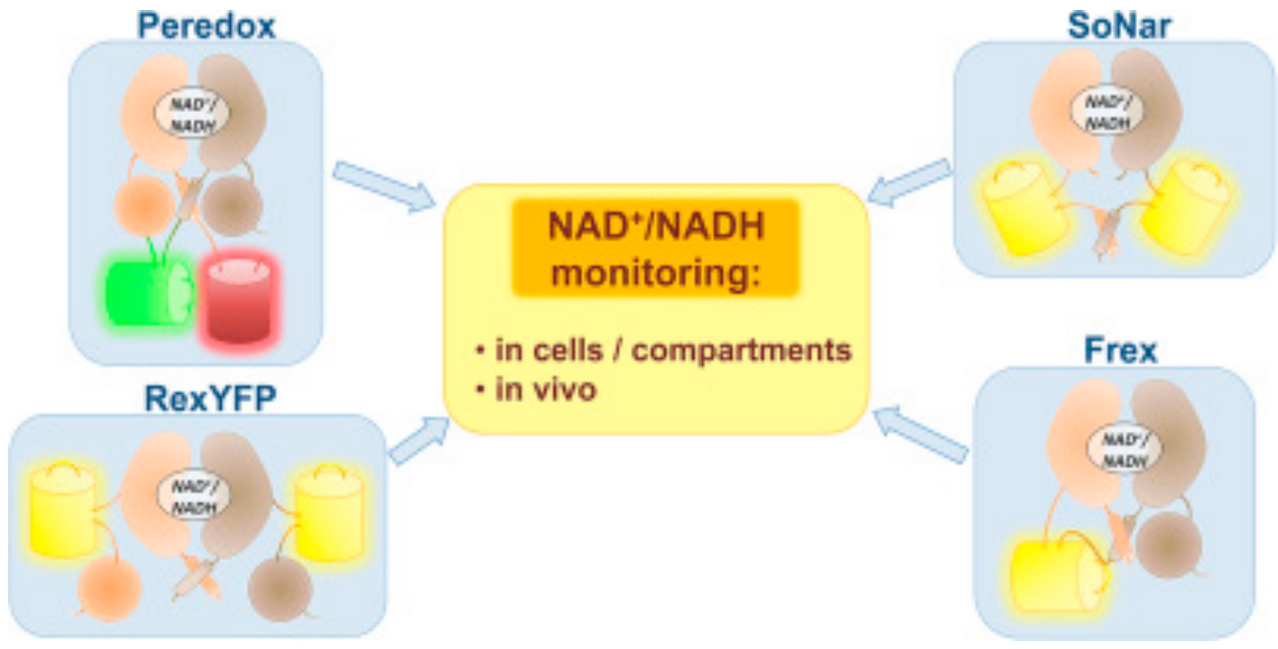



# Measure energy equivalents and co-factors

Fluorescent biosensors allow the monitoring of NAD(P)/H in space and time.

They allow in vivo analyses.

Generally consist of two basic components: substrate-binding proteins and one or two fluorescent proteins.

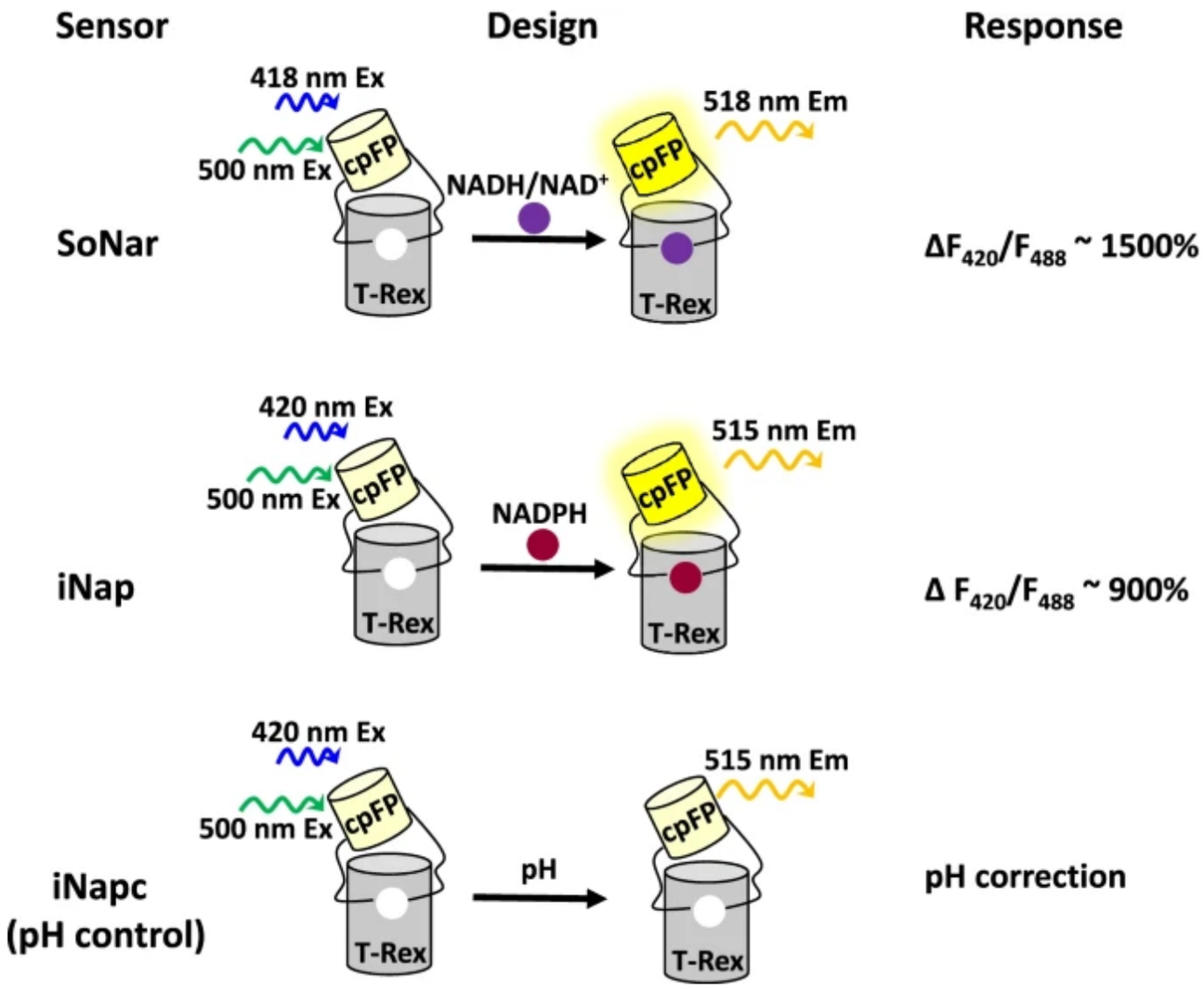



Fluorescent biosensors must meet several criteria for live-cell and in vivo developmental studies, including high specificity, large responsiveness, appropriate affinity, strong brightness, and ratiometric readout, which allows reliable and convenient capture of subtle changes in physiological contexts.

Various transcription factors and regulatory proteins specifically sense intracellular biomolecules from bacteria to mammals. Substrate-sensing proteins often trigger conformational changes upon biomolecule binding, which induces fluorescence changes in fluorescent proteins. Fluorescence can be readily measured by routine instruments such as plate readers, flow cytometry or fluorescence microscopy.

# Fluorescence biosensors for the measuring of energy equivalents and co-factors

SoNar was designed by inserting circularly permutated yellow fluorescent protein (cpYFP) into the truncated *Thermus aquaticus* T-Rex protein. The sensor has two excitation peaks, which enable an intrinsically ratiometric measurement. SoNar responds to the NAD+/NADH ratio but does not depend on either individual NAD+ or NADH concentrations alone.



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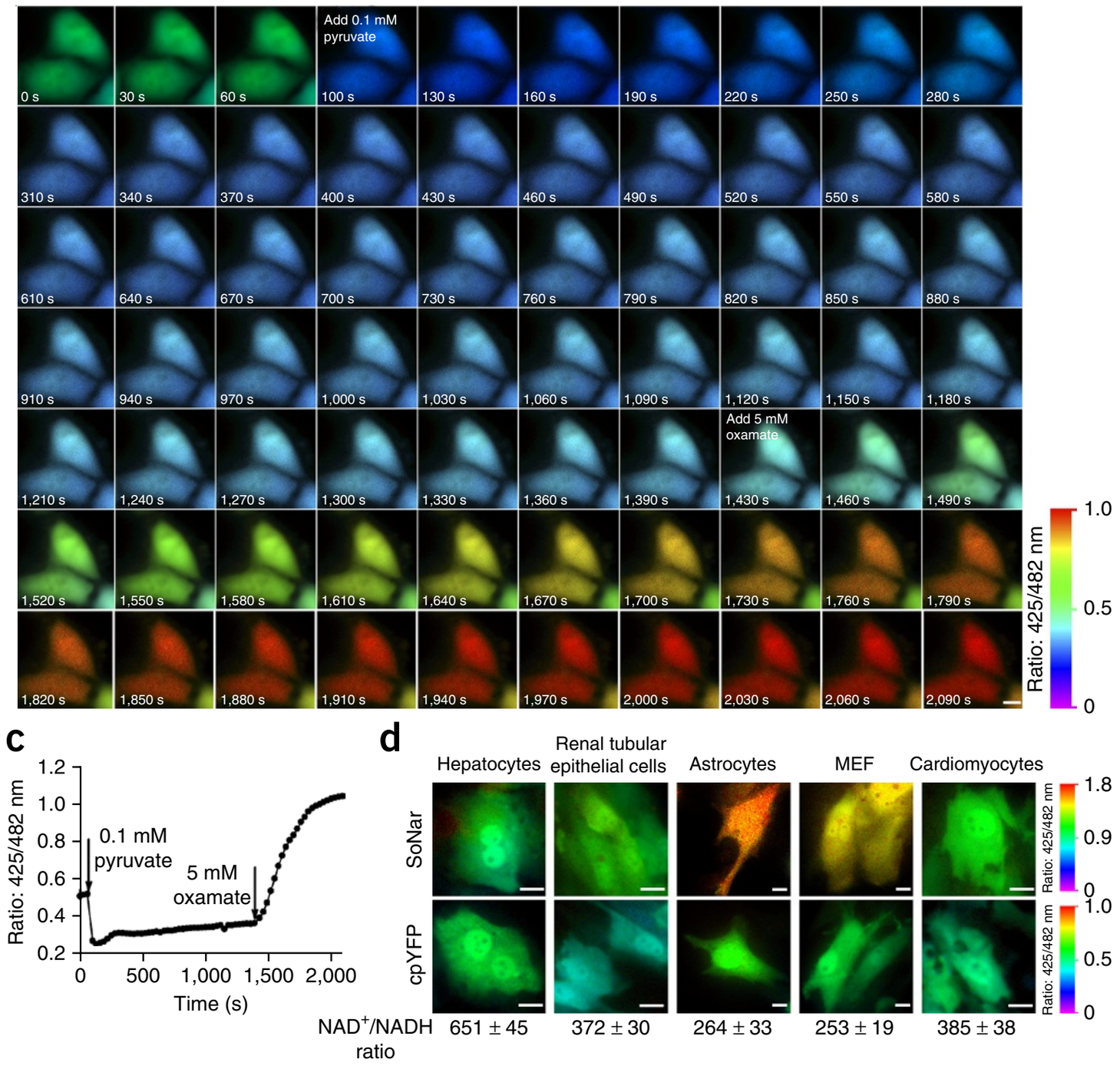


*Zhao et al,* ***Nat Protocol****, 2016*

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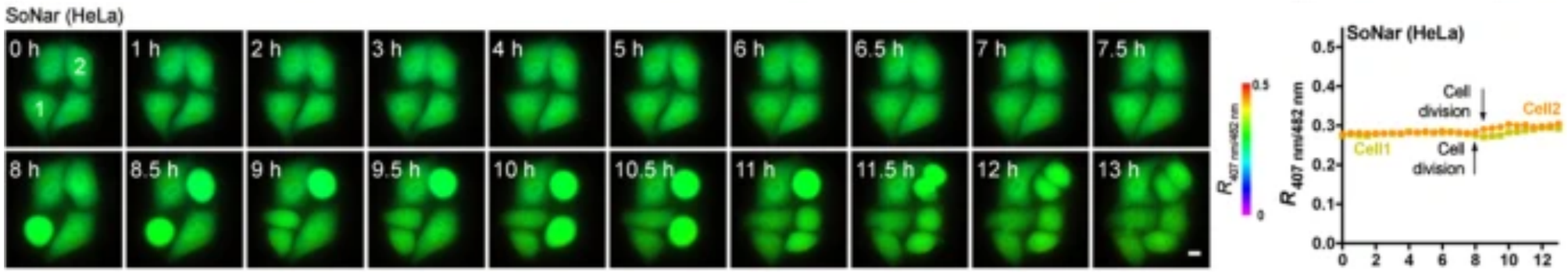


Real-time tracking NADH/$NAD_+$ and NADPH dynamics during the cell cycle

*Li et al,* ***Cell Regener****, 2022*

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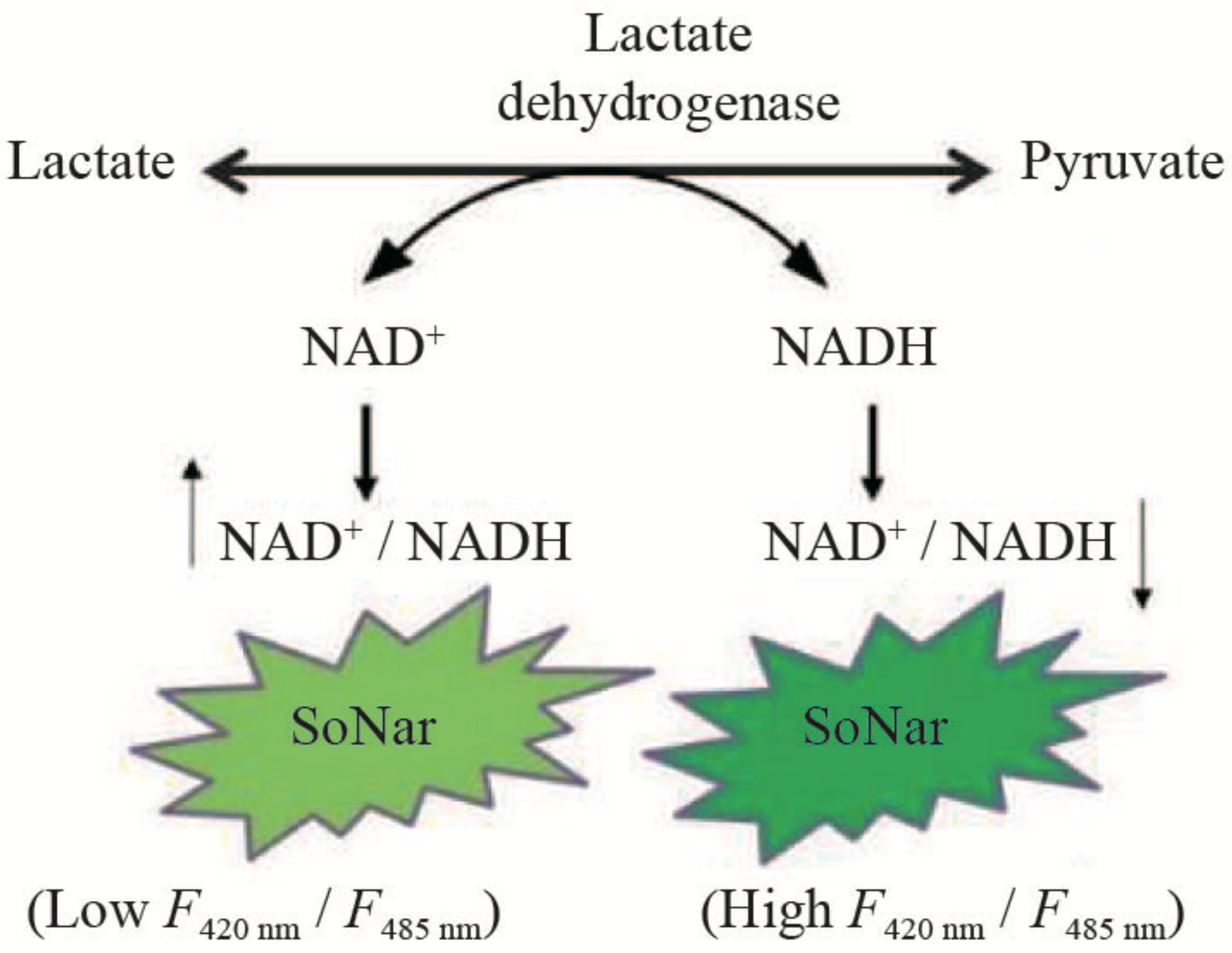
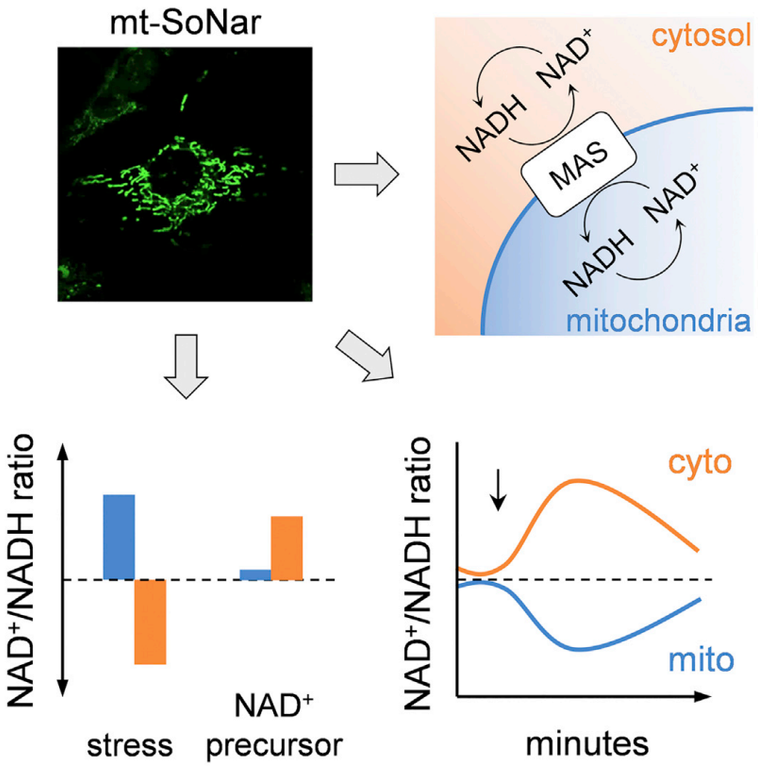
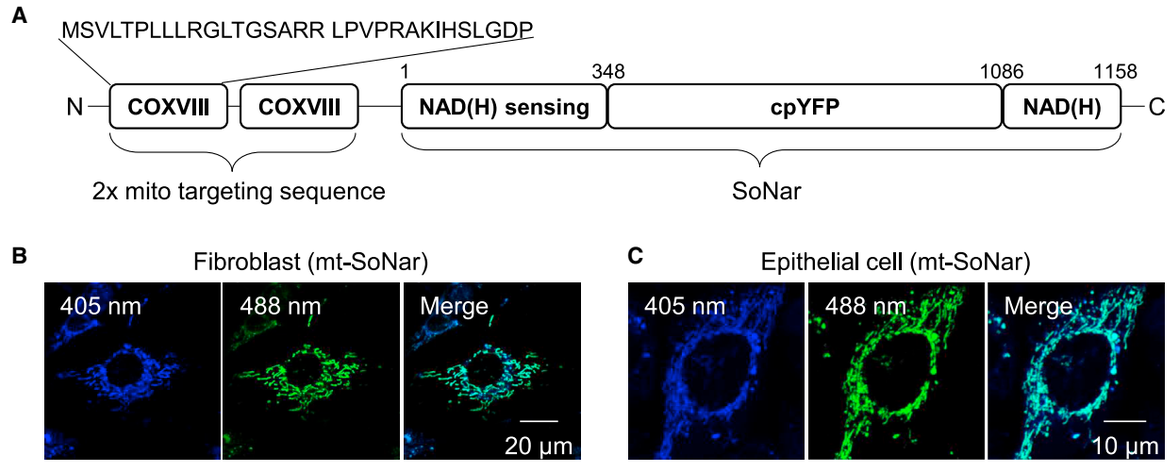


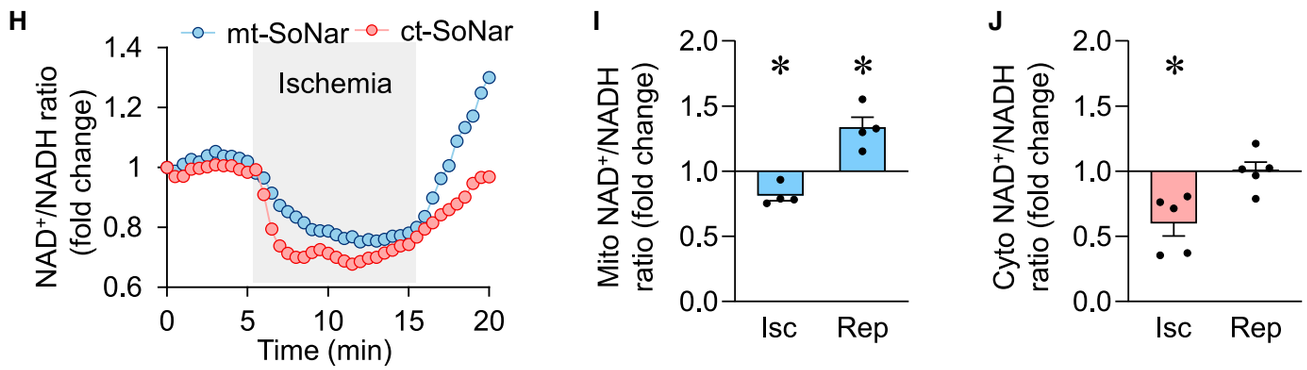
Cell Reports
Methods

Article

# Genetically encoded biosensors for evaluating NAD$^+$/NADH ratio in cytosolic and mitochondrial compartments

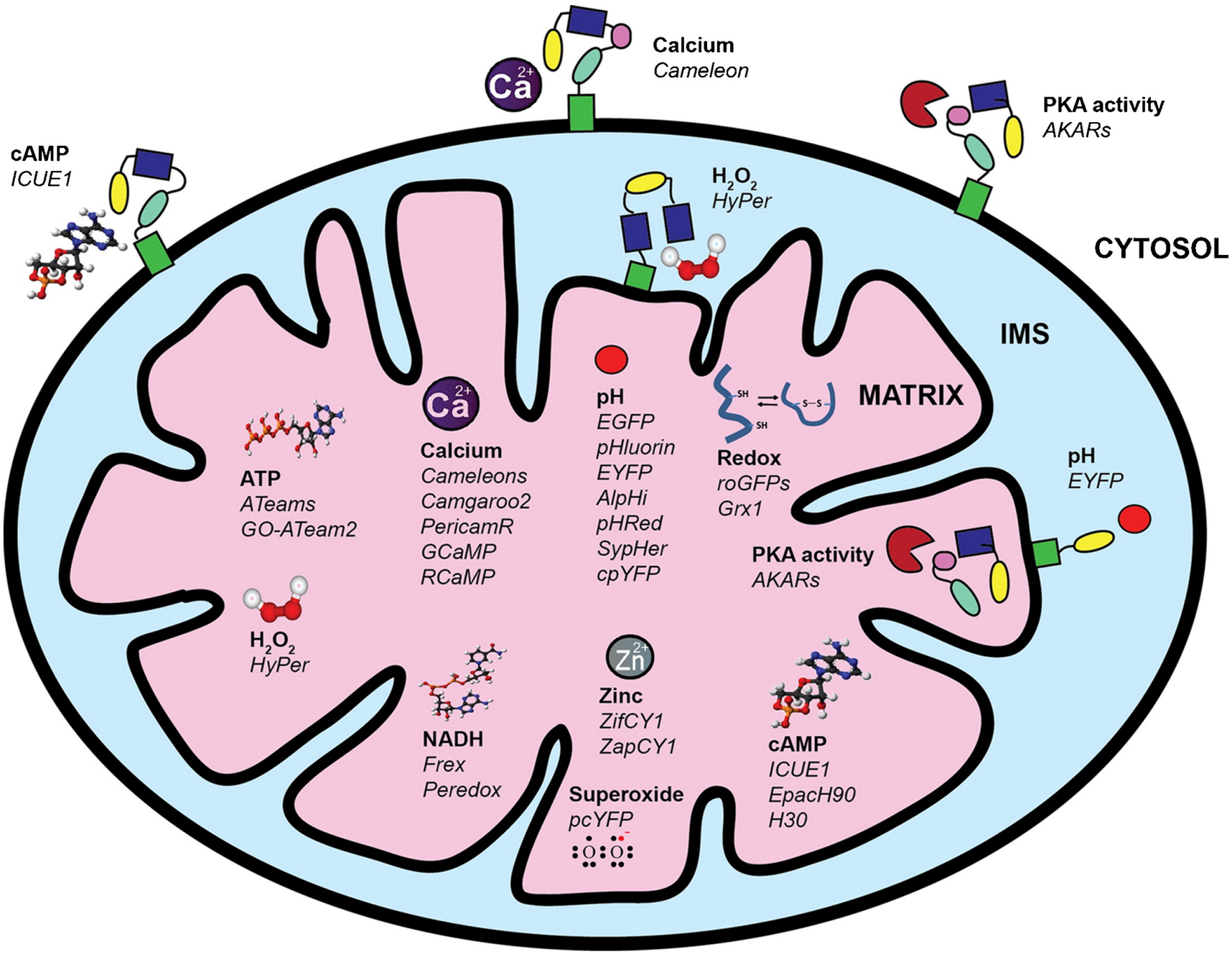



*Hu et al, **Cell Rep Methods**, 2021*

# Fluorescence biosensors for the measuring of energy equivalents and co-factors




*De Michele et al,* ***Int J Biochem Cell Biol****, 2014*

# Metabolic assays seek to:

Measure metabolites and macromolecules

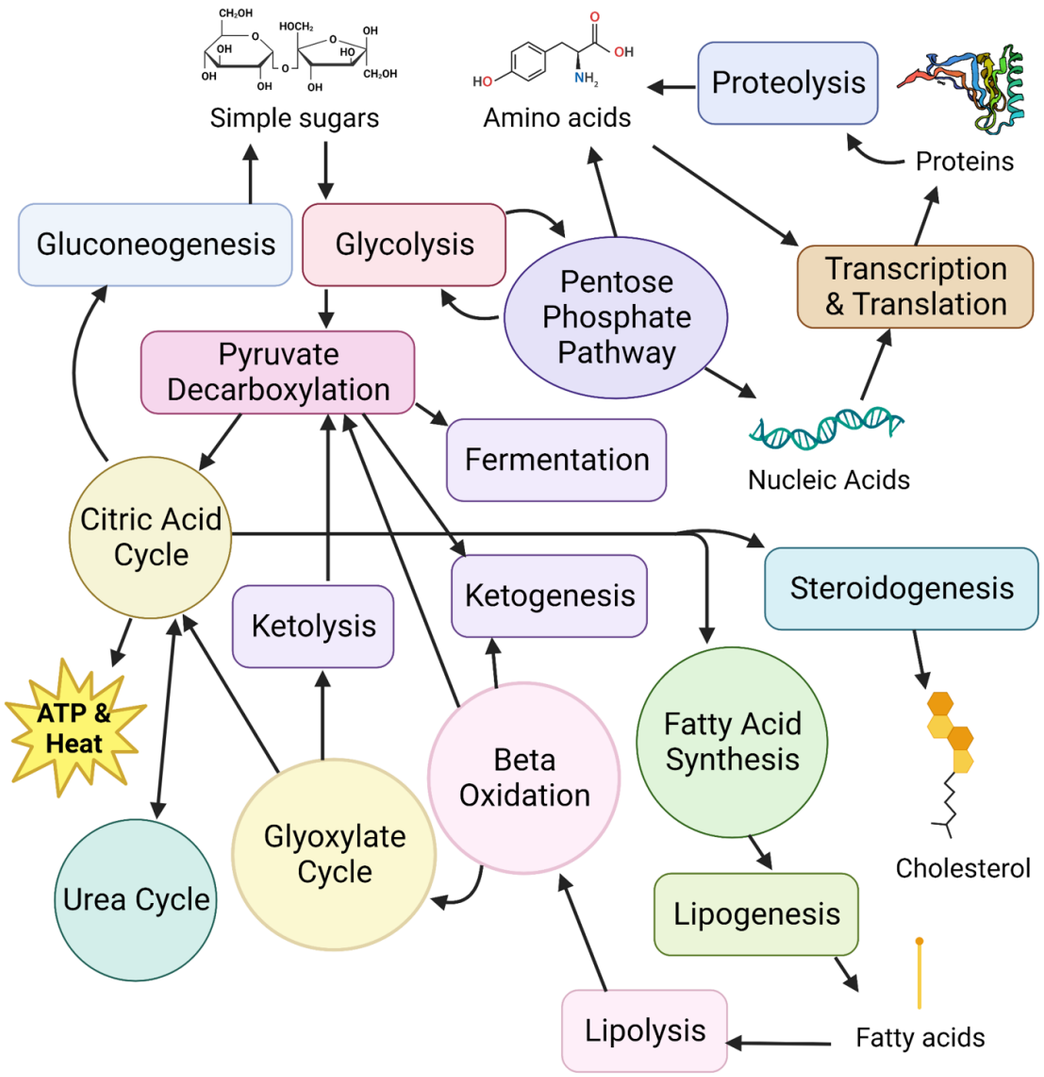
Measure energy equivalents and co-facors

Measure enzymatic activities

Measure activity of metabolic organelles

# Enzyme activity assays

Enzymatic assays can be used to probe the activity of specific pathways



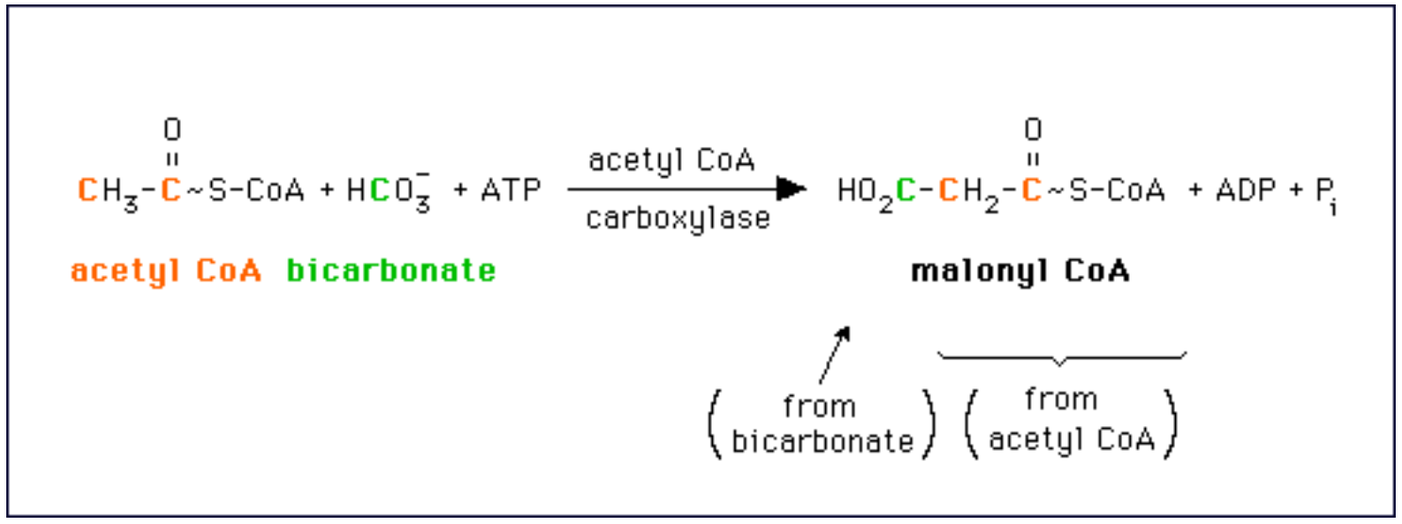

….or test new inhibitors!

# Acetyl-Coenzyme A Carboxylase 1 (ACC1) Assay Kit

#79315
96 reactions

**Description**

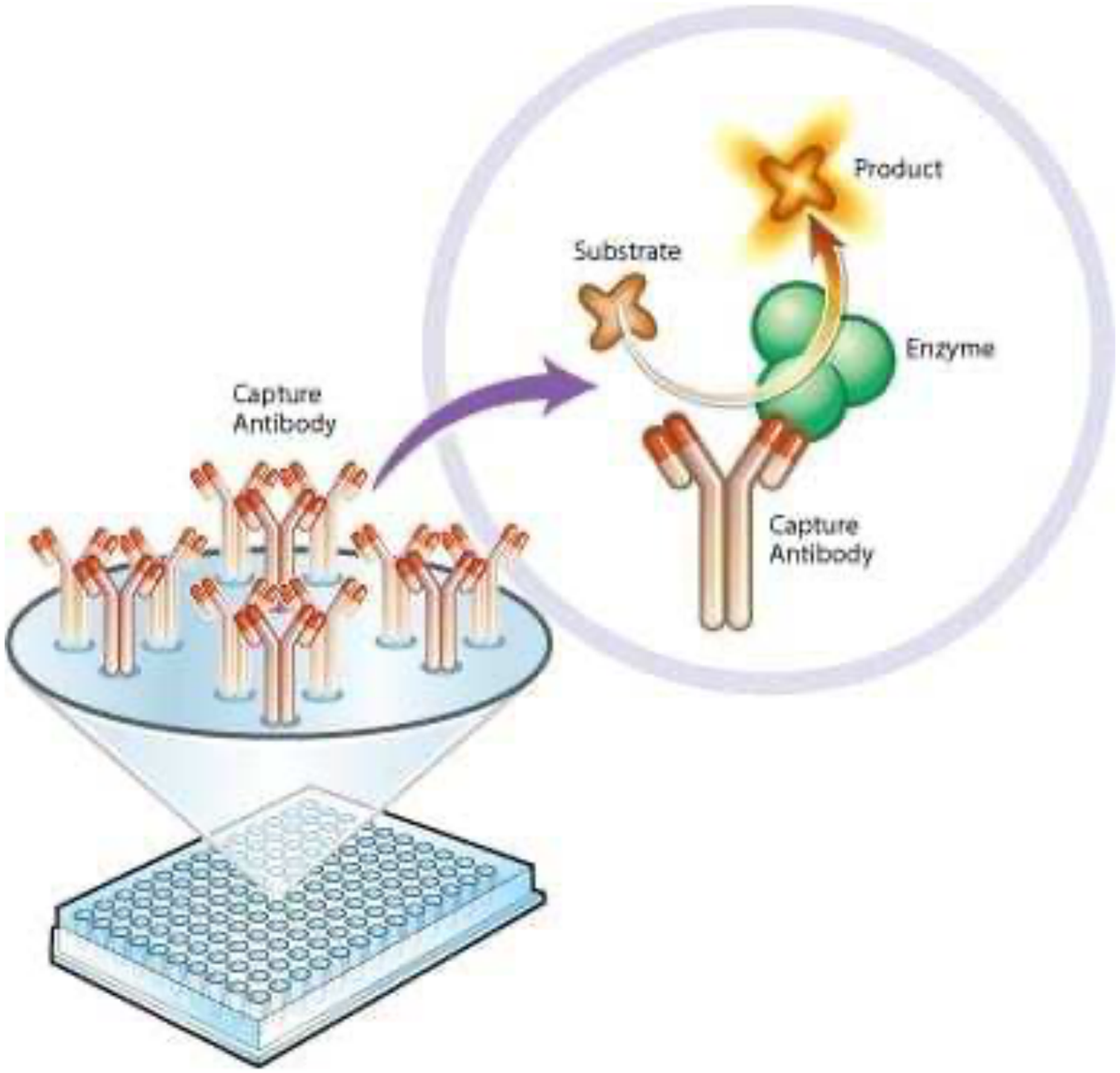
The Acetyl-Coenzyme A Carboxylase (ACC1) Assay Kit is designed to measure ACC1 activity for screening and profiling applications using ADP-Glo™ as a detection reagent. The assay kit comes in a convenient 96-well format, with enough purified recombinant ACC1, ATP, acetyl-CoA, sodium bicarbonate and assay buffer for 100 enzyme reactions.




**Supplied Materials**

| Catalog # | Name | Amount | Storage |
|---|---|---|---|
| 50202 | ACC1, FLAG-His-Tags* | 10 µg | -80°C |
| 79283 | 5x ACC Assay Buffer | 1 ml | -20°C |
| 79686 | 500 µM ATP | 100 µl | -20°C |
| | 2 mM Acetyl-CoA | 25 µl | -20°C |
| | 1M Sodium Bicarbonate | 75 µl | -20°C |

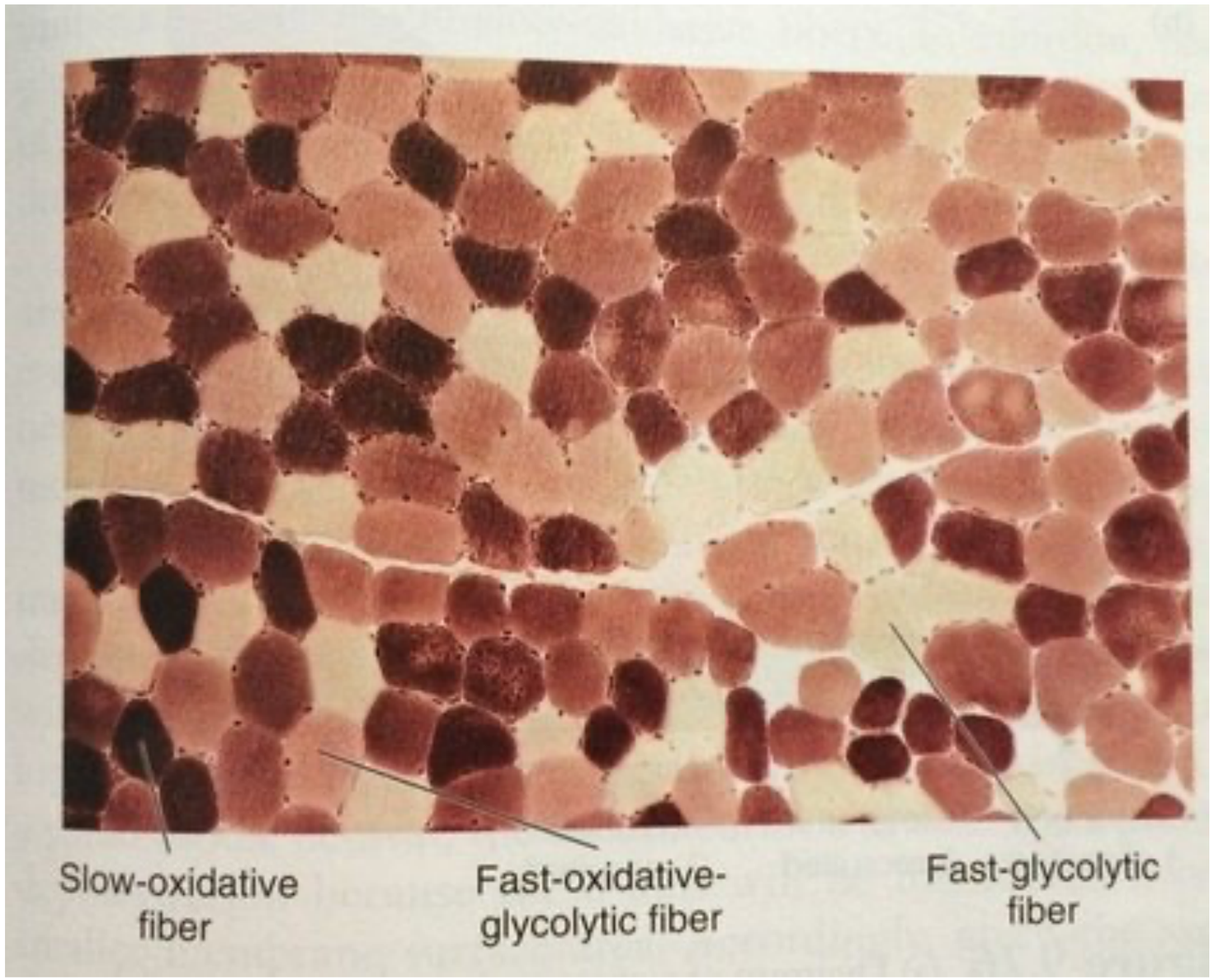
# Enzyme activity assays




Enzyme can be immune-purified and activity probed in vitro.

Yet, all problems related to in vitro settings are still there

# Enzyme activity assays - ex vivo

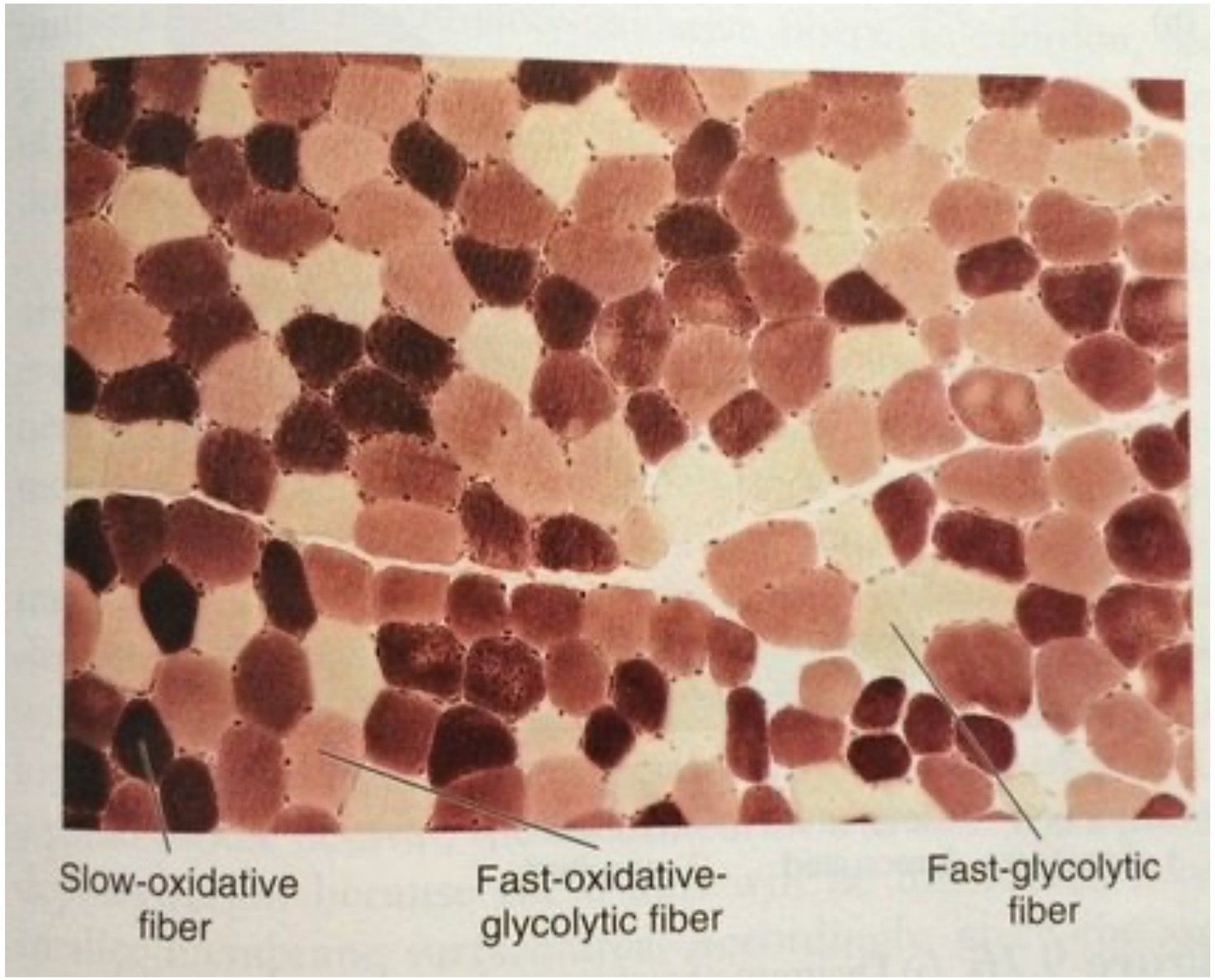



Metabolic enzymes retain activity post harvesting and post fixation.

Activity of most abundant enzymes can be assessed in fixed cells or tissues.

E.g.: Citrate Synthase

# Enzyme activity assays - ex vivo

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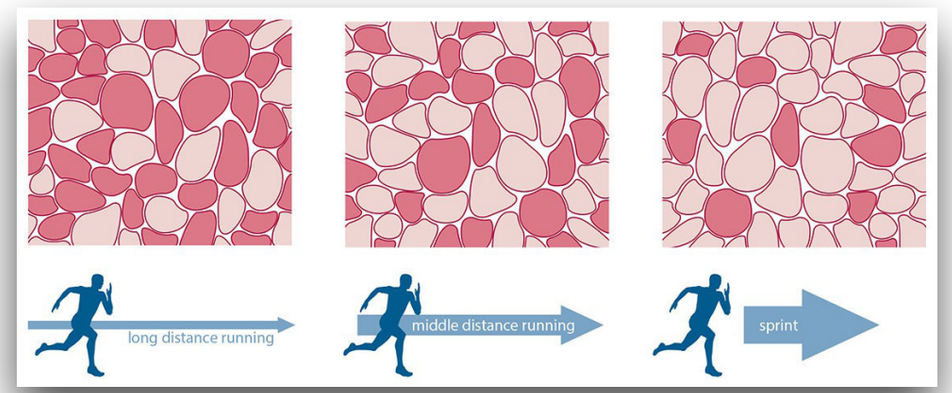


# Metabolic assays seek to:

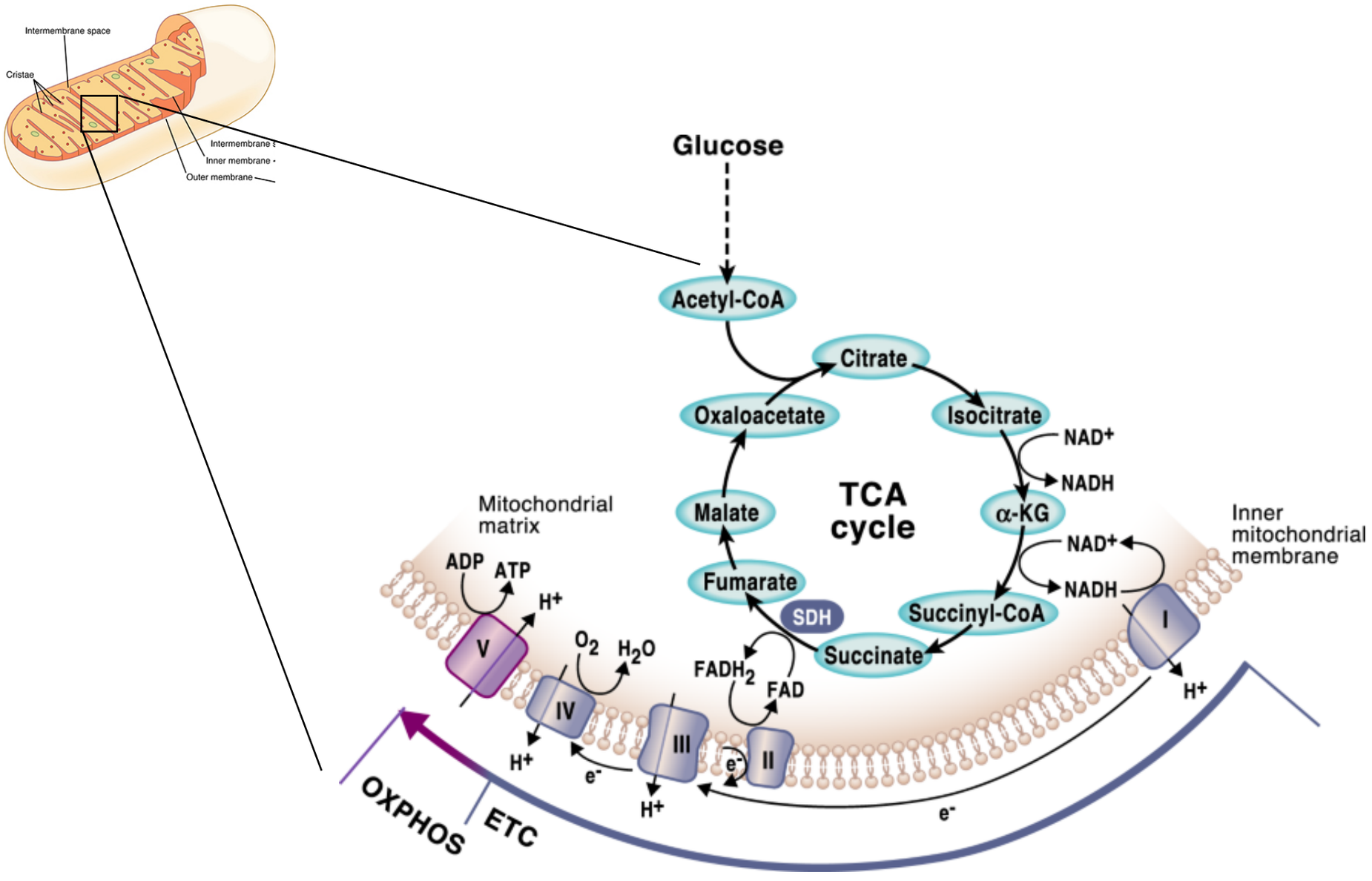
Measure metabolites and macromolecules

Measure energy equivalents and co-facors

Measure enzymatic activities

Measure activity of metabolic organelles

# Mitochondria couple pyruvate oxidation, electron transport and oxidative phosphorylation

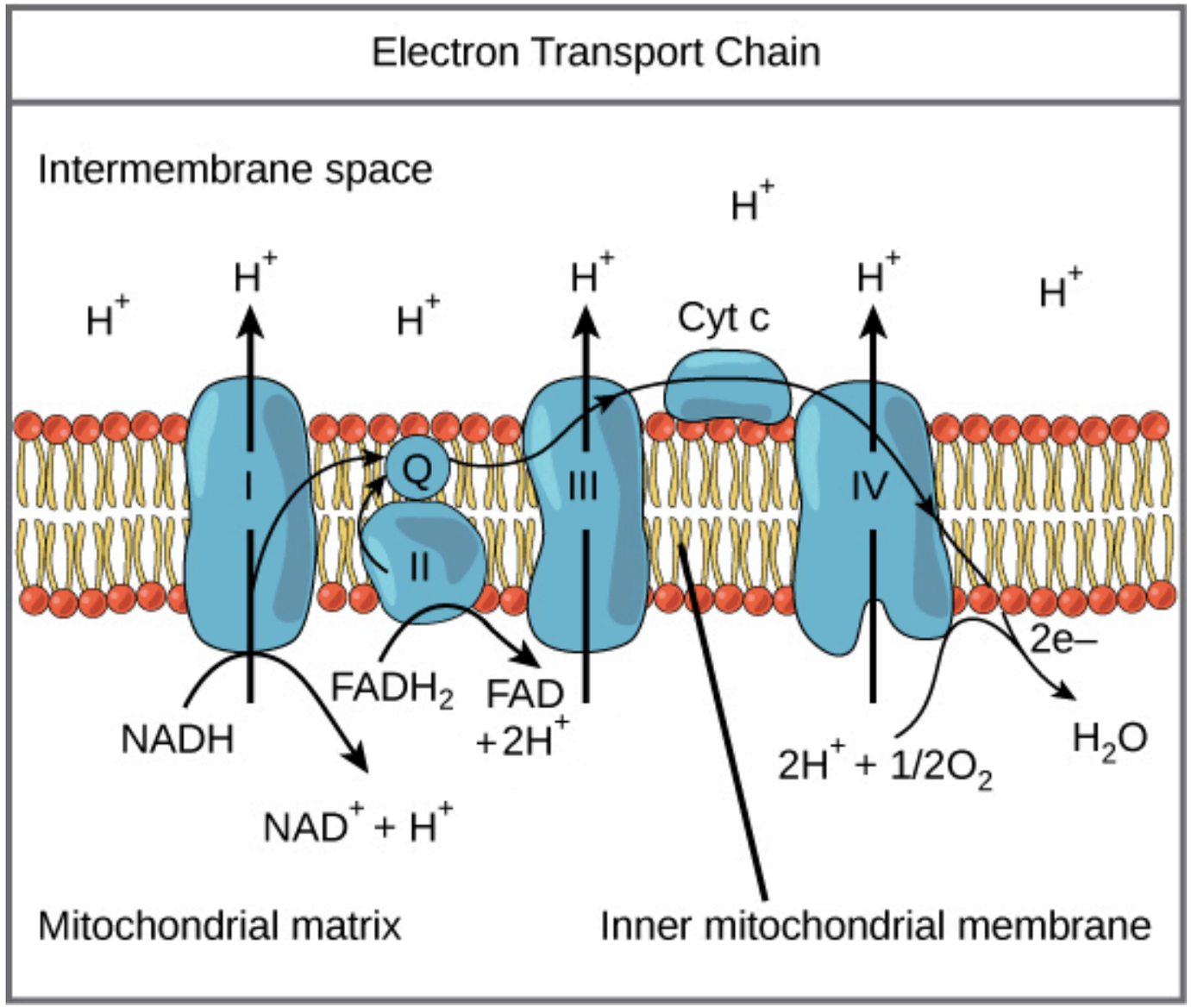


# Components of the Respiratory Chain

1. $NAD^+$-linked dehydrogenases
2. Flavin-linked dehydrogenases
3. Iron-sulphur proteins
4. Ubiquinone
5. Cytochromes

# The complexes of the ETC

- Complex I
  - NADH dehydrogenase
- Complex II
  - Succinate dehydrogenase
- Complex III
  - Cytochrome $bc_1$ complex
- Complex IV
  - Cytochrome $aa_3$ oxidase
- Complex V
  - ATP synthase



# Assessing “mitochondrial activity” means:

Complex assembly

Complex activity

NAD oxidation

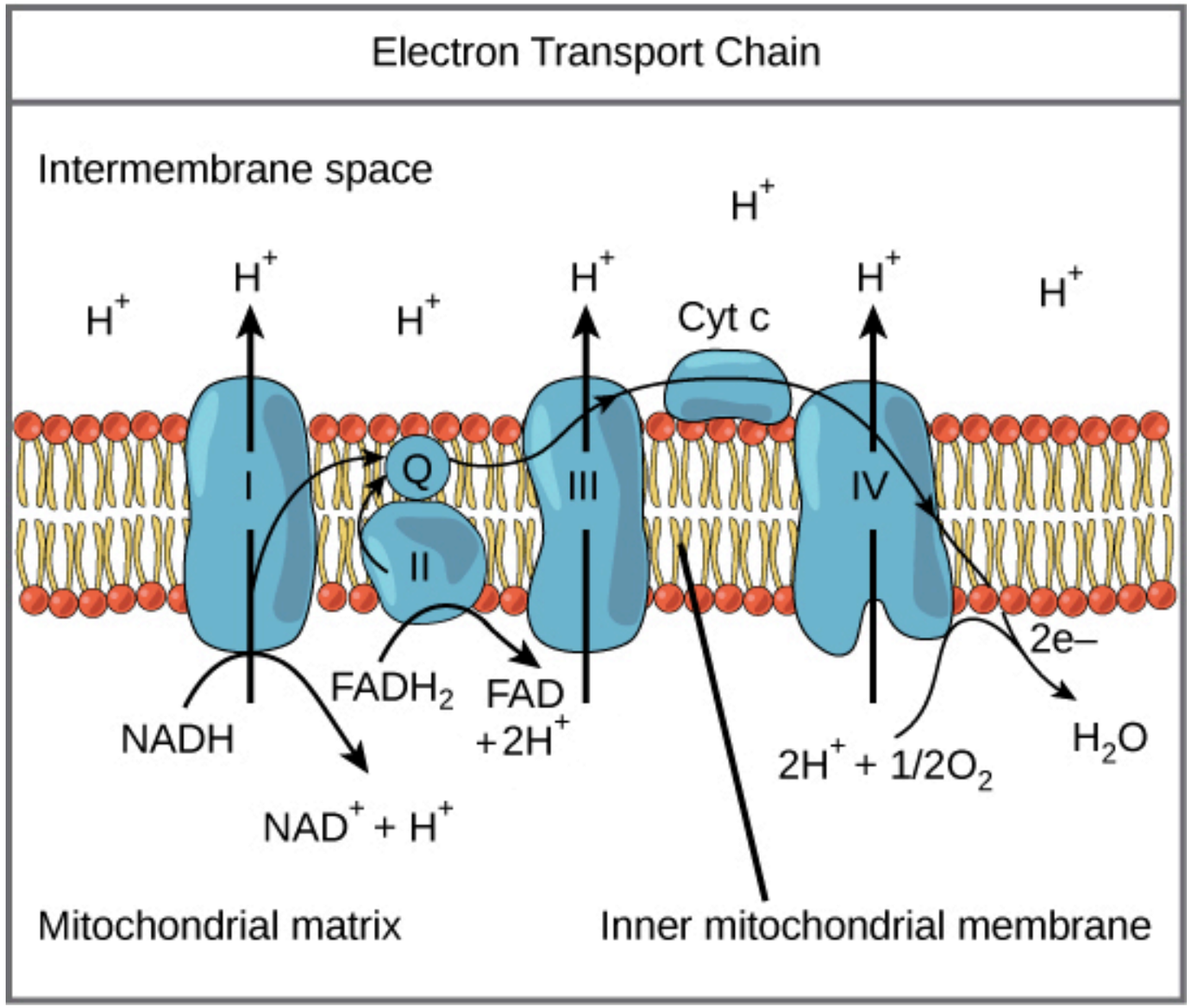
FAD oxidation

CoQ oxidation

Proton pumping

Cytochrome composition

Oxygen consumption



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| Complex assembly | ← | “Blue Native” gels |
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| FAD oxidation | ← | Spectrometry |
| CoQ oxidation | ← | Mass spectroscopy |
| Proton pumping | ← | Fluorescent probes |
| Cytochrome composition | ← | Spectrometry |
| Oxygen consumption | ← | Respirometry |

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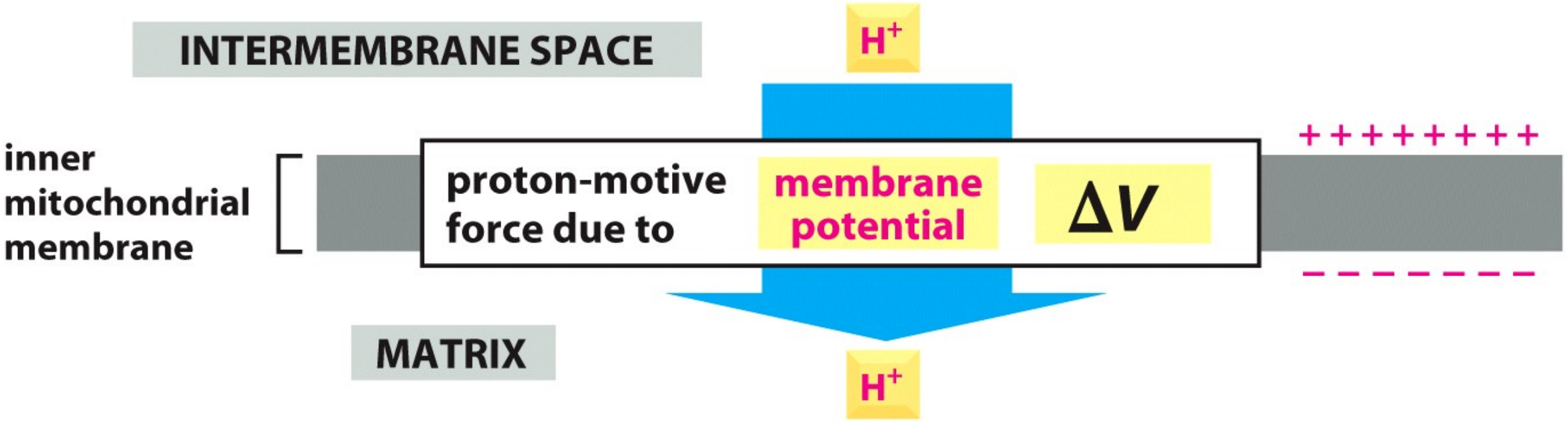
# Membrane potential is a proxy for proton pumping



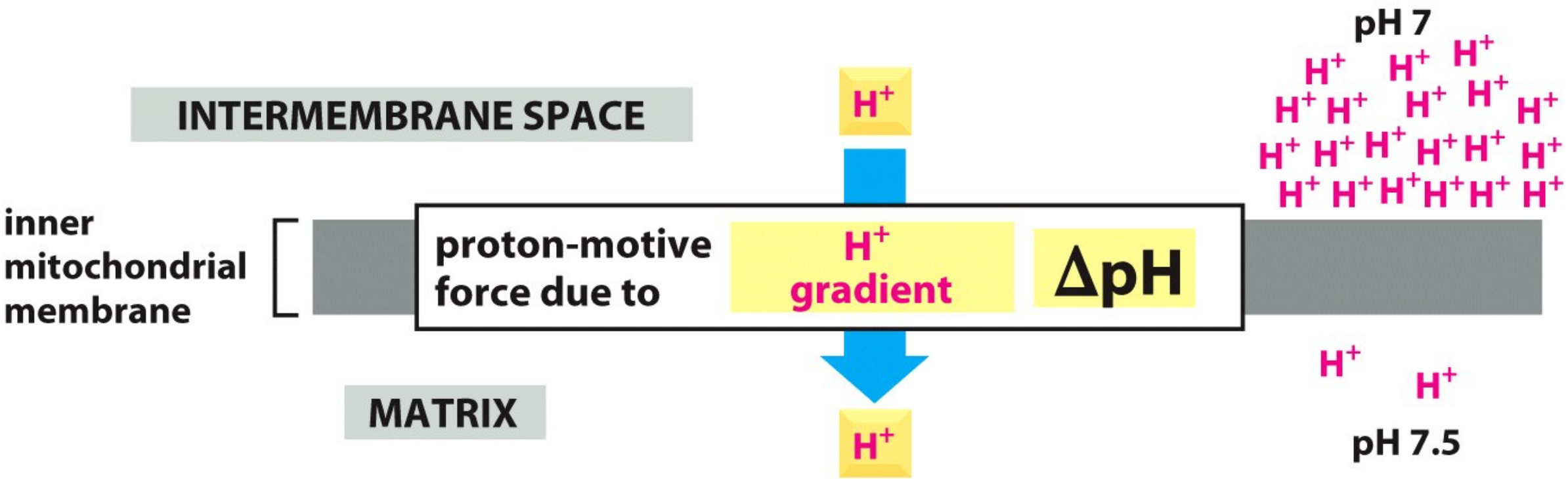
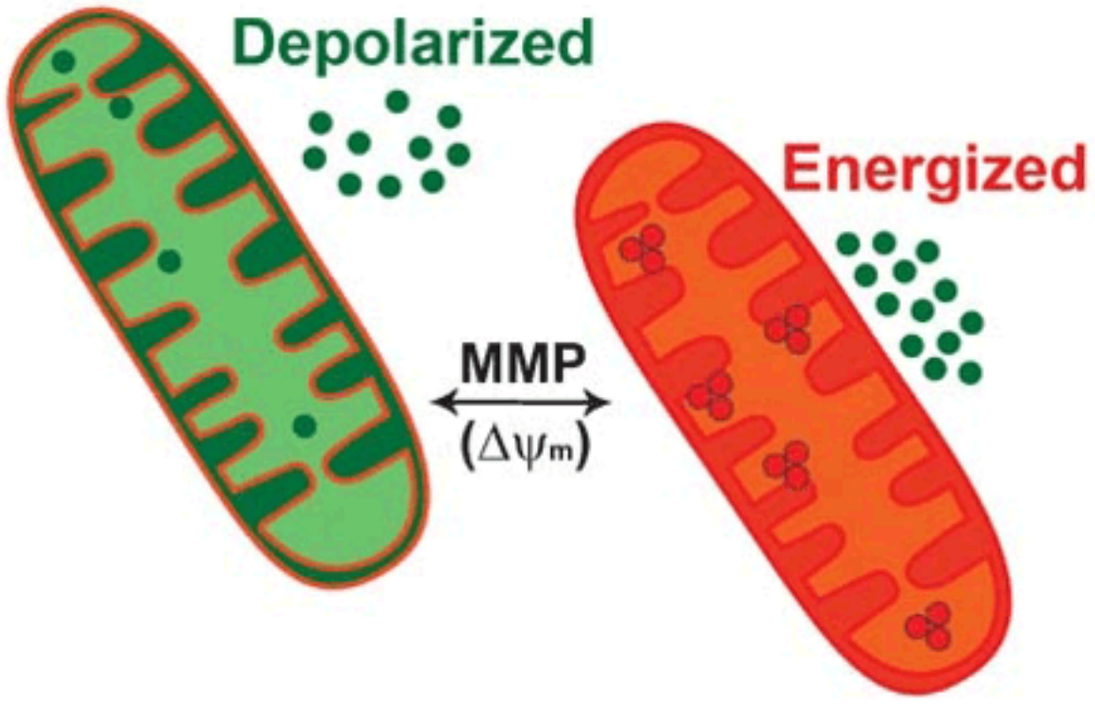




Figure 14-10 *Essential Cell Biology* (© Garland Science 2010)

# Membrane potential can be measured with fluorescent probes




**Probes:**

TMRE

TMRM

Rhodamine123

JC-1 (not ratiometric)

DiOC (toxic!!)

Probes must be:
- Targeted onto mitochondria (exploiting MMP)
- Fluorescent
- Change status according to acidity/membrane polarization

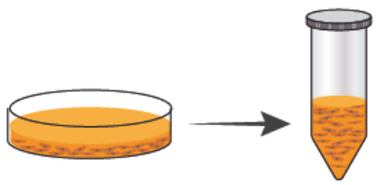
Readout:
- Flow cytometry
- Plate reader
- Imaging

# Membrane potential can be measured with fluorescent probes

## Imaging / Plate reader

### Day 1: Transduce and Plate Cells

**Step 1** Prepare Cells



**Step 2** Prepare Transduction Reaction

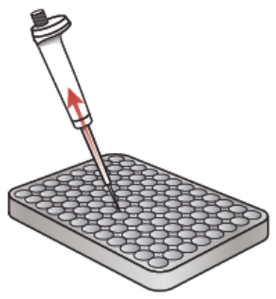


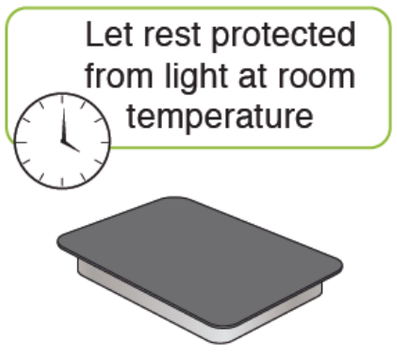

**Step 3** Mix Cells & Transduction Mix




### Day 2: Live Cell Assay

**Step 4** Add TMRE/TMRM






**Step 5** Measure Fluorescence

Standard Plate Reader

Microscope

Imaging Plate Reader

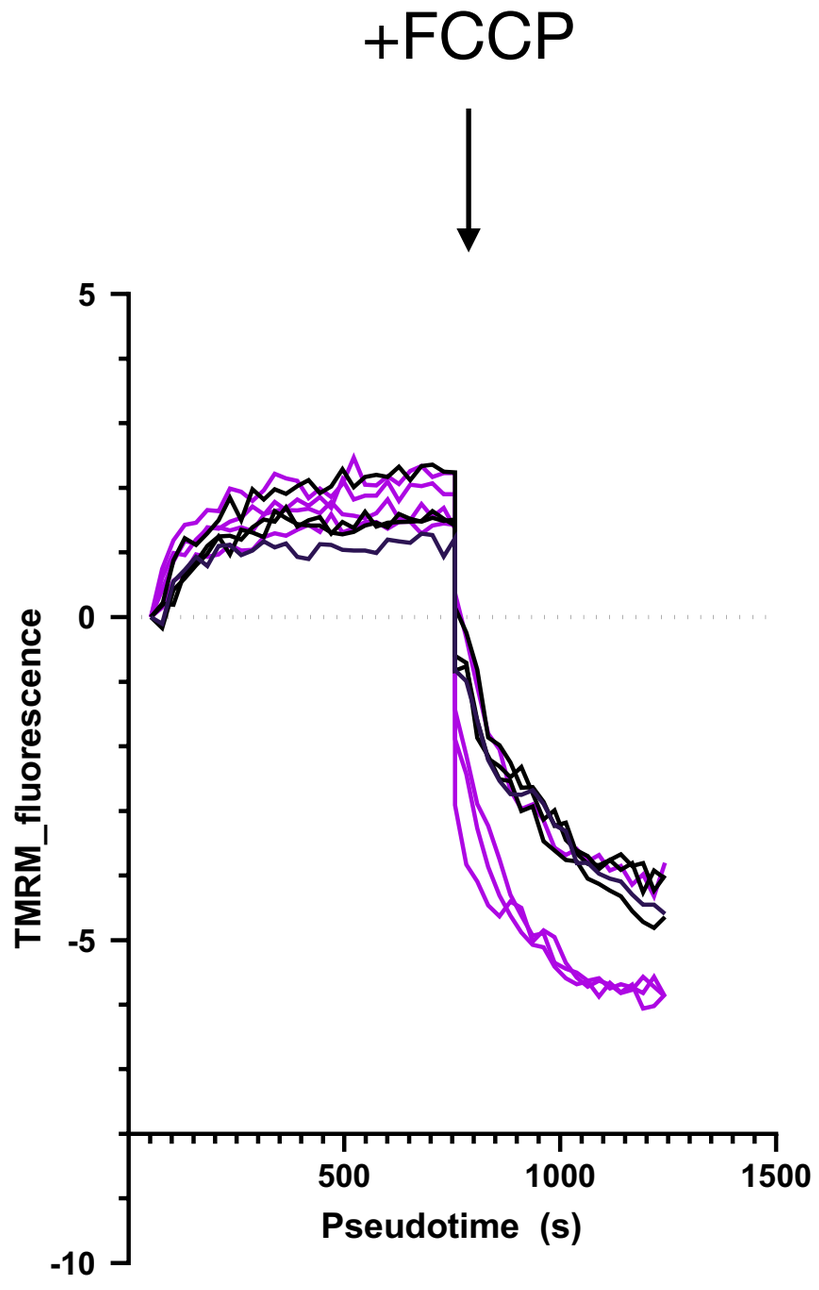
# Membrane potential can be measured with fluorescent probes

**Imaging:**

Distinguish single cells

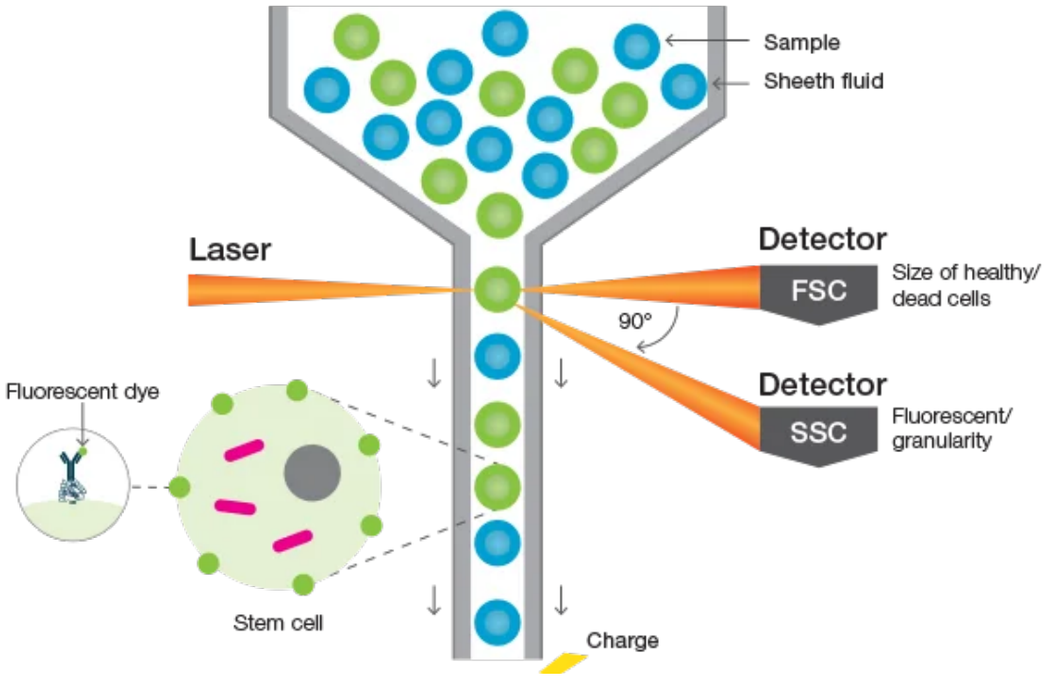
**Plate reader:**

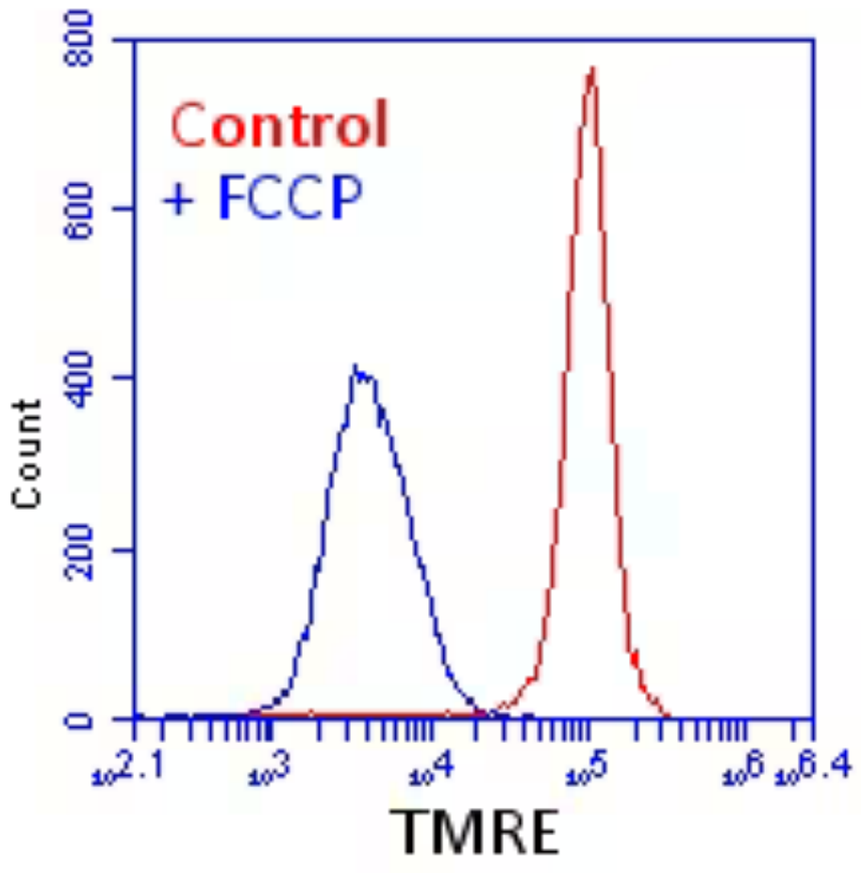
Quick!!



# Membrane potential can be measured with fluorescent probes

## Flow cytometry



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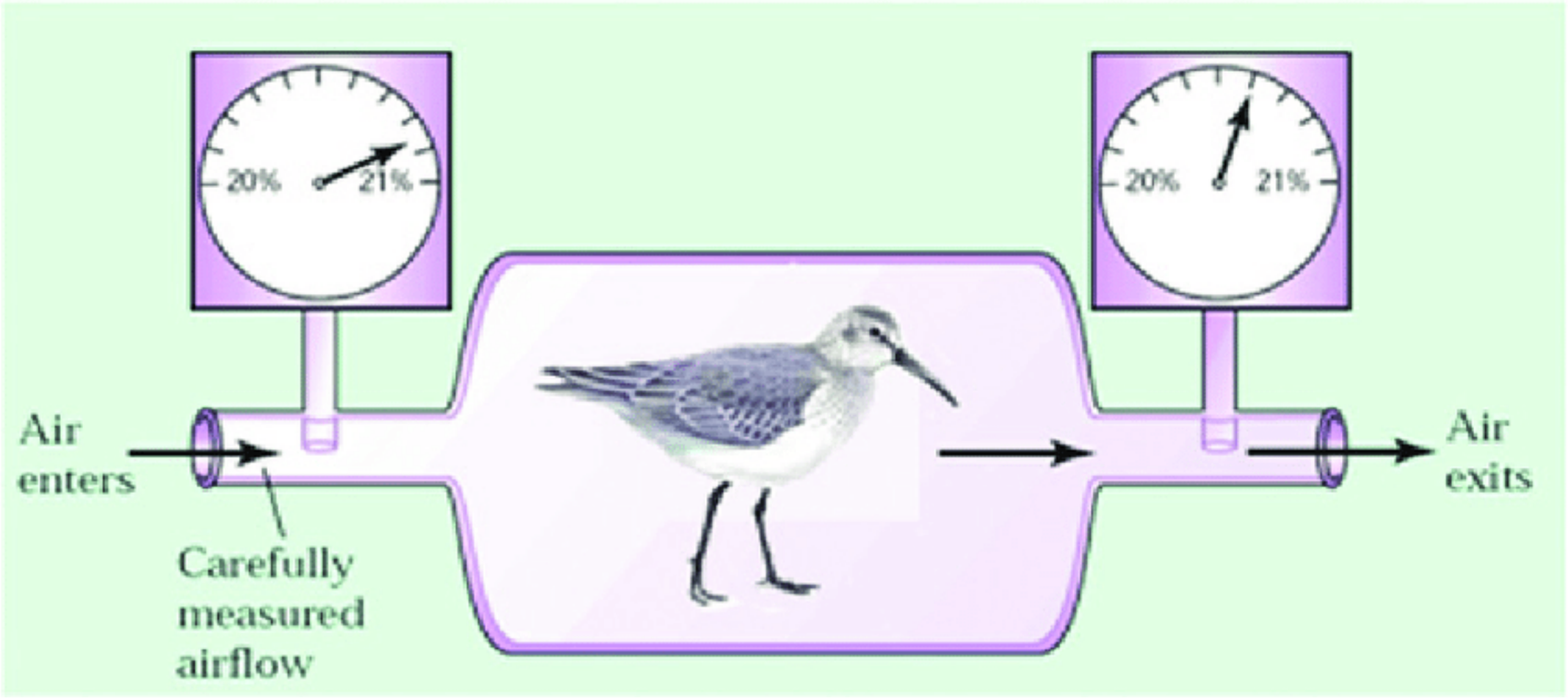
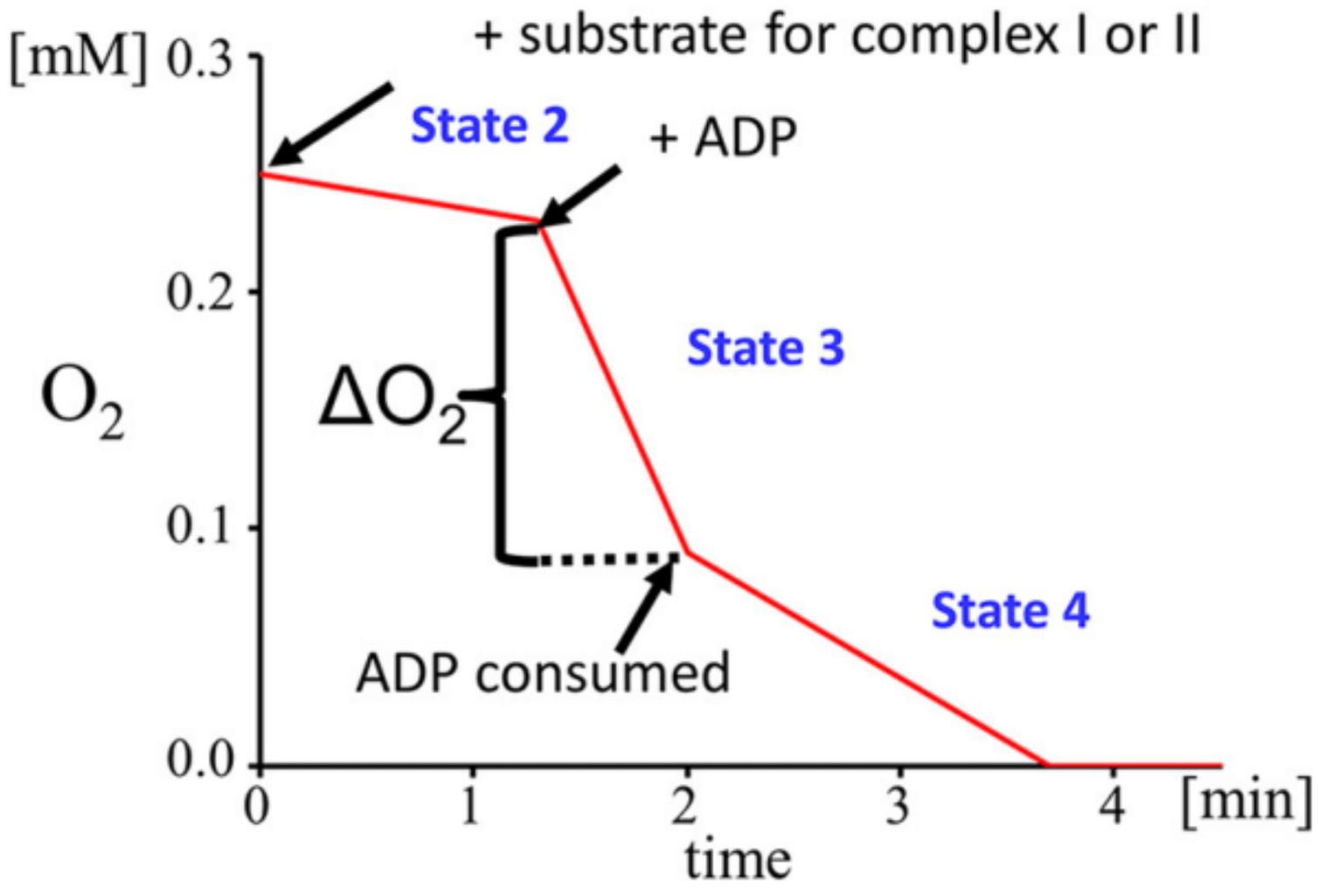
# The Clark electrode

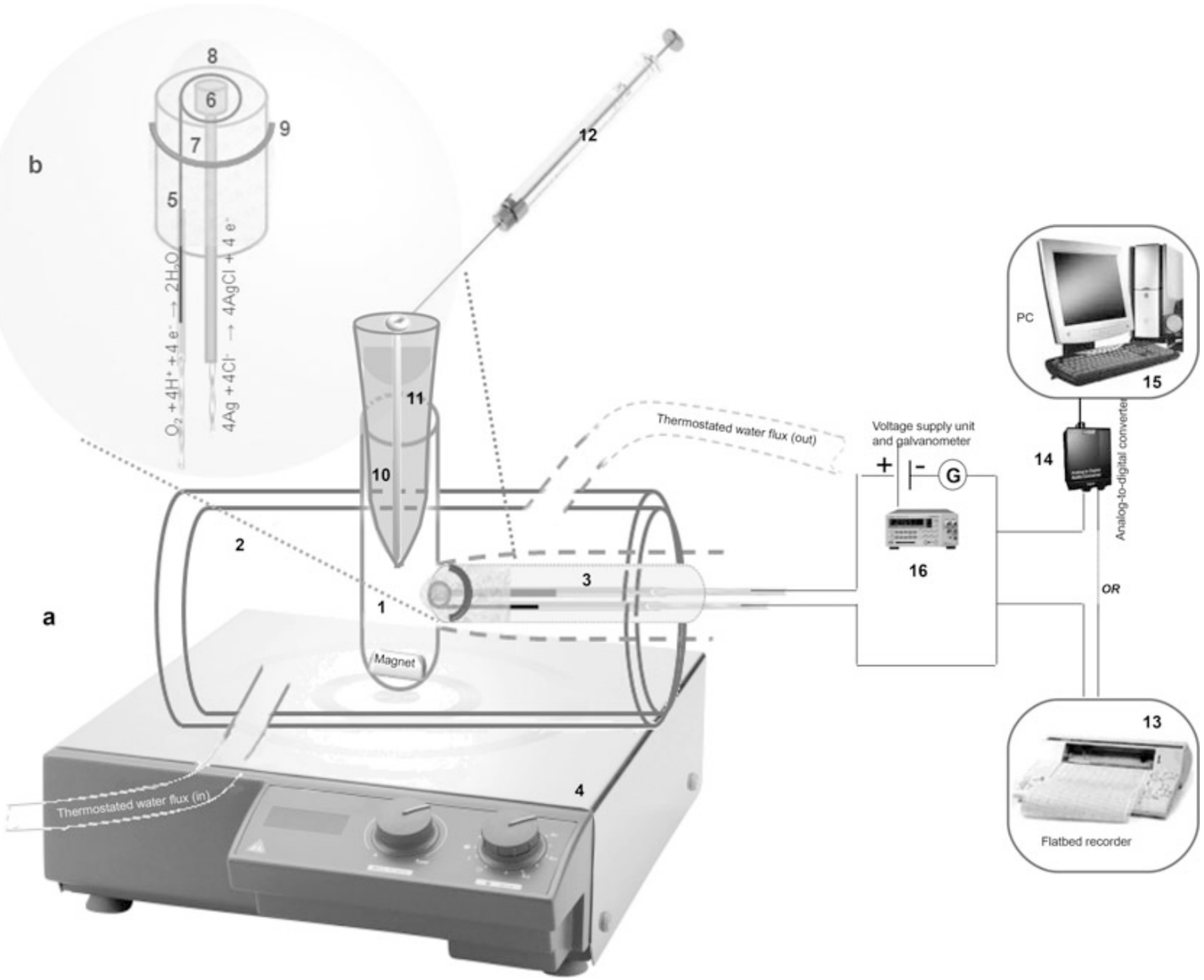

20%
21%
20%
21%
Air
enters
Carefully
measured
airflow
Air
exits

# The Clark electrode




The slopes of different mitochondrial states can be used to infer Complex activity and NAD/FAD oxidation

# The Clark electrode




General representation of the Clark-type electrode and experimental setup.

(**a**) The biological preparations (mitochondrial or cell suspensions) are introduced in a well-defined volume of media (1) with an oxygen electrode inserted (3) and magnetic stirrer coupled (4). The electrode determines O2 concentration in aqueous solutions over a period of time.

(**b**) Oxygen electrode inset. The electrode itself is located inside the chamber. The platinum cathode (5) is located surrounding a rod-like center anode (6) made by silver (reference Ag/AgCl electrode).

The O2 recordings can be done in open or closed chamber mode. In the last case, the reciprocal air solution O2 diffusion is avoided, which allows for better determination of the respiratory rates. Experimental additions of solutions, substrates, mitochondria, and substrates/inhibitors are done through the top of the chamber (10) (open mode) or through the small hole (11) inside the stopper, respectively, using a glass syringe (12).

# OROBOROS


Oroboros O2k
Titration-Injection microPump TIP2k
Barometric pressure transducer
Peltier temperature control, ±0.001 °C
Stainless steel housing
Syringe A
Needle A
Stopper A
Window Chamber A
Smart Fluo-Sensor B
O2 Sensor POS B
ISS
O2k-Titration Set
A
B
O2 Sensor POS A
Smart Fluo-Sensor A
Insulated copper block
Stirrer control light B
Smart Fluo-Sensor plug B
Ion-Selective Electrode plug B
POS-Connector B

# Respirometry

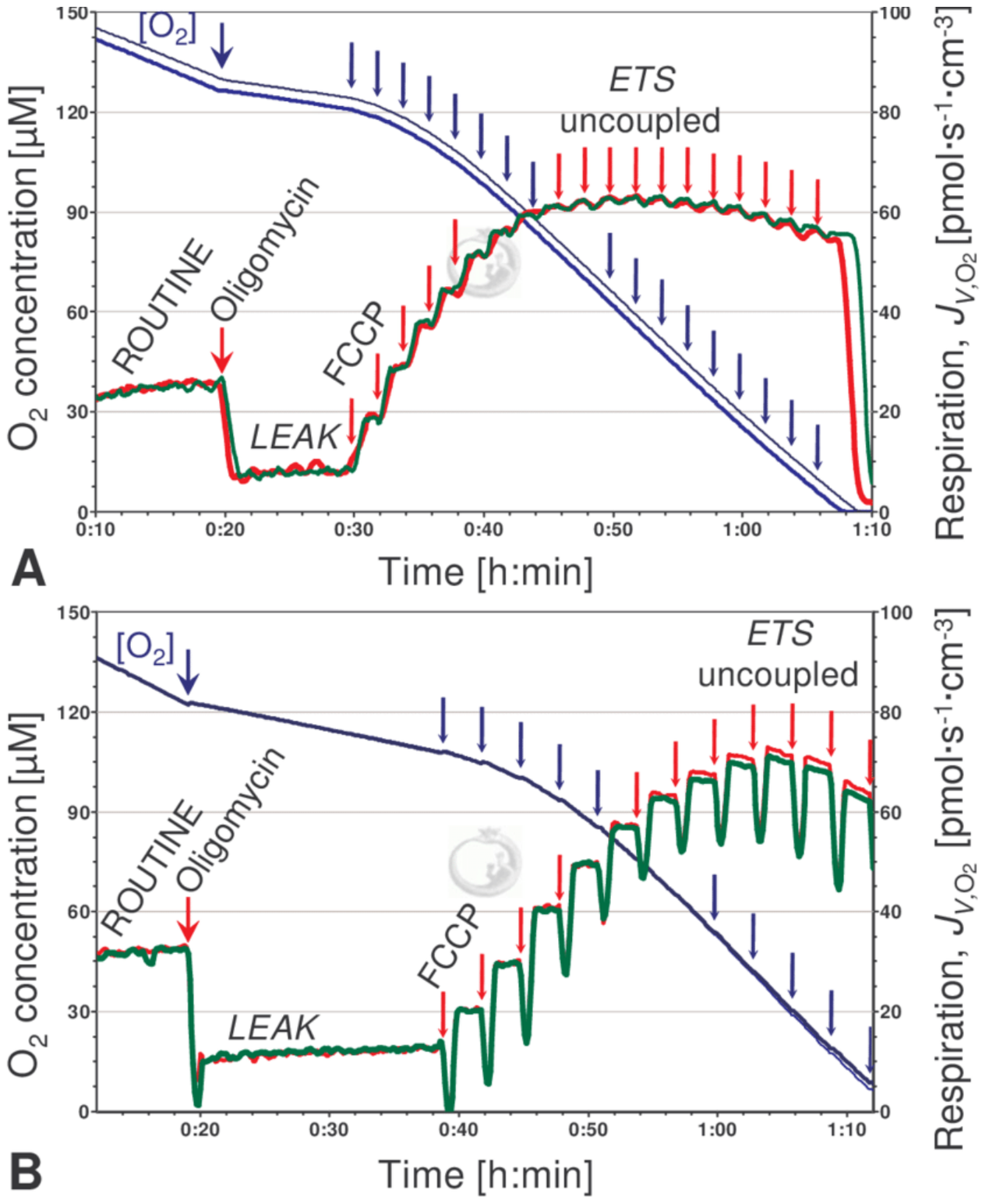
Respirometry reflects the function of mitochondria as structurally intact organelles. It provides a dynamic measurement of metabolic flux (rates), in contrast to static determination (states) of molecular components, such as metabolite and enzyme levels

Mitochondrial respiratory function cannot be measured on frozen tissue samples but usually requires minimum storage times of biological samples and delicate handling procedures to preserve structure and function or highly specific cryopreservation.

Mitochondrial respiration yields an integrative measure of the dynamics of complex coupled metabolic pathways, in contrast to monitoring activities of isolated enzymes.

Understanding mitochondrial respiratory control, in turn, requires experimental modulation of metabolite levels, electrochemical potentials, and enzyme activities.

# Respirometry




Oxygen consumption rate is an integration of oxygen levels over a certain period of time

# Respirometry: stress test

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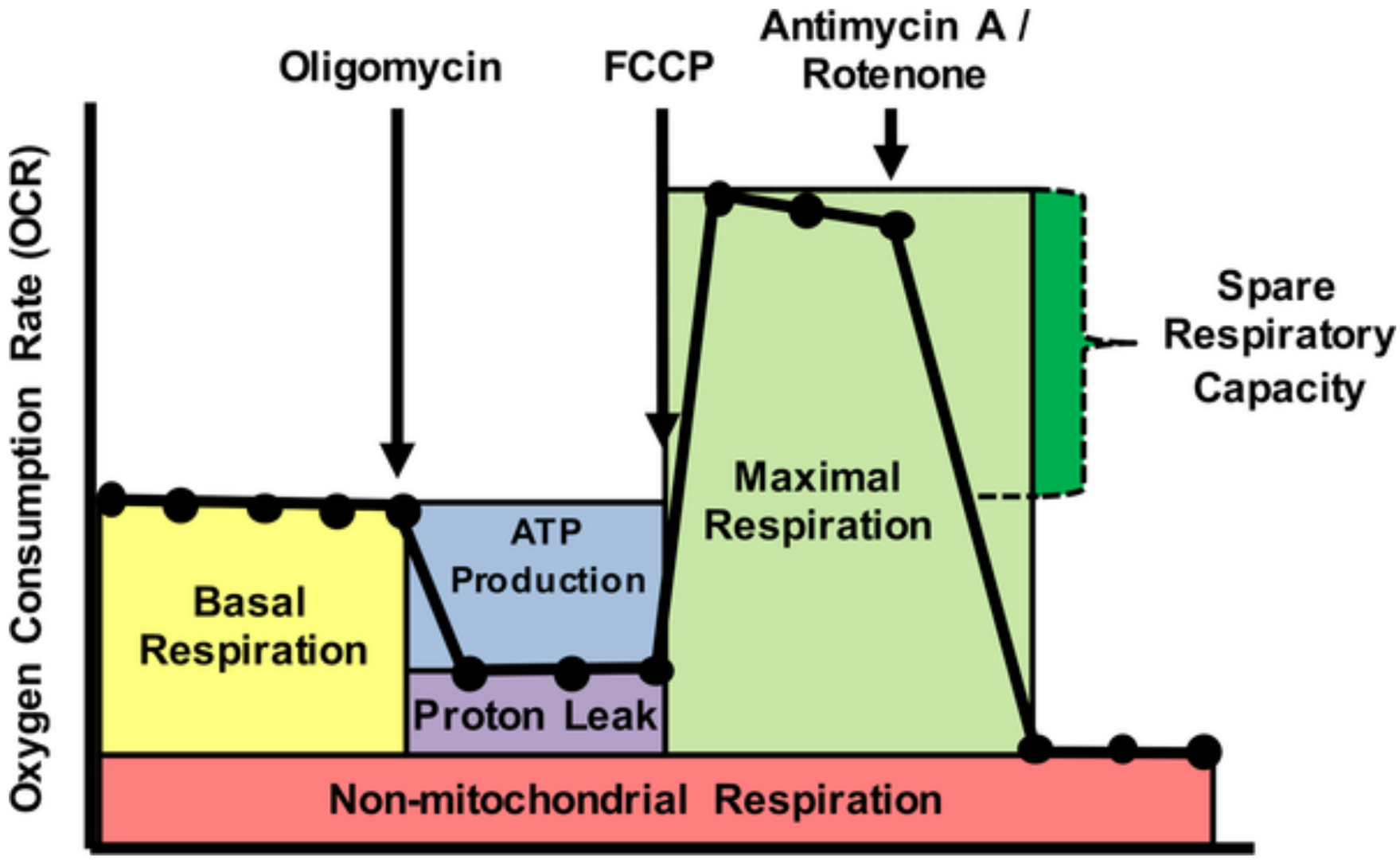

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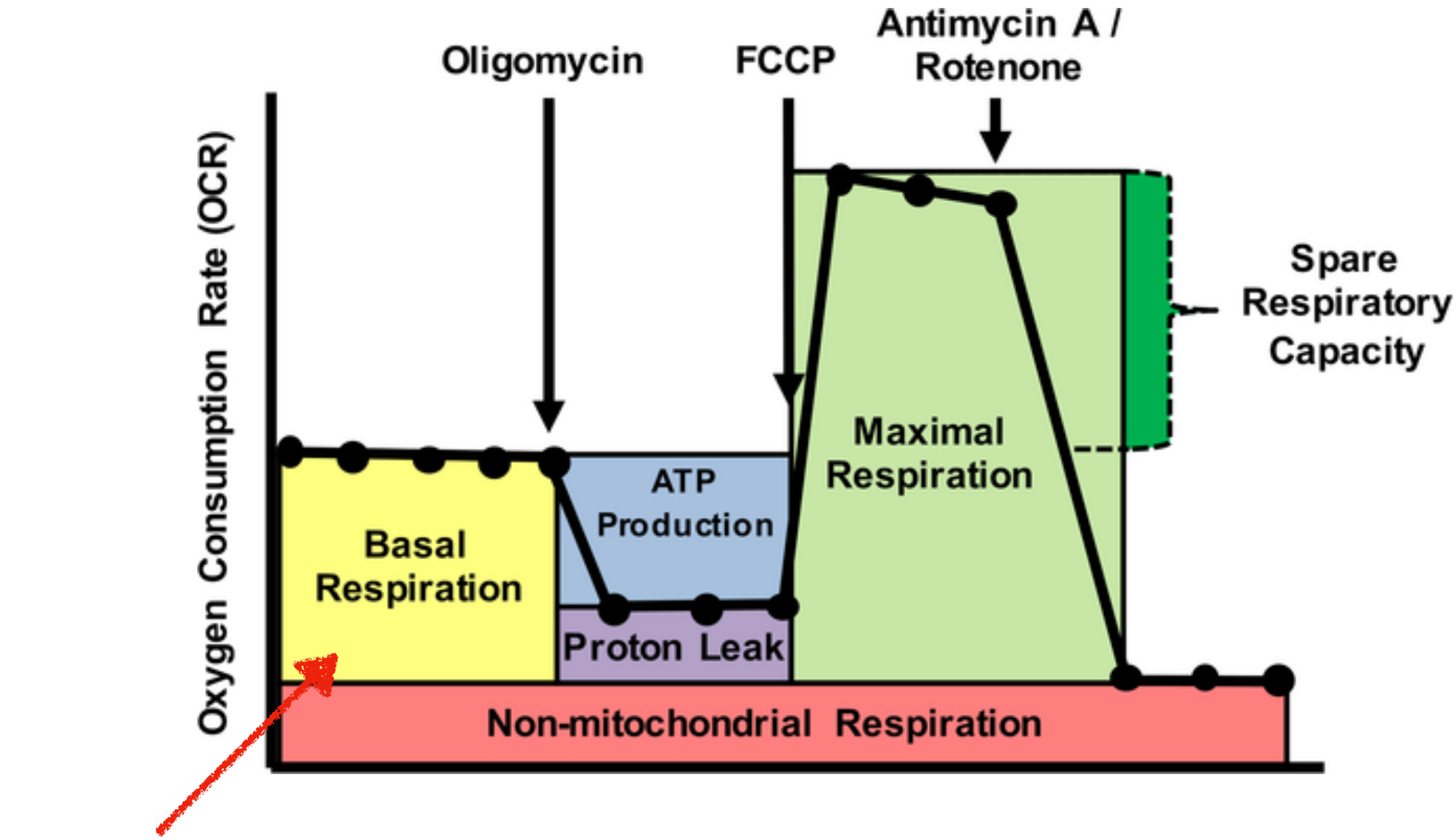

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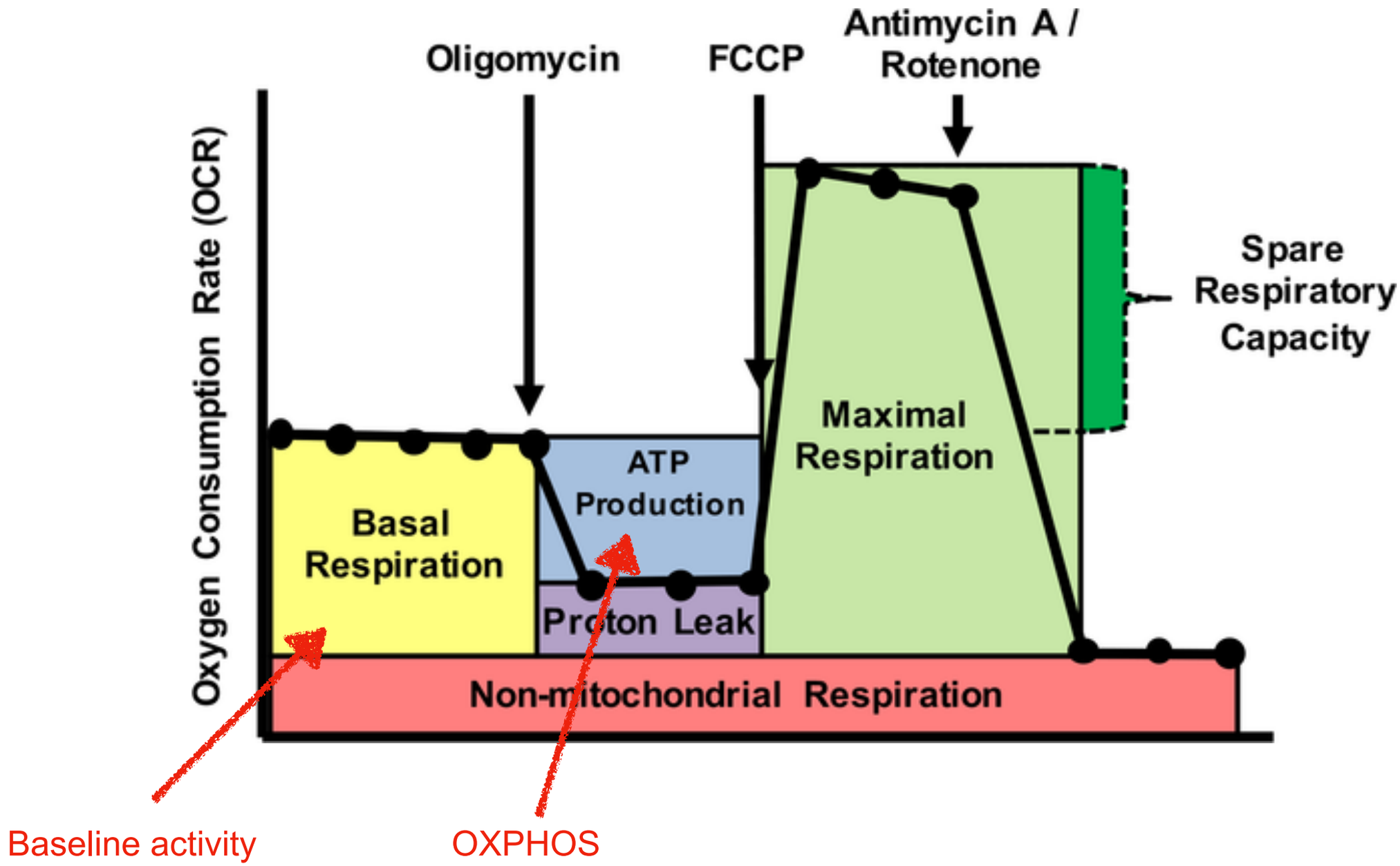

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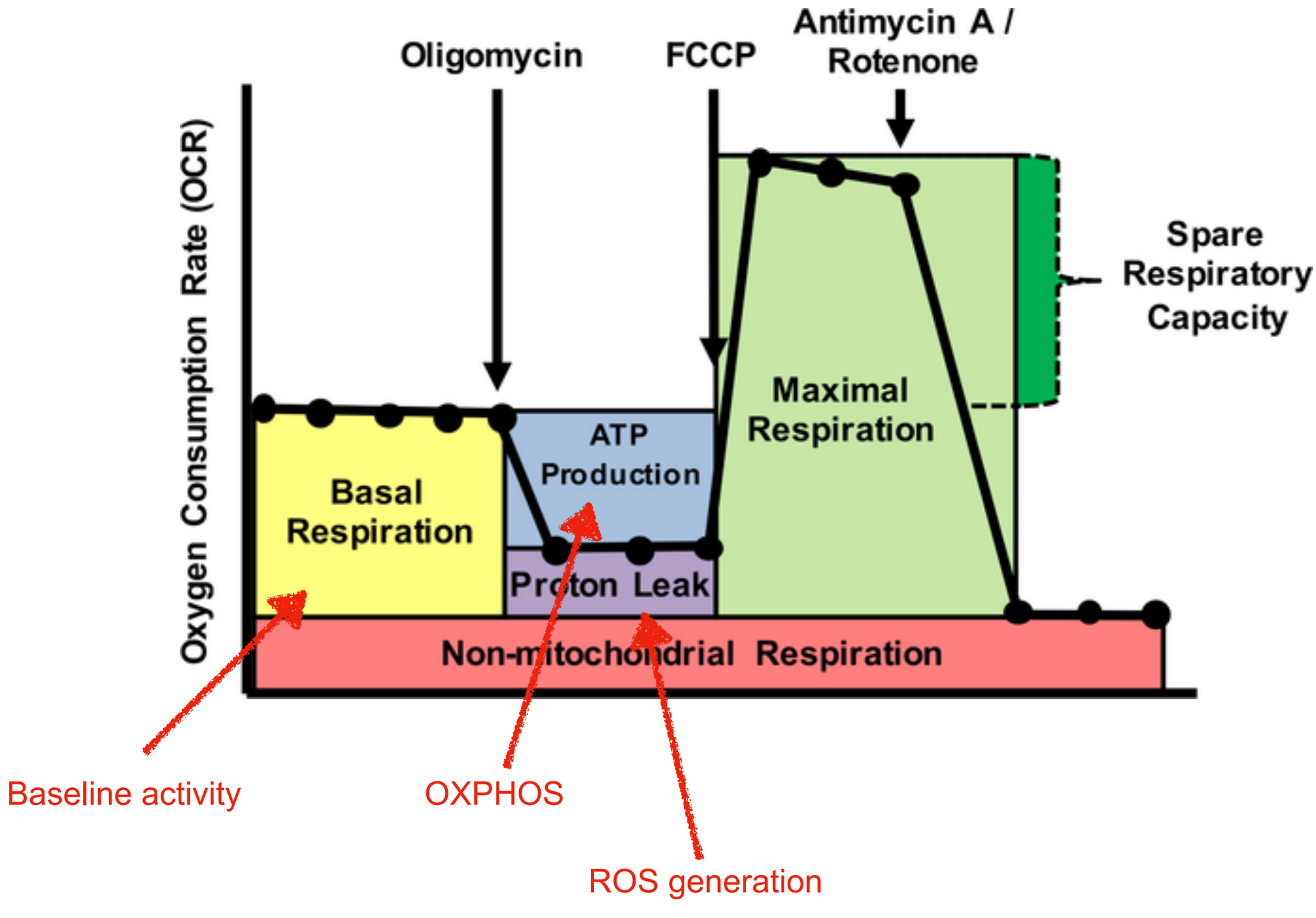

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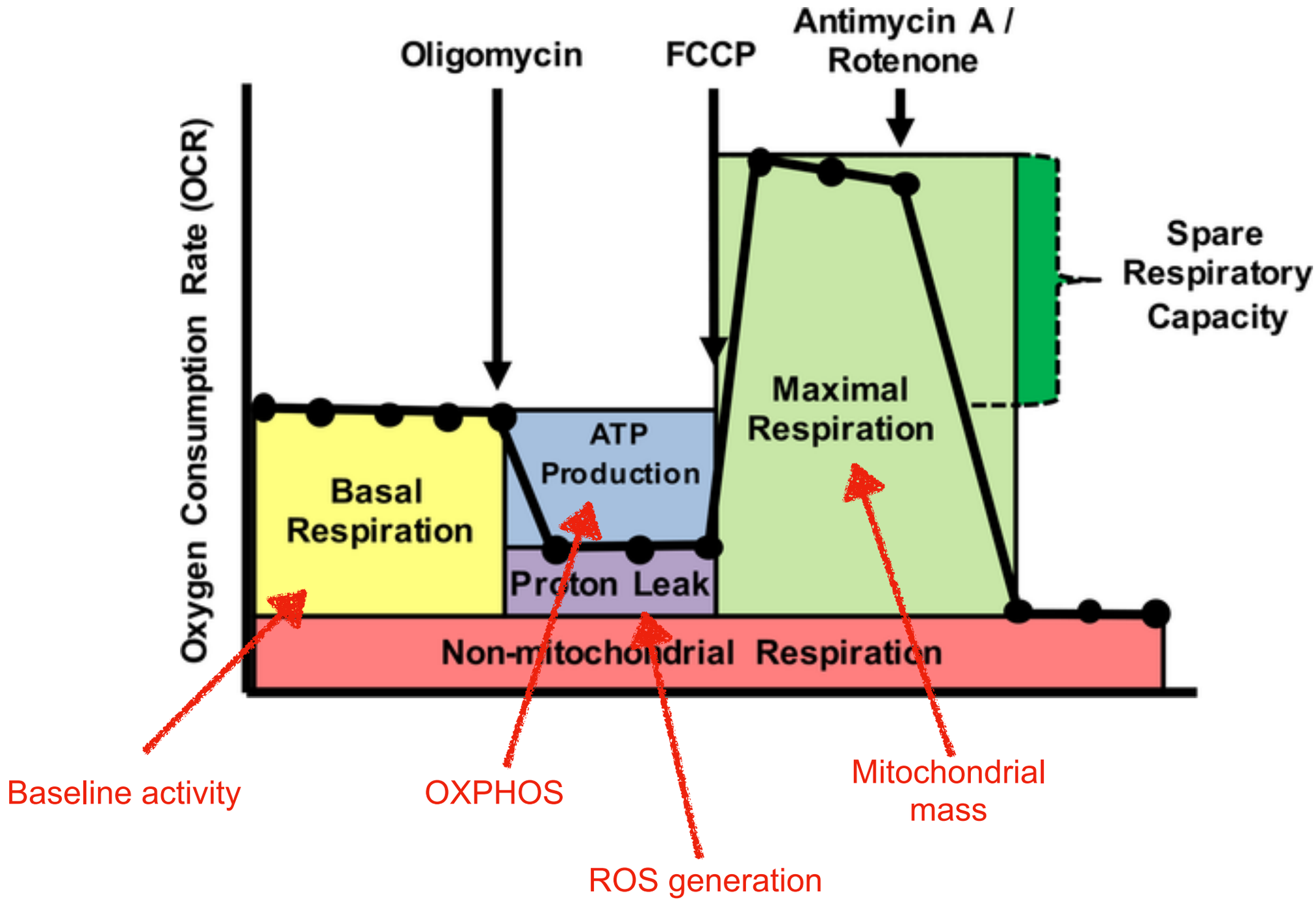

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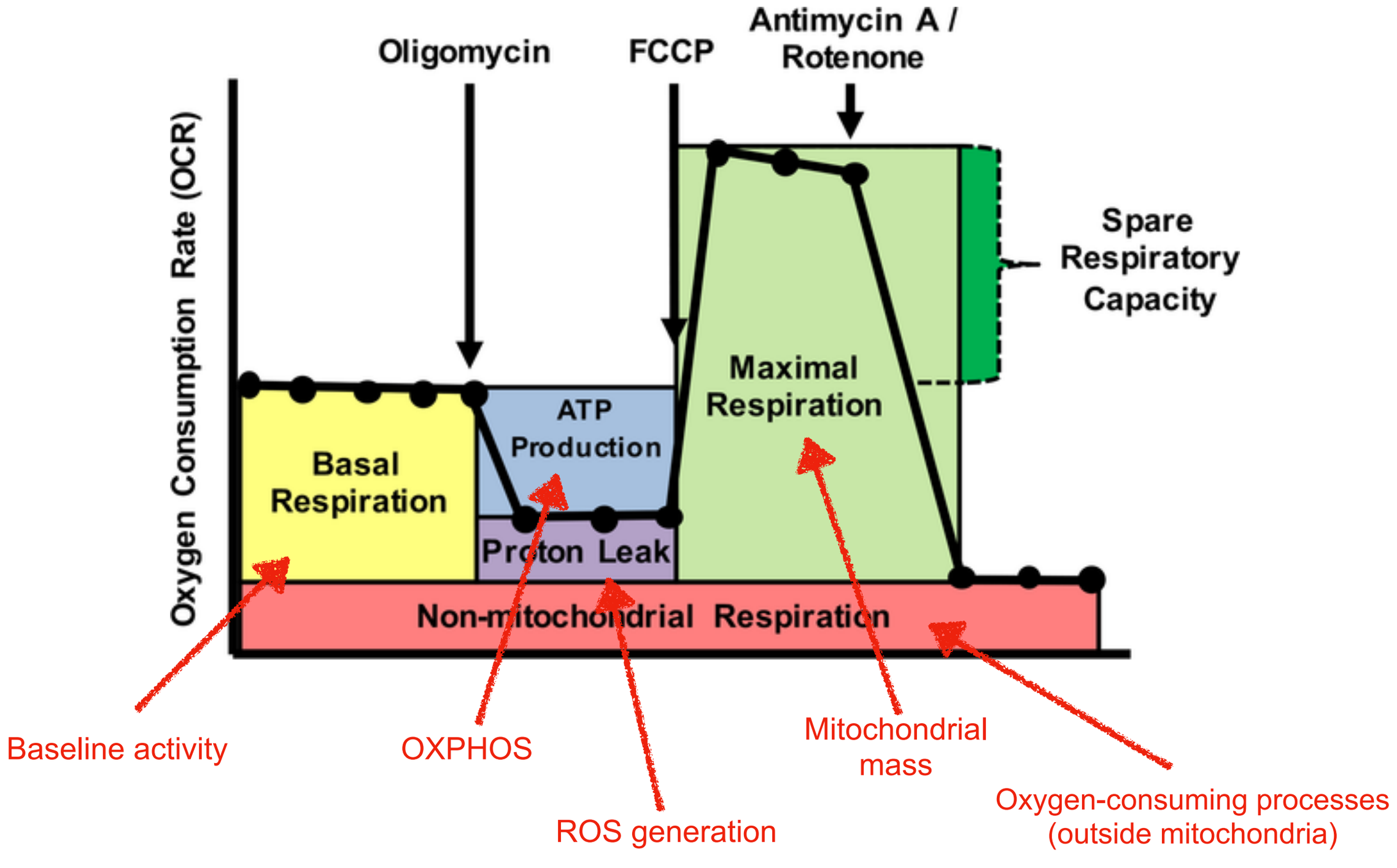

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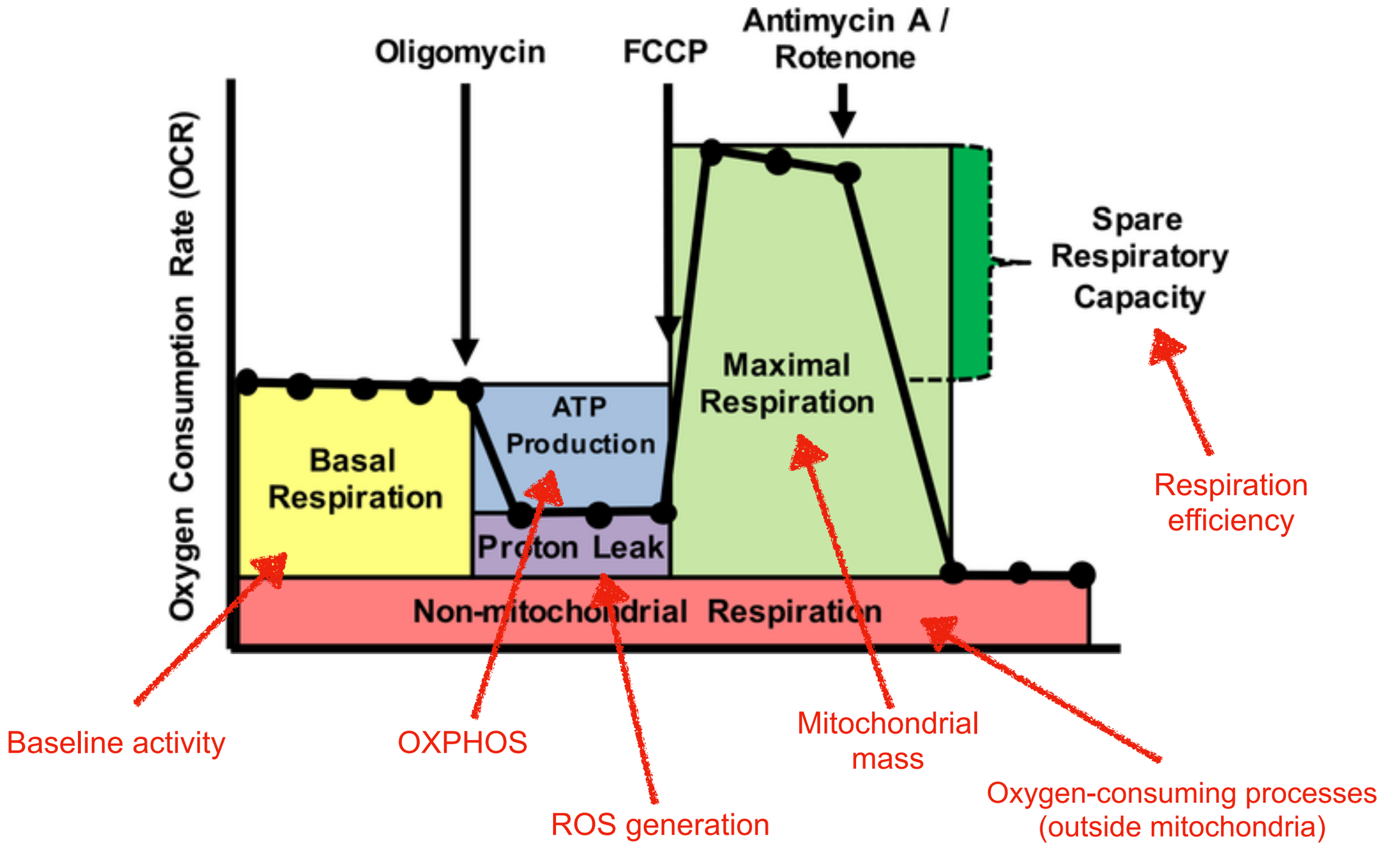
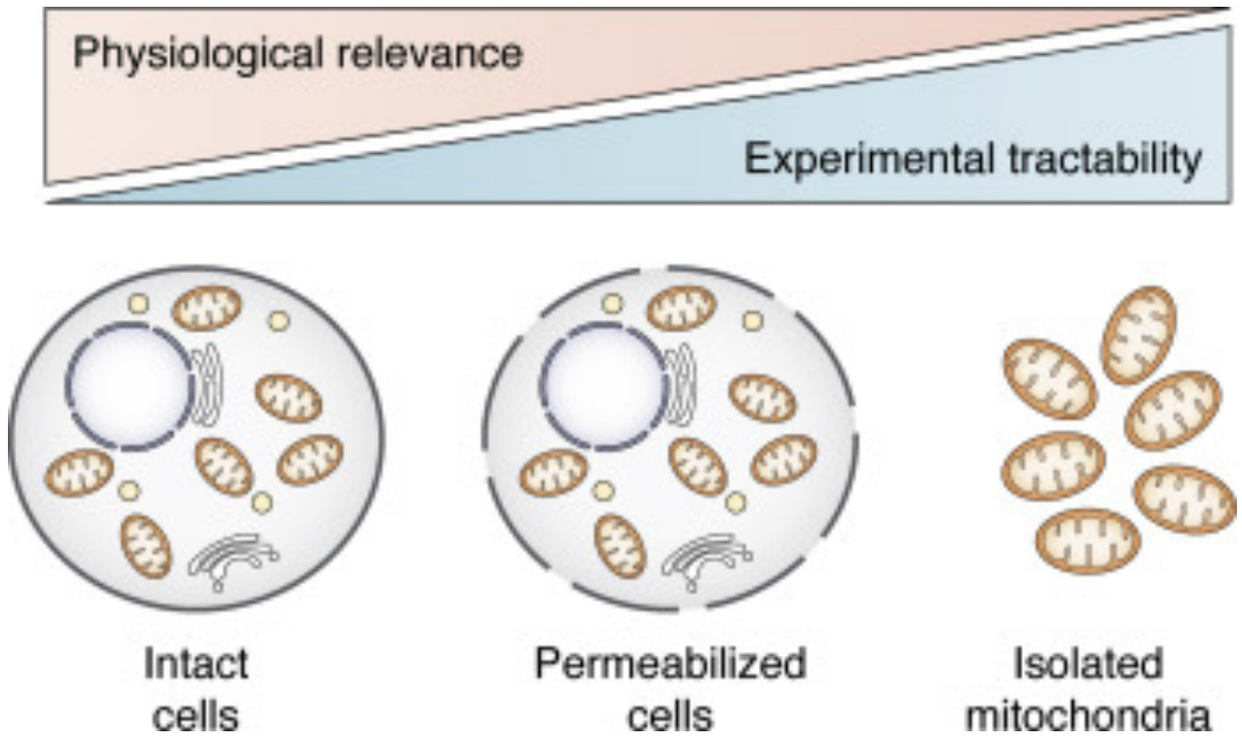


# Respirometry (issues)




*Schmidt et al, **JBC**, 2021*

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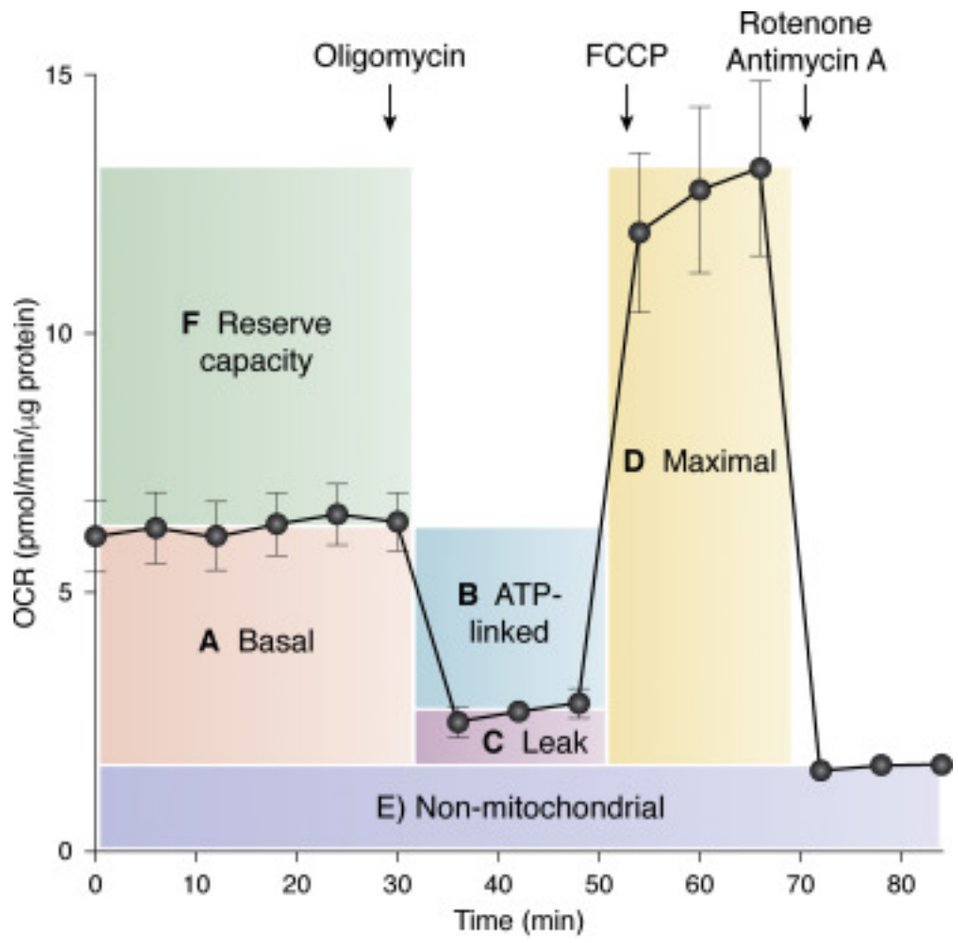
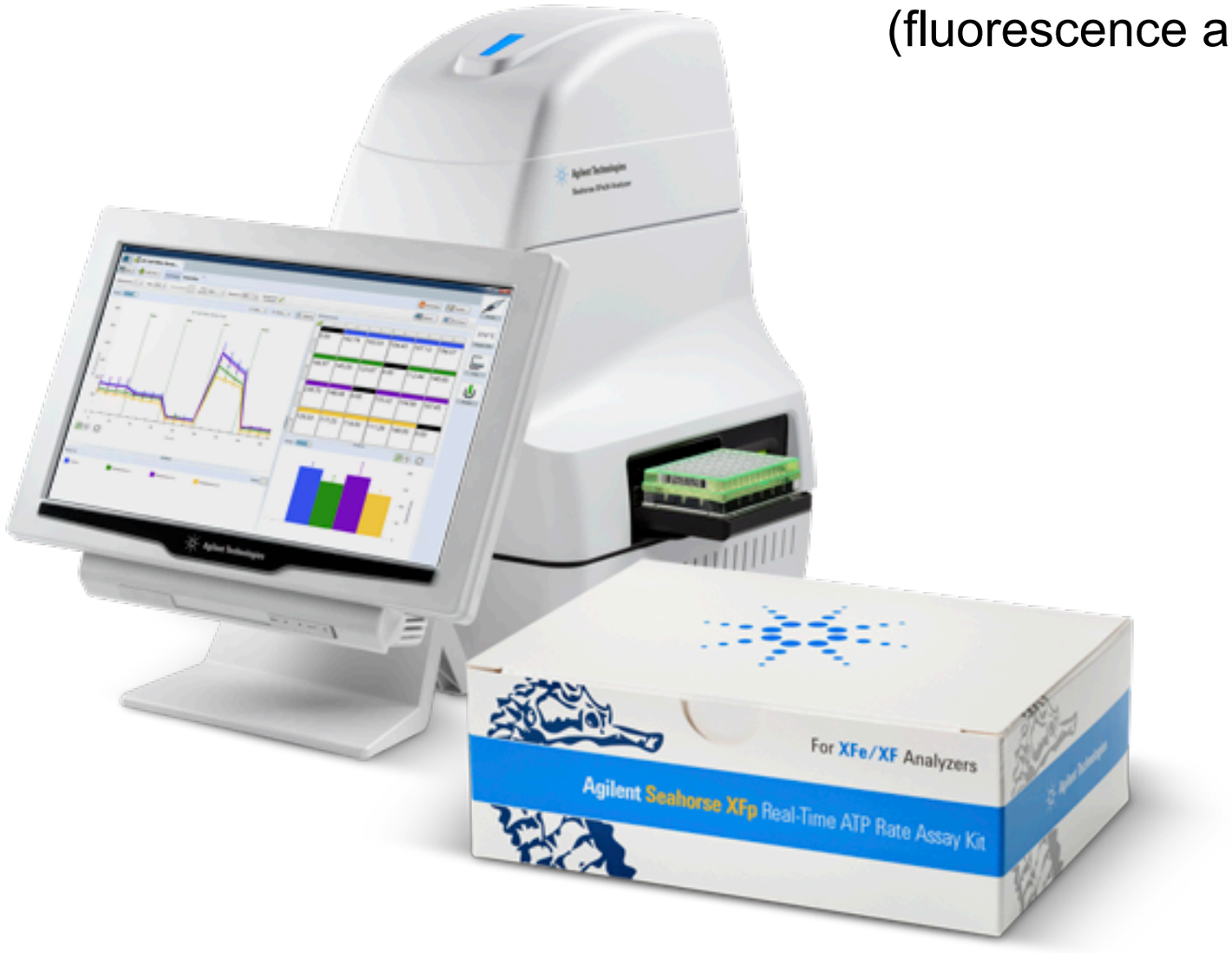



| | |
|---|---|
| **A** | Ambiguous; mixed patterns of substrate oxidation |
| **B** | Ambiguous; subject to complex caveats |
| **C** | Ambiguous; lacks information about non-oxphos-linked respiration |
| **D** | Non-physiological; may interfere with transport processes |
| **E** | Ambiguous; may change as a result of conditions |
| **F** | Non-physiological; likely limited to very specific cell/tissue types |

*Schmidt et al, **JBC**, 2021*

# Respirometry: SeaHorse®




Based on different technology (fluorescence analyzers)

PRO: easy to use

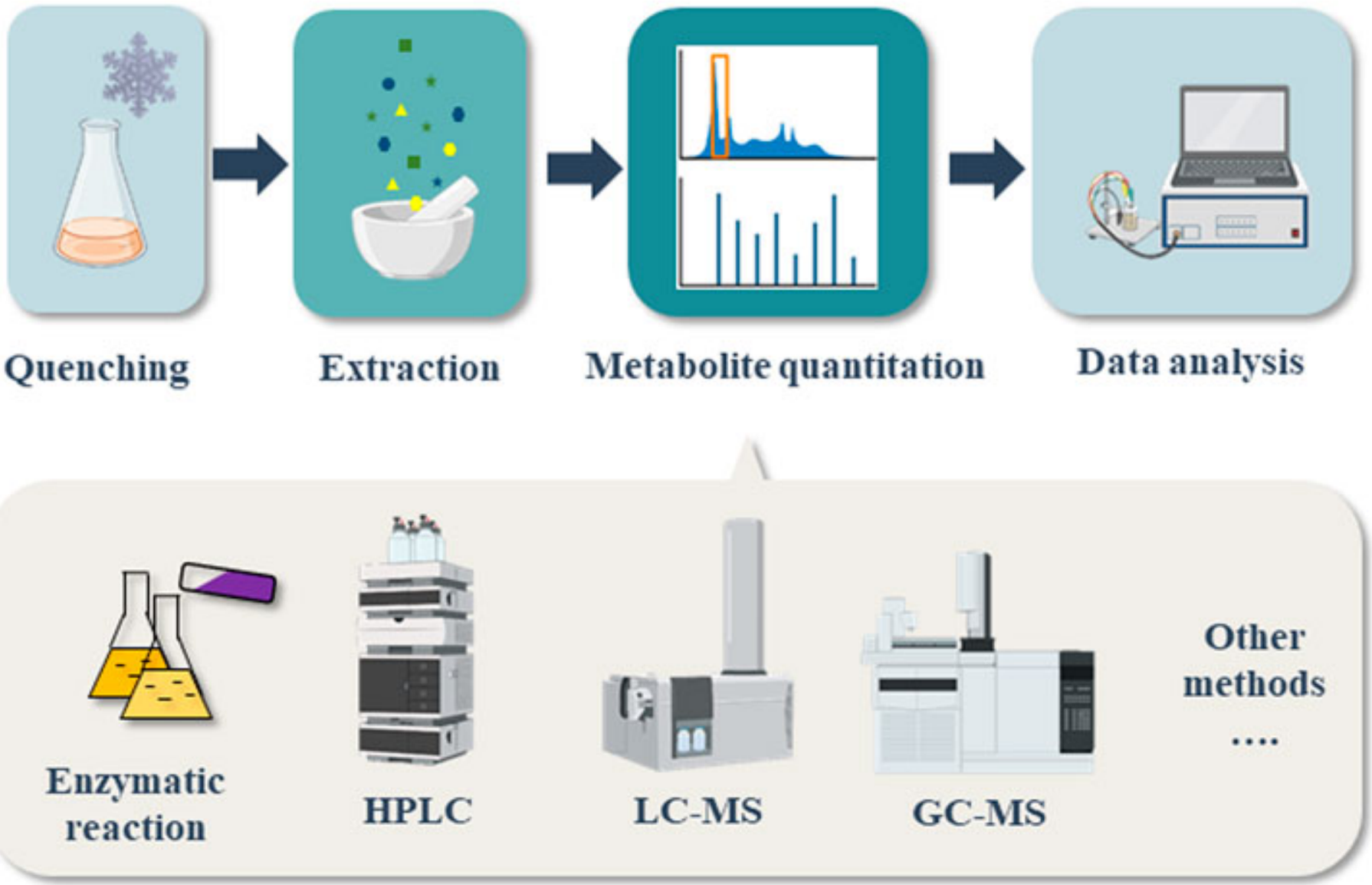
Oxygen consumption + extracellular acidification rate (EACR)

CON: not a direct measure

Works best on intact cells

# Measure metabolites and macromolecules

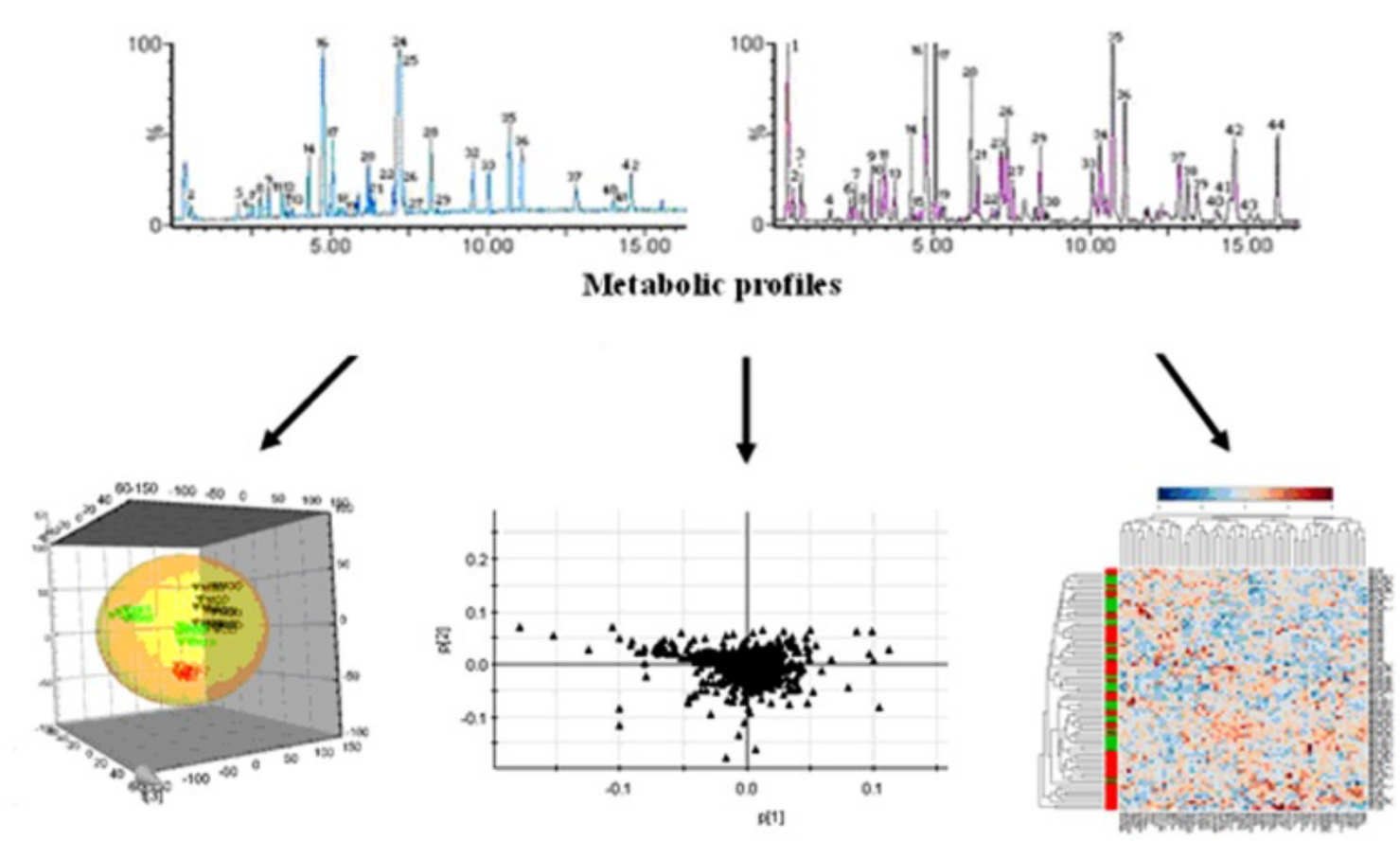
Quantitative measures rely on biochemical analytical approaches:




*Wang & Luo,* ***Front Bioeng Biotech****, 2022*

# Spectrometry (NMR or MS)

Analytical approaches that resolve a mixture of components into spectra defined by multiple peaks (where each peak correspond to a different molecule).
These peaks are separated according to different physical properties (mass, charge, solubility, etc).
You obtain a “fingerprint” that is a snapshot of cell metabolism at a given time.




Common types of analysis include those that quantify principal components, associate hierarchical clusters, create partial least squares, discriminant function, or even form artificial neural networks. Collectively, this analysis helps identify and discriminate the function of the metabolites in the sample, where databases can secondarily be used to validate specific pathway activity.

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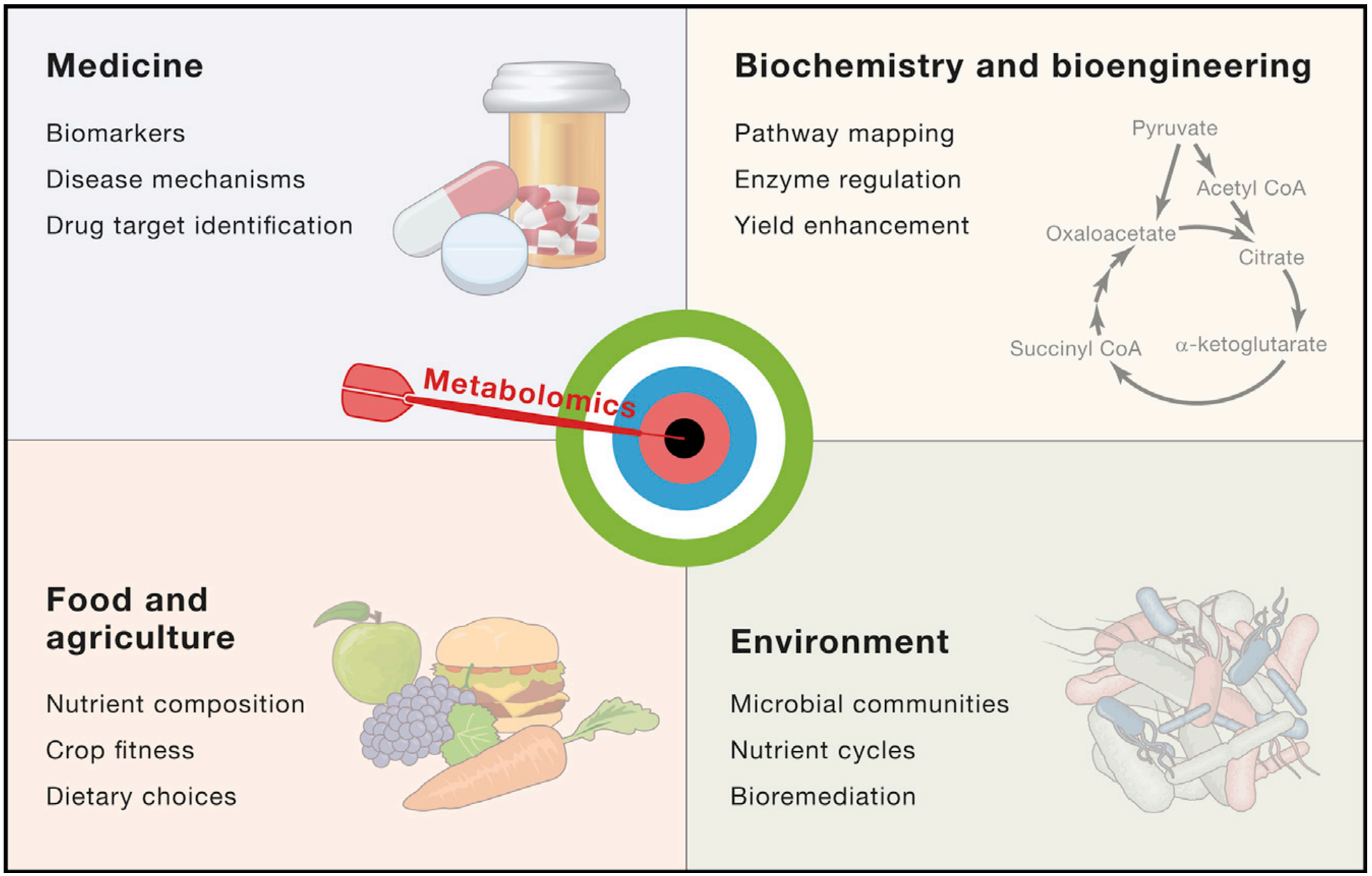
**Pros:**

- Sensible
- Robust
- Large-scale approach (METABOLOMICS)

**Cons:**

- Difficult (requires extensive training)

# Metabolomics




Metabolism plays a central role in all areas of biology, from ecology to bioengineering to cancer. Each of these areas is now being increasingly examined from a metabolic viewpoint and there is high value to taking a big-picture perspective. This is feasible due to advances in metabolite measurement technologies like NMR and mass spectrometry (Fiehn, 2002; Beckonert et al., 2007).

# Metabolic flux analysis

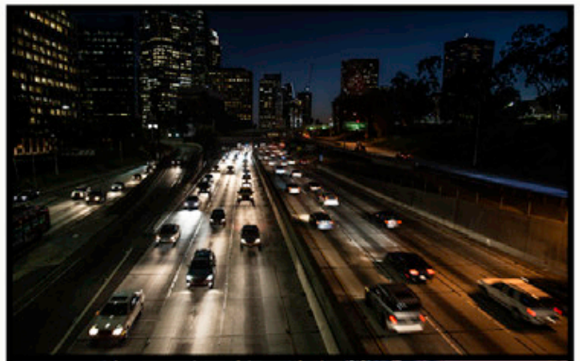
Measurement of metabolite concentrations by metabolomics, however, tells only half the story. Equally important is understanding pathway activity, which can be quantified in terms of material flow per unit time, i.e., metabolic flux.

## <u>Concentrations and fluxes do not reliably align.</u>

This is intuitive to drivers: although flux increases with car density until traffic slows, a high concentration of cars on the road does not reliably indicate high flux. Similarly, in metabolism, metabolite build-up can occur not only due to increased production, but also due to decreased consumption.

Metabolomics
→ measures concentration

Isotope tracing
→ probes flux

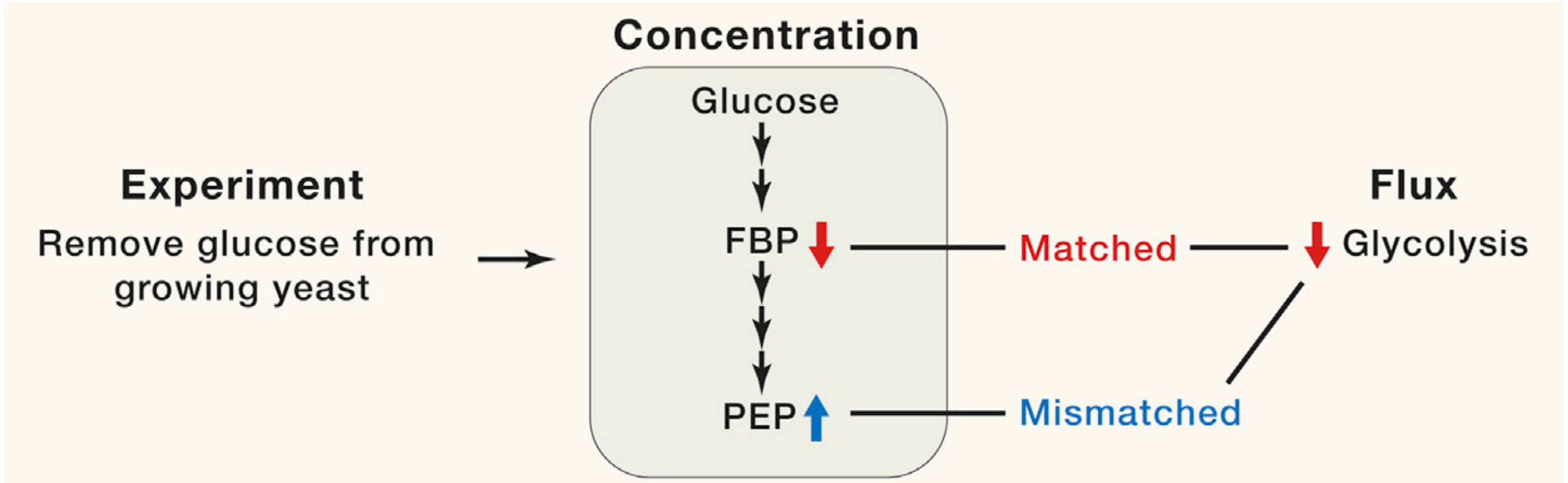


Flux increases with car density (concentration) until traffic slows

Very high car density but low flux

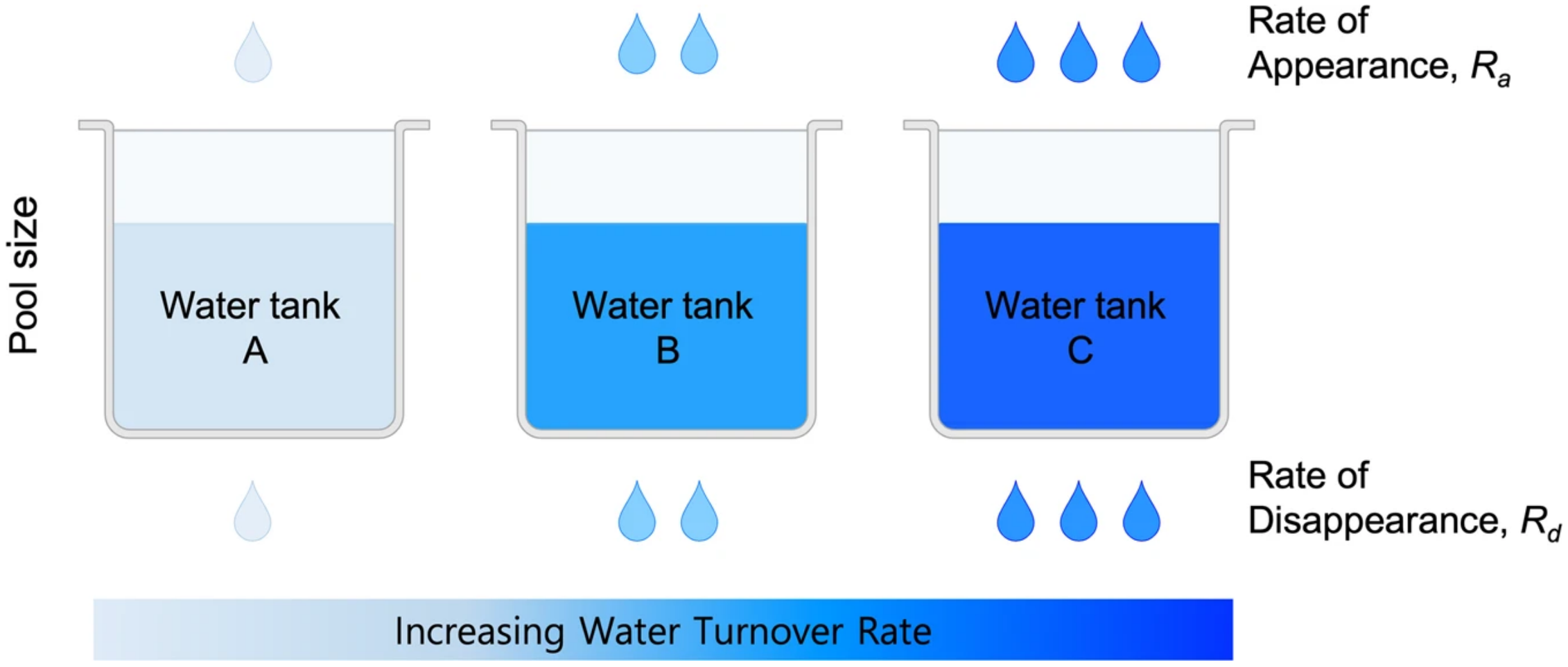
# Metabolic flux analysis

*For example, when glucose is removed from yeast, glycolytic efflux drops sharply, leading to build-up of lower glycolytic intermediates even though pathway influx is decreased (Lowry et al., 1971; Xu et al., 2012).*




**Because metabolite levels and fluxes provide complementary information, metabolic understanding is best achieved by investigating both.**

# Metabolic flux analysis




While the water pool size does not change if the two rates are identical, regardless of their absolute rates, differences in the water turnover rate may affect the quality of the water.

*Kim et al,* ***EMM****, 2022*

# Metabolic tracing

Metabolomics measures metabolite abundances. While informative, metabolite abundances do not reveal pathway activities: metabolite levels are determined by the balance of production and consumption in a nonlinear way. Accordingly, **there is great value in probing pathway fluxes with isotope tracers.**

**This can be achieved by introducing the tracer and measuring the dynamics of downstream metabolite labeling.**

# What is a tracer?

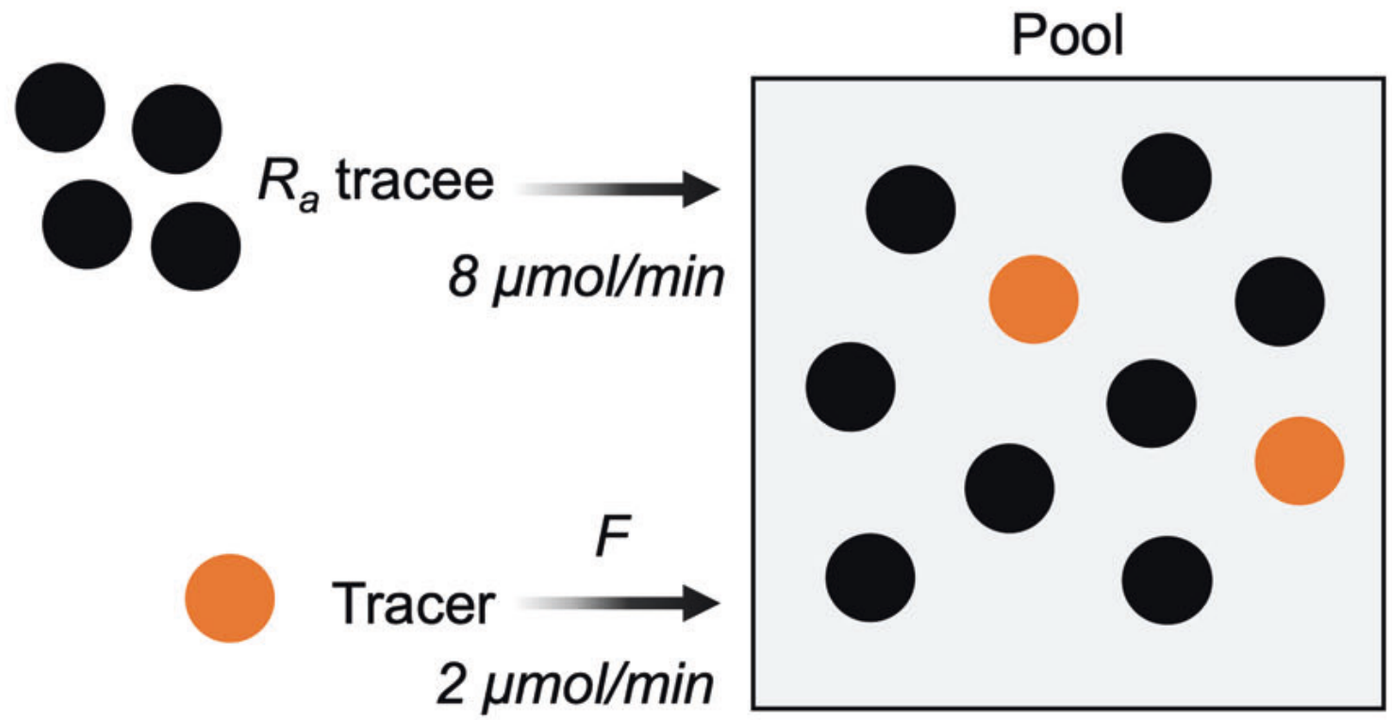
Metabolic tracers are molecules that can be introduced into a biological system, enters the metabolite pool and is processed by metabolic enzymes.

**These molecules and their derivatives can be measured** (imaging, MS, spatial MS).

This allows the monitoring of metabolic fluxes. Intuitively, faster labeling implies higher flux. Indeed, for a metabolite made directly from the tracer, initial rate of label accumulation (measured in molarity or moles per cell, not labeling fraction) equals the reaction’s flux.

# Metabolic tracing

The calculation of substrate kinetics is predicated on two basic tracer models: (1) tracer dilution and (2) tracer incorporation.

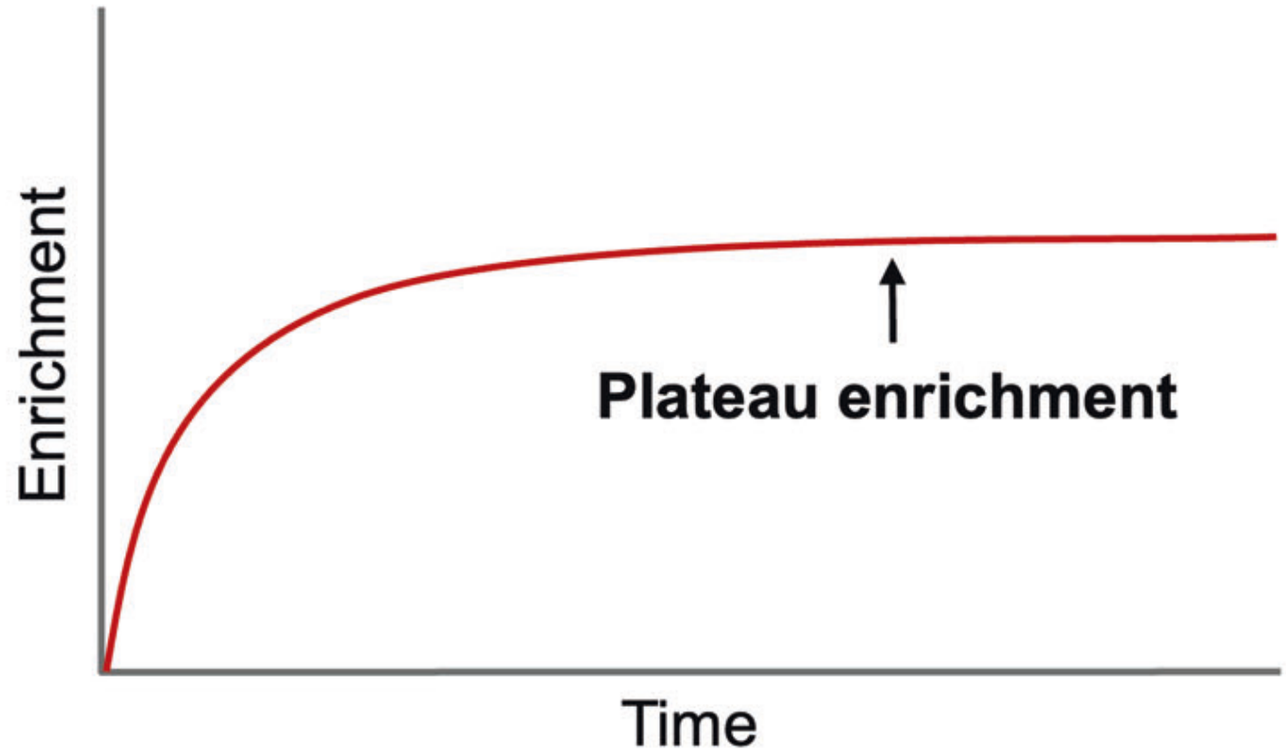



Models of tracer introductions:

- Pulse administration (decreases w/ its uptake)
- Constant infusion (constant over time)

*Kim et al, **EMM**, 2022*

# Metabolic tracing



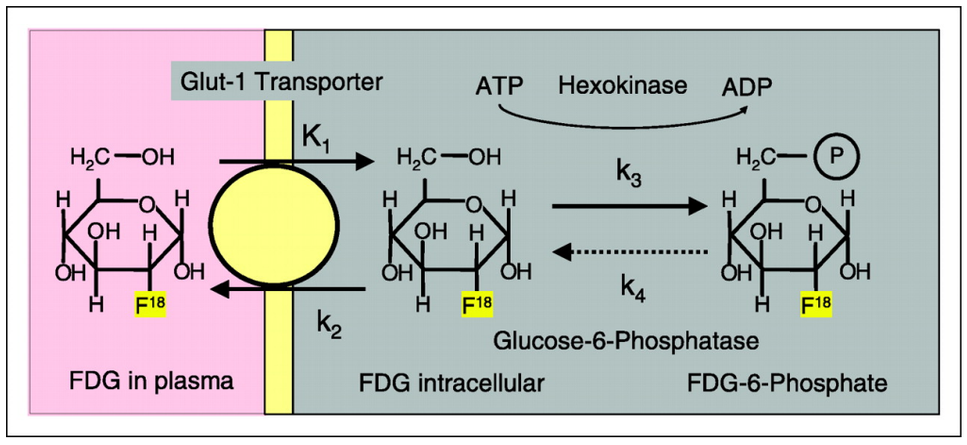

The tracer dilution model is based on the dilution of tracer administered into the system by the appearance in the same pool of unlabeled tracees. When an isotopic steady state is achieved, meaning that the rates of tracer and tracee appearance are constant over time and that there is a steady-state enrichment of the tracer in the body pools of the tracee, substrate kinetics can be calculated by the same method, regardless of the number of metabolic pools.

*Kim et al,* ***EMM****, 2022*

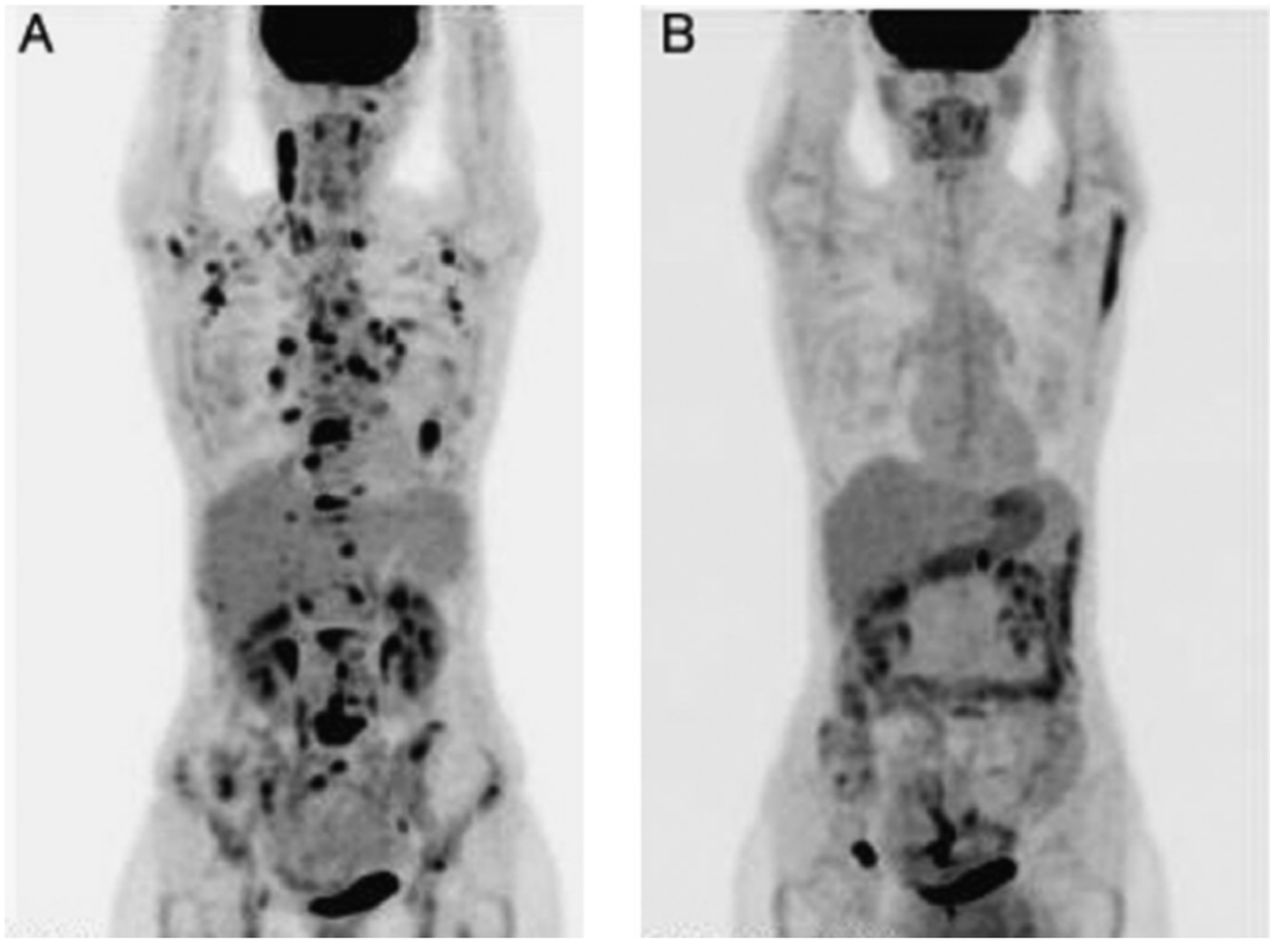
# Metabolic tracing

Several types of molecules monitor the metabolic activity of the cell.

FDG-sensible glucose entered the clinical practice long time ago.



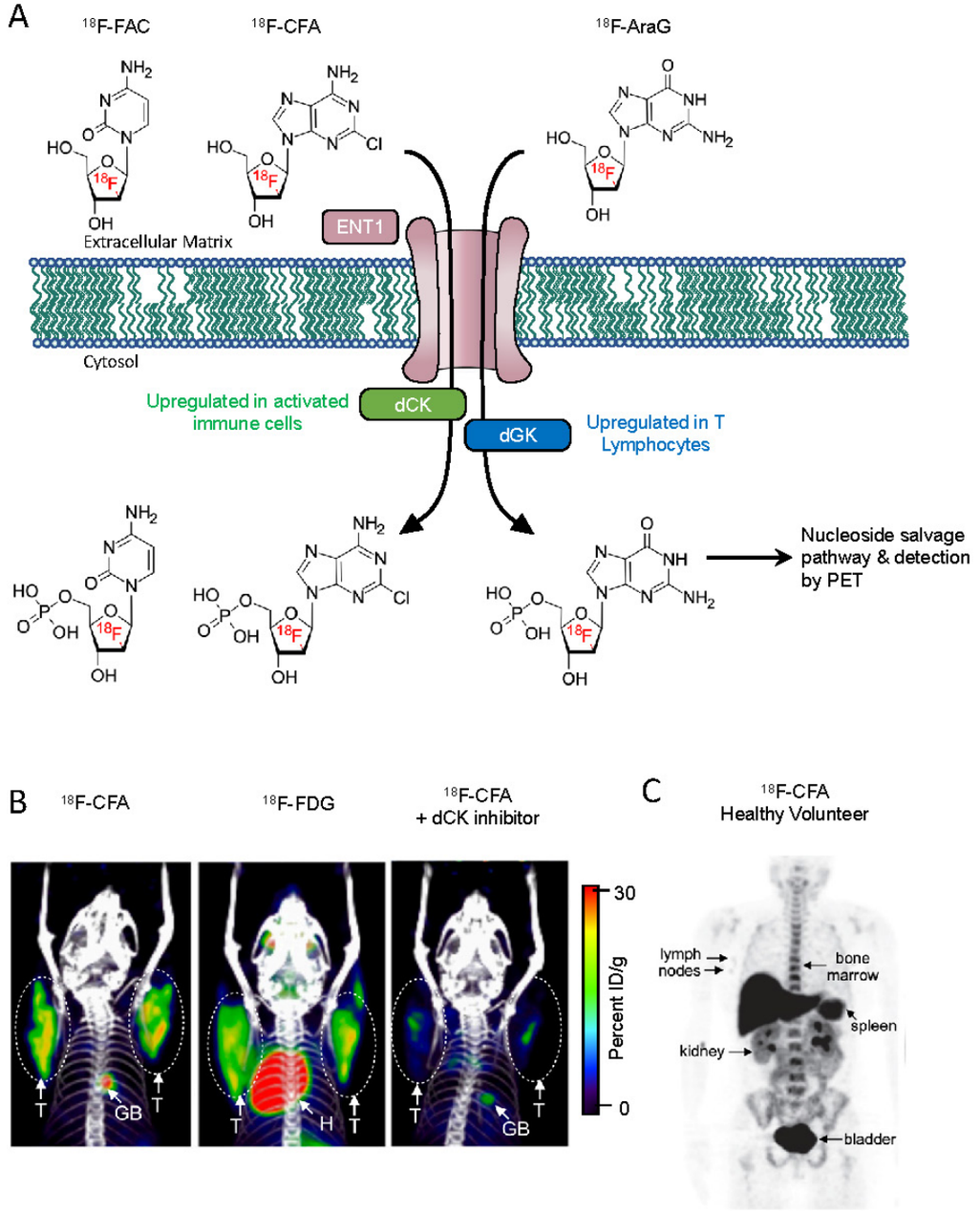

**Fluoro(18F)-deoxyGlucose**




*Kimura H et al,* ***Lung Cancer****,*

Positron-emitting glc analog to visualize neoplastic lesions in the body using PET.

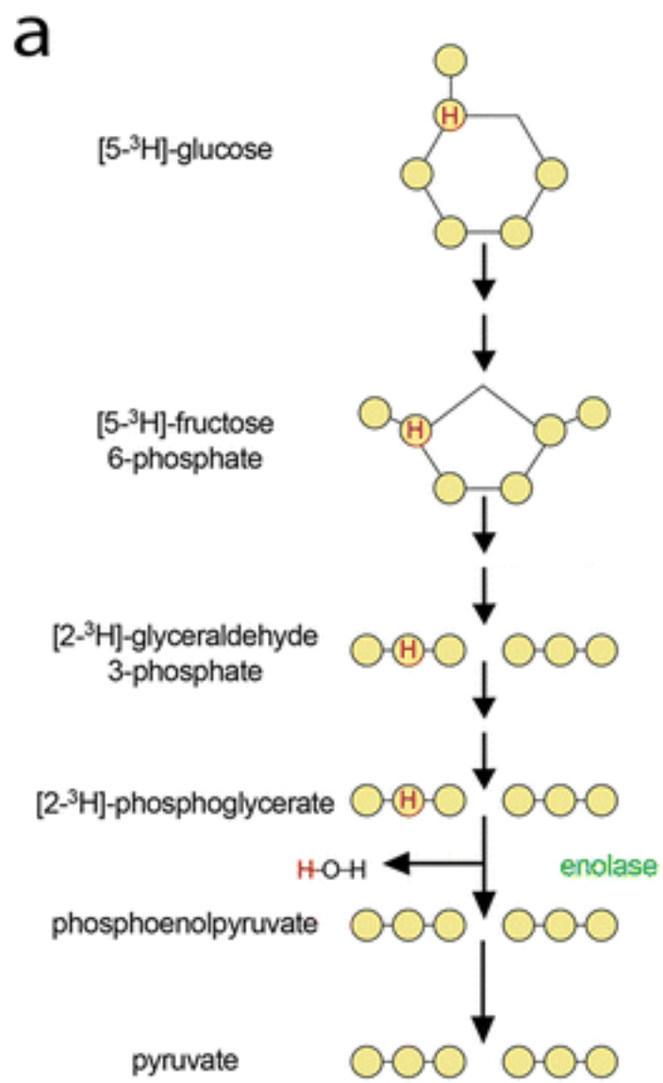
# Metabolic tracing




Nucleoside analog-based PET imaging. (A) PET tracers $^{18}$F-AraG and $^{18}$F-CFA are transported intracellularly by equilibrative nucleoside transporter 1 (ENT1). The tracers are then phosphorylated by their targets deoxyguanosine kinase and deoxycytosine kinase, trapping them intracellularly within cells. The tracers are then further metabolized along the nucleoside salvage pathway and incorporated into newly synthesized DNA. (B) PET/CT images of mice bearing CEM tumors, a human lymphoblastic leukemia, (circled) transduced to express Cytidine Deaminase (CDA). $^{18}$F-CFA was used in the left and right images, while $^{18}$F-FDG was used for the center image. A small molecule inhibitor of dCK, DI-82, was used in the right image to confirm the specificity of $^{18}$F-CFA for dCK. Adapted from. (C) $^{18}$F-CFA PET of a healthy human volunteer. Organs with high uptake are indicated with arrows.

# Metabolic tracing

Radioactive isotopes have been used to study metabolic activity of the cells



# Metabolic tracing

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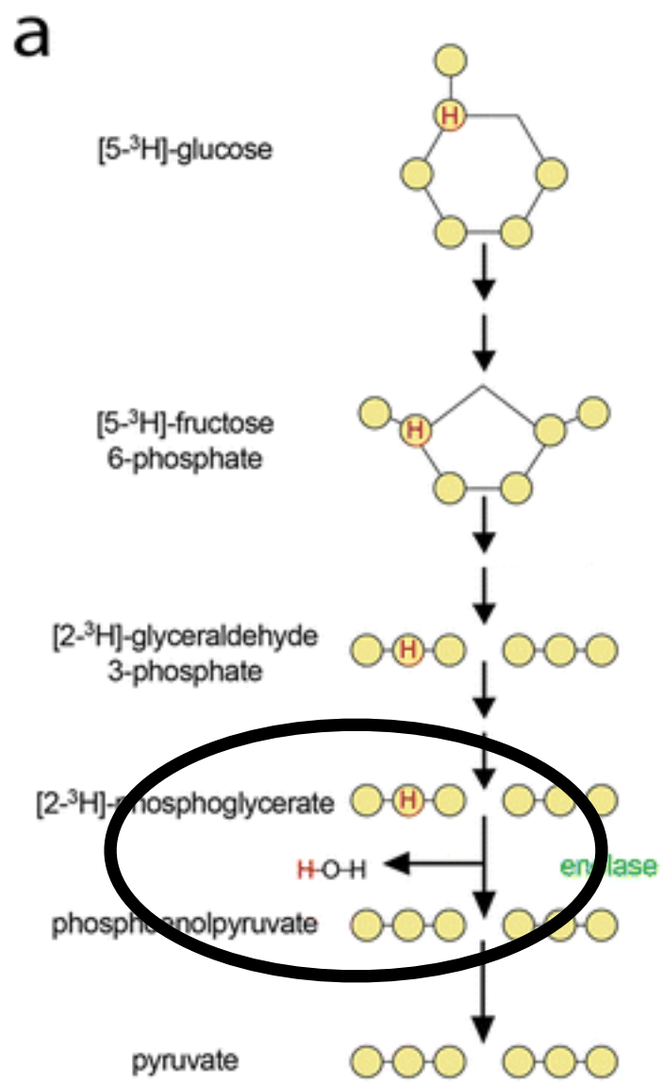
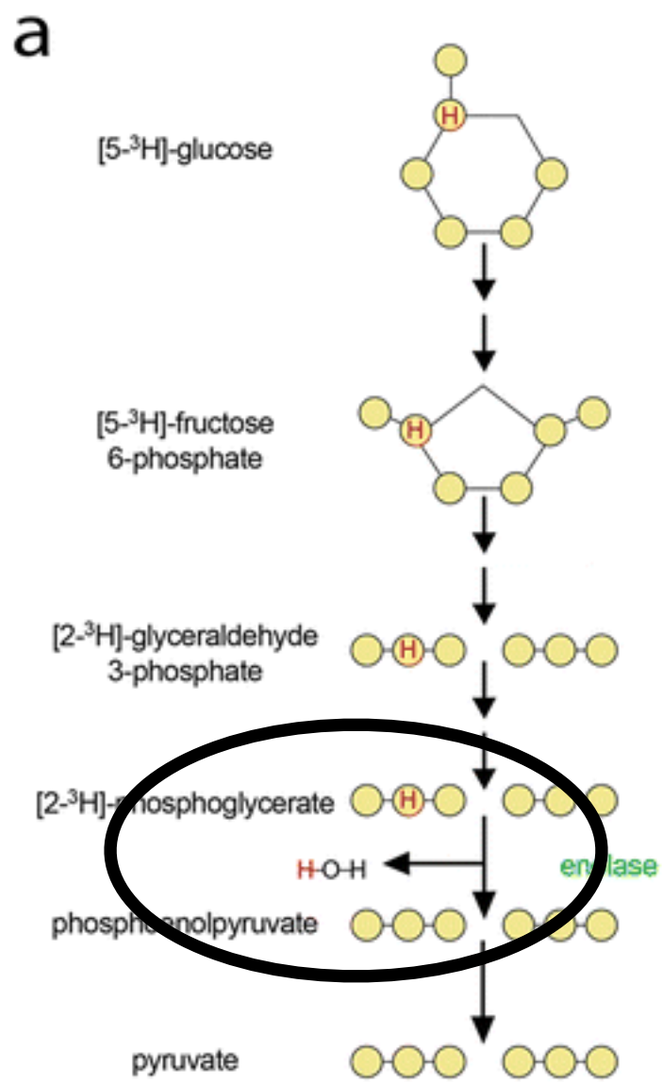


# Metabolic tracing

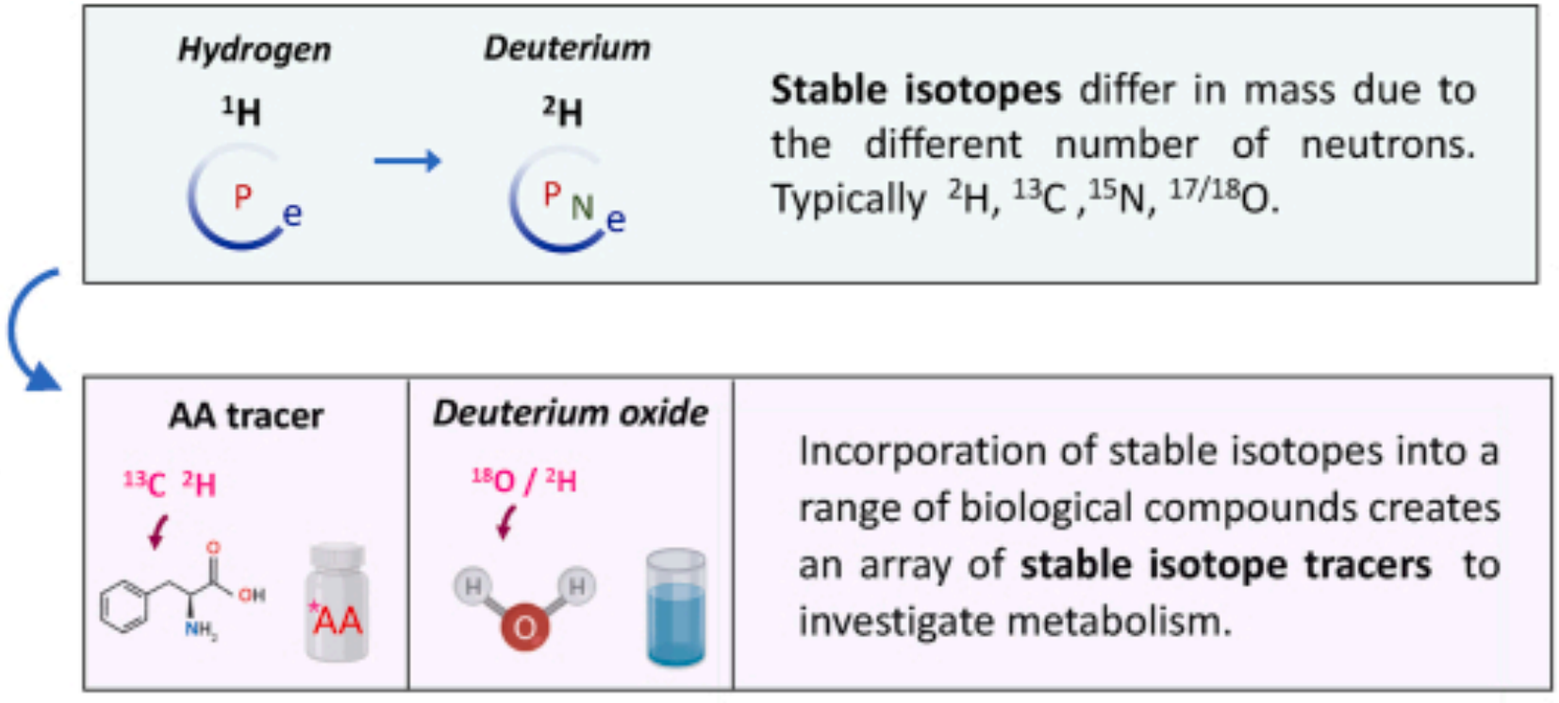
Radioactive isotopes have been used to study metabolic activity of the cells




Radio-emission in culture broth can be measured with a Geiger machine

# Metabolic tracing

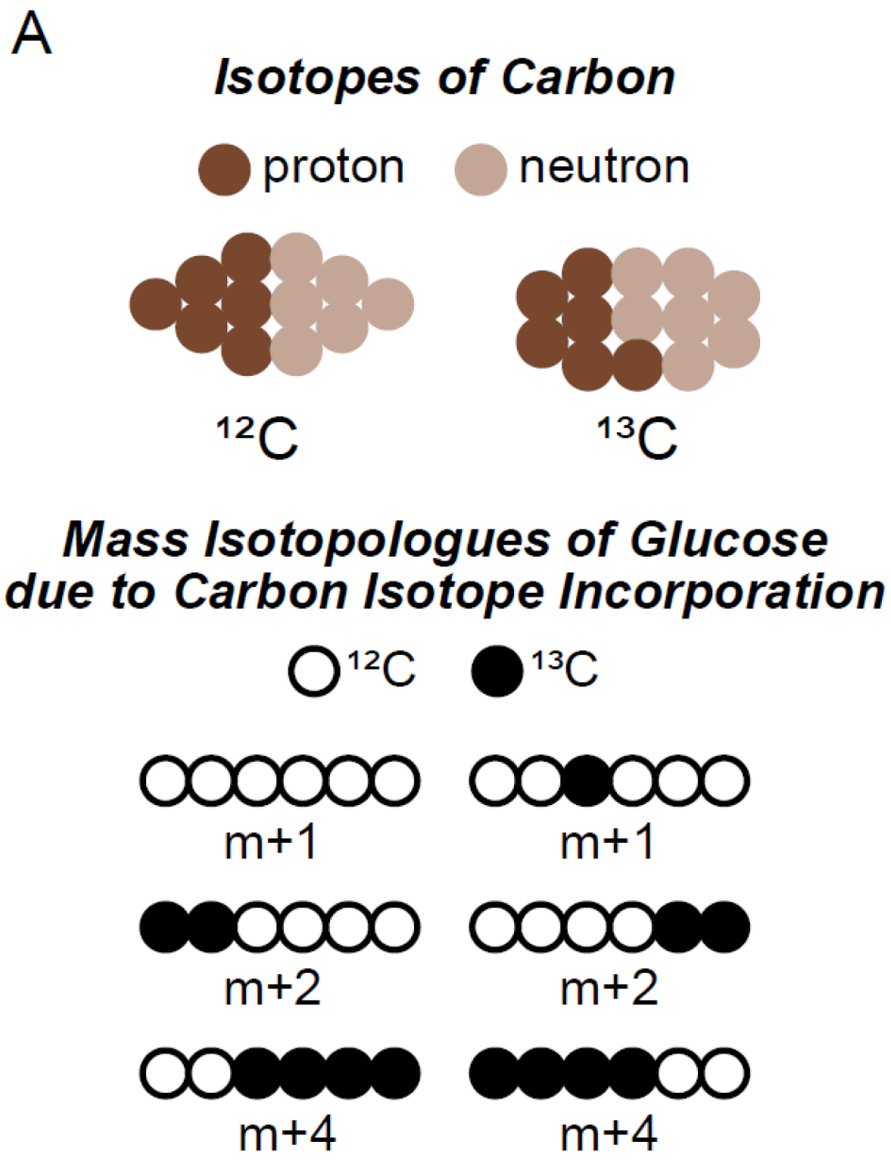
Stable isotopes are species of an element which whilst chemically and functionally identical, differ in mass due to the different number of neutrons in the atomic nucleus. This difference in mass, measured using mass spectrometry, makes them analytically distinguishable from each other and allows them to be used to ‘trace’ metabolism.




Stable isotopically labeled tracers are any molecules with one or more heavier stable isotopes (e.g., 13C, 2H, or 15N isotopes) incorporated somewhere in the molecule. Stable isotope tracers may be administered in the chemical form of the tracer (e.g., 13C glucose) or as heavy water (deuterium oxide, 2H2O) that will produce the desired metabolic tracer in vivo.

*Wilkinson et al,* ***Curt Nutr Open Sci****, 2021*

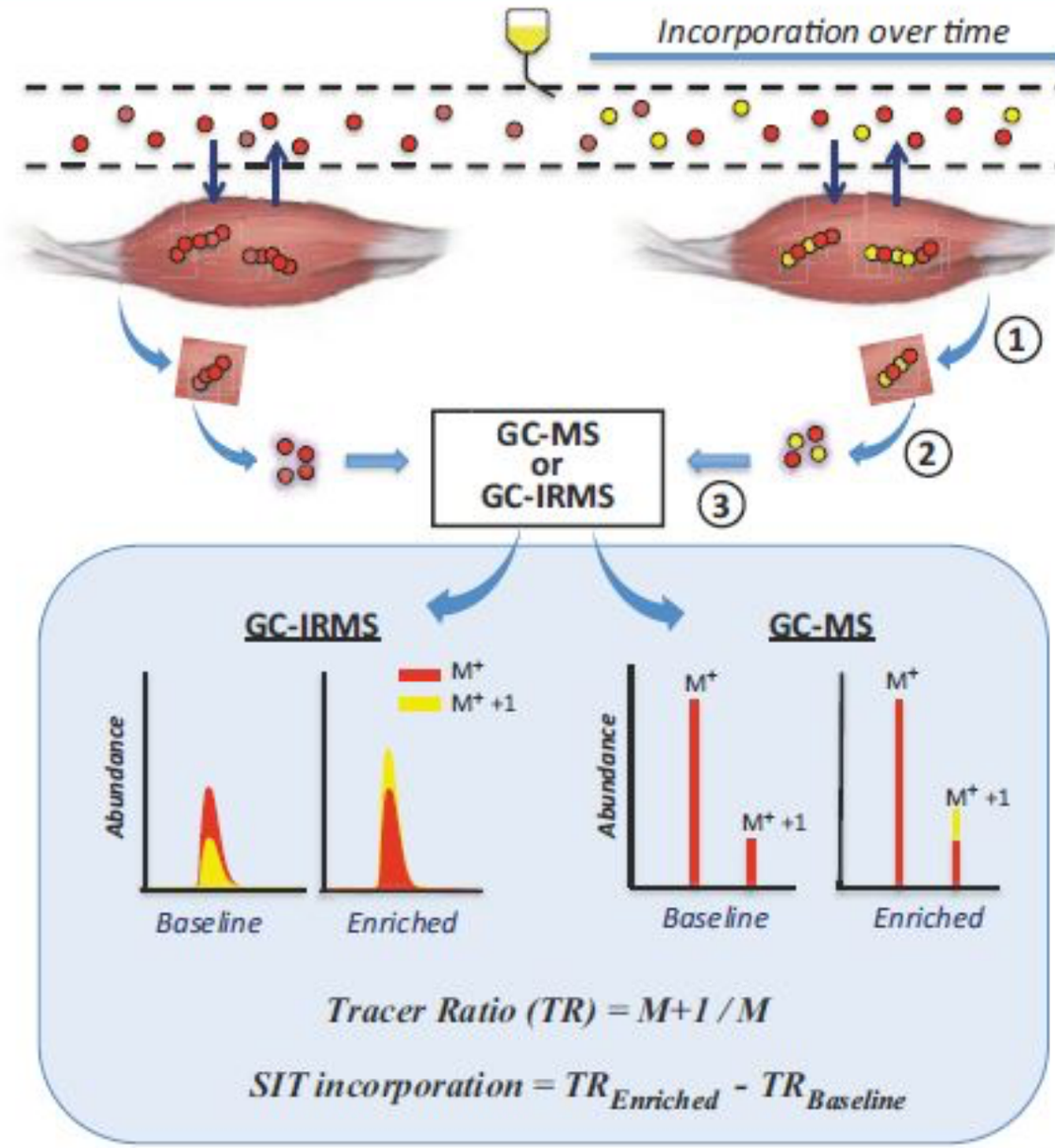
# Carbon isotope tracing




Use of [13]-Carbon isotopes creates multiple isotopologues of carbon-containing molecules (most nutrients)

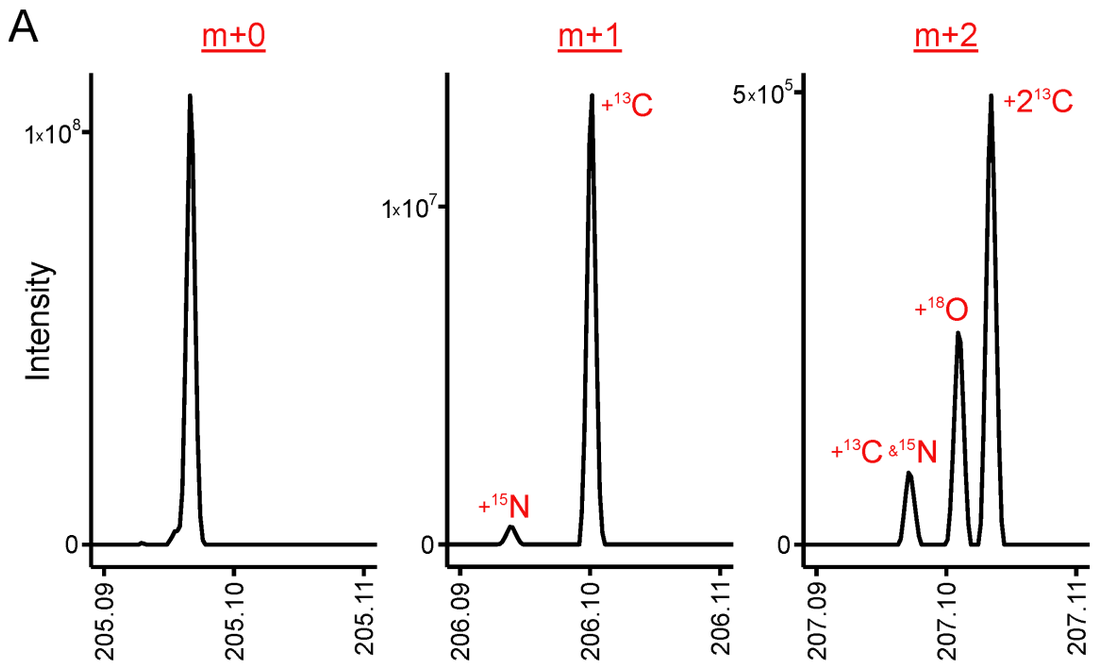
# Stable isotope tracing

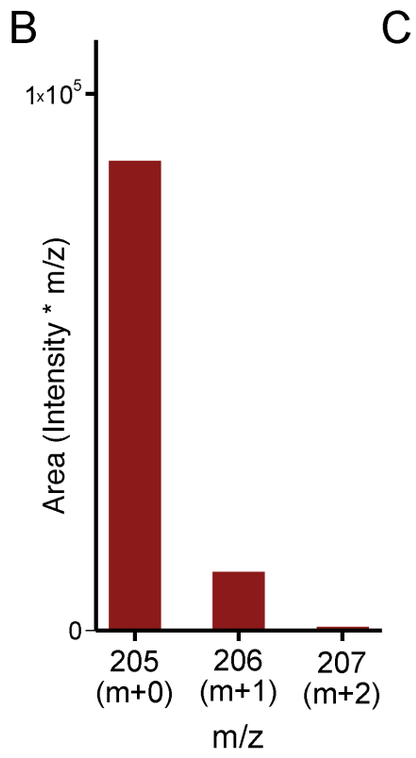
*Figure 2.* The rate of biosynthetic pathways can be calculated by acquiring a muscle biopsy both before (baseline) and after (enriched) the incorporation of a SIT (1). After hydrolysis, purification and derivatisation (2) substrates can be measured using GC-MS techniques (3). GC-MS measures the molecular mass (M) and the abundance of that molecule containing heavier isotopes (M+1). Comparatively IRMS combusts the whole molecule and measures the lighter and heavier isotope in the resulting gas produced e.g. $13CO_2$ vs. $12CO_2$. After SIT infusion, the 'heavier' isotope is increased more than what occurs naturally and the resulting difference represents the rate of SIT incorporation over that time.




Stable isotopically labeled tracers are metabolized (as normal nutrients) inside cells and generate heavy (M+1, M+2, M+3,..) metabolites that can be measured analytically.

# Stable isotope tracing



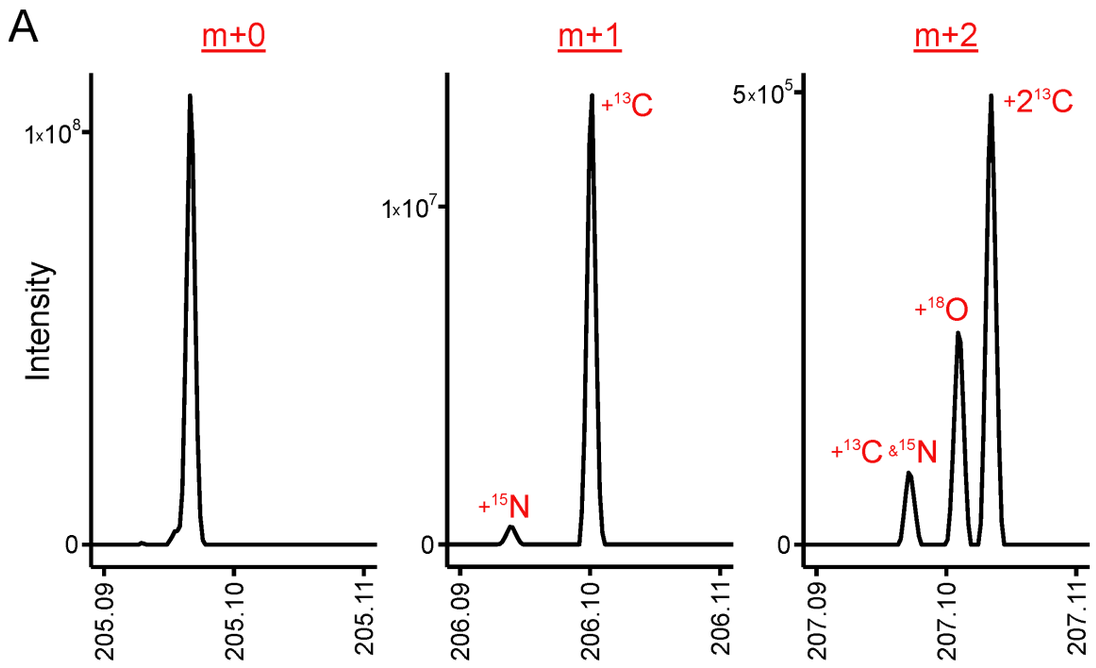

C

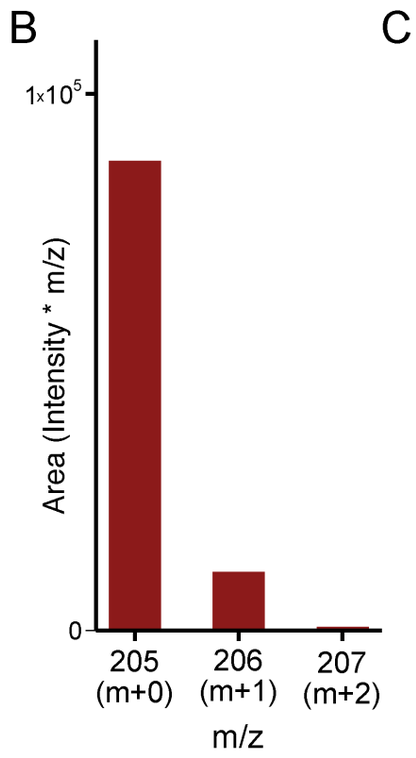
| | High Resolution | | Low Resolution | |
|---|---|---|---|---|
| **mass** | **meas** | **corr** | **meas** | **corr** |
| m+0 | 0.889 | 1.000 | 0.882 | 1.007 |
| m+1 | 0.107 | 0.001 | 0.111 | −0.003 |
| m+2 | 0.004 | −0.002 | 0.007 | −0.003 |
| m+3 | - | 0.000 | - | 0.000 |
| m+4 | - | 0.000 | - | 0.000 |
| m+5 | - | 0.000 | - | 0.000 |
| m+6 | - | 0.000 | - | 0.000 |
| m+7 | - | 0.000 | - | 0.000 |
| m+8 | - | 0.000 | - | 0.000 |
| m+9 | - | 0.000 | - | 0.000 |
| m+10 | - | 0.000 | - | 0.000 |
| m+11 | - | 0.000 | - | 0.000 |

Heavier isotopologues have distinctive spectra

e.g.: (M+1) is 1 Da heavier = contains extra 1 proton

# Stable isotope tracing




C

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| m+4 | - | 0.000 | - | 0.000 |
| m+5 | - | 0.000 | - | 0.000 |
| m+6 | - | 0.000 | - | 0.000 |
| m+7 | - | 0.000 | - | 0.000 |
| m+8 | - | 0.000 | - | 0.000 |
| m+9 | - | 0.000 | - | 0.000 |
| m+10 | - | 0.000 | - | 0.000 |
| m+11 | - | 0.000 | - | 0.000 |

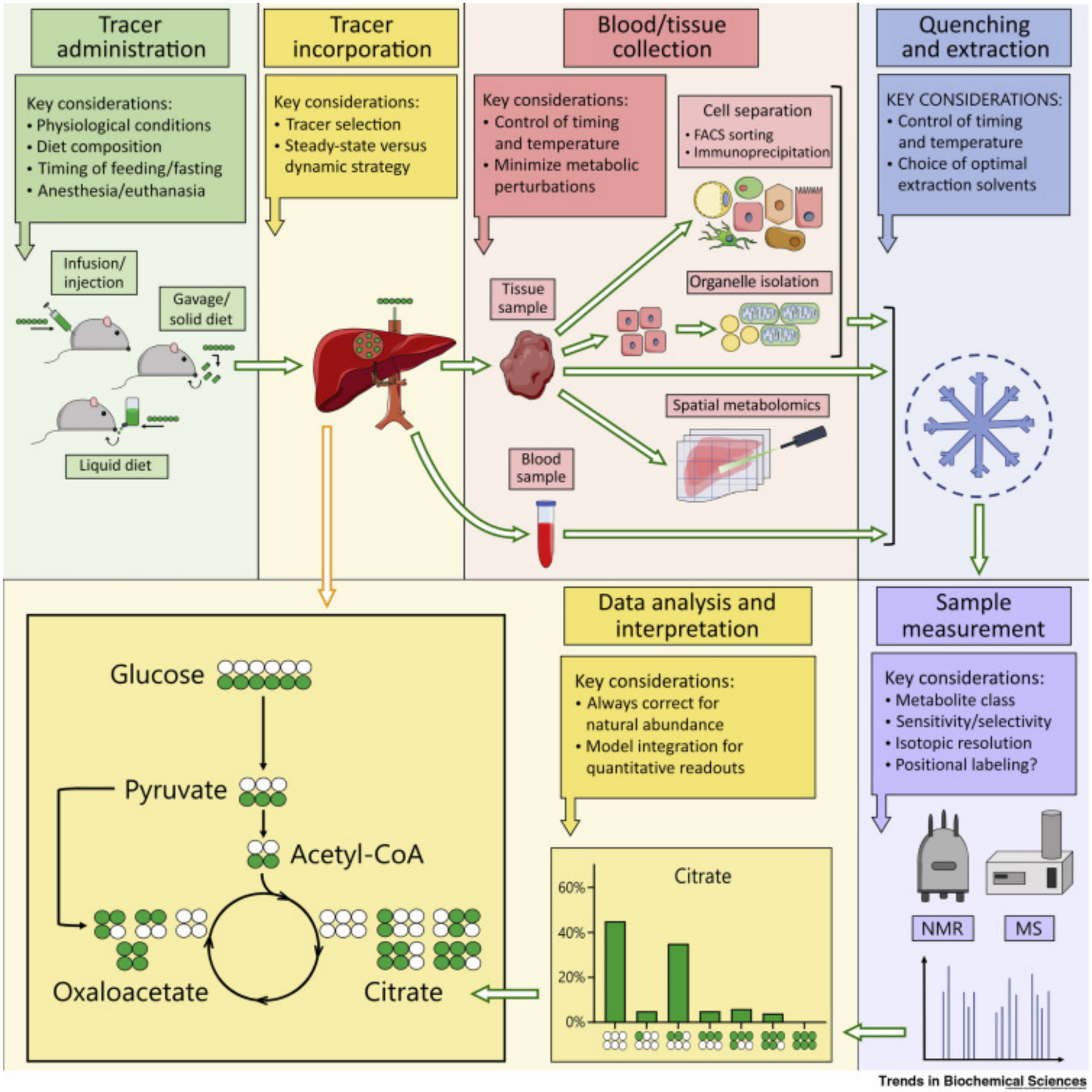
Heavier isotopologues have distinctive spectra

e.g.: (M+1) is 1 Da heavier = contains extra 1 proton

Heavier isotopologues are naturally occurring in nature.

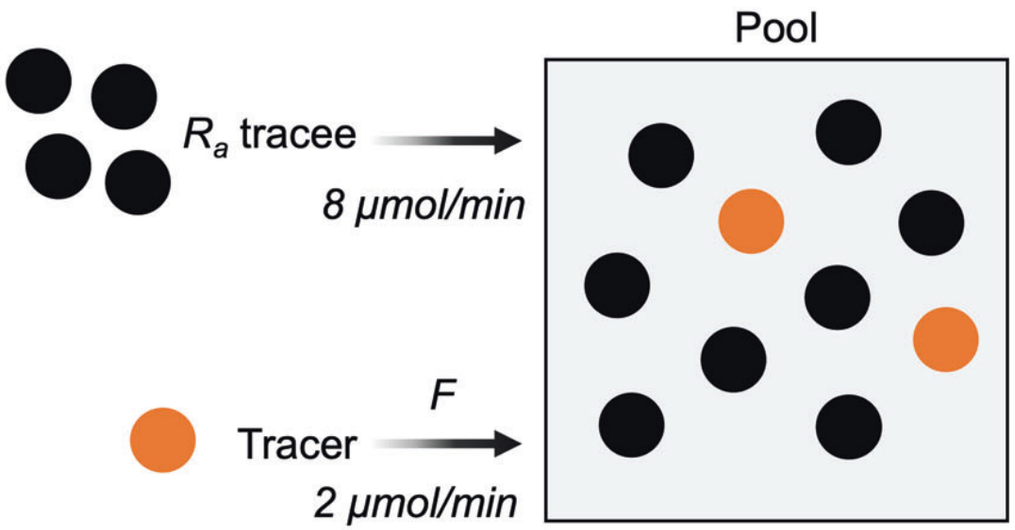
Always present in MS spectra (normalization)

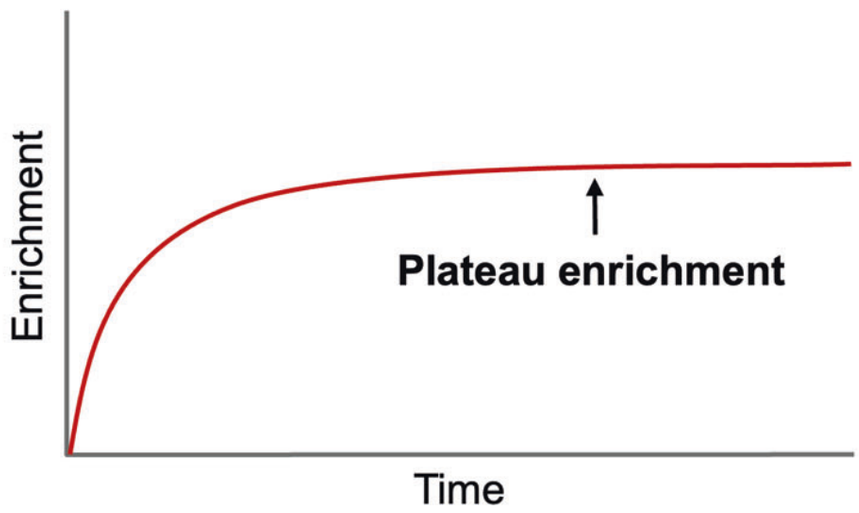
# Stable isotope tracing




*Fernandez-Garcia et al, **TiBS**, 2020*

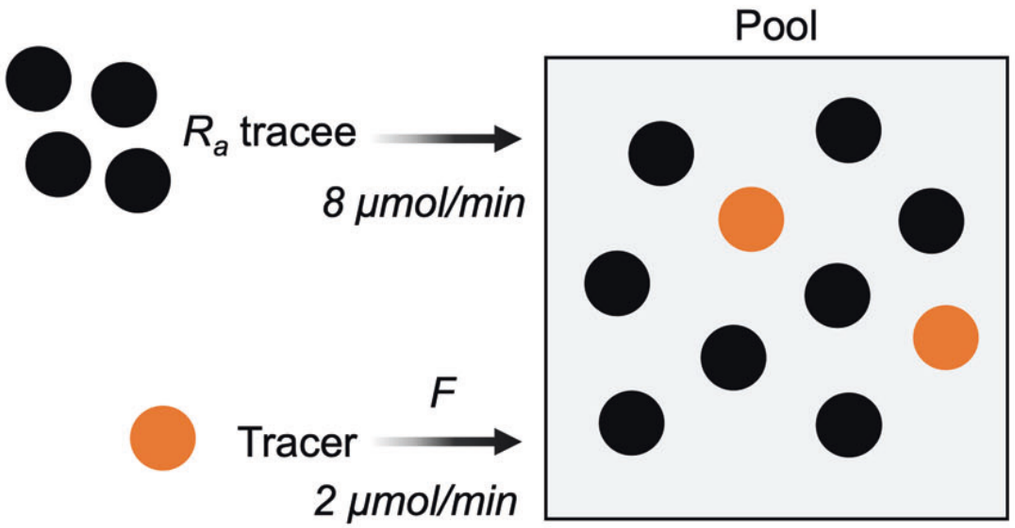
# Molar enrichment

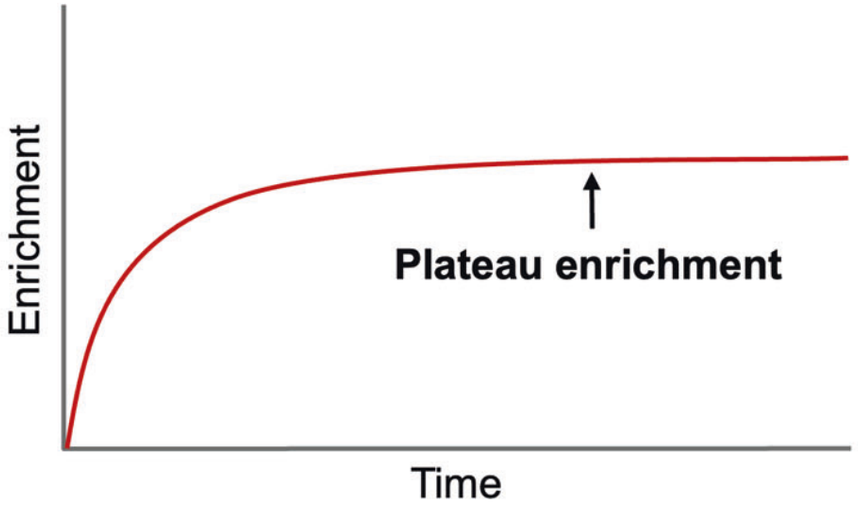


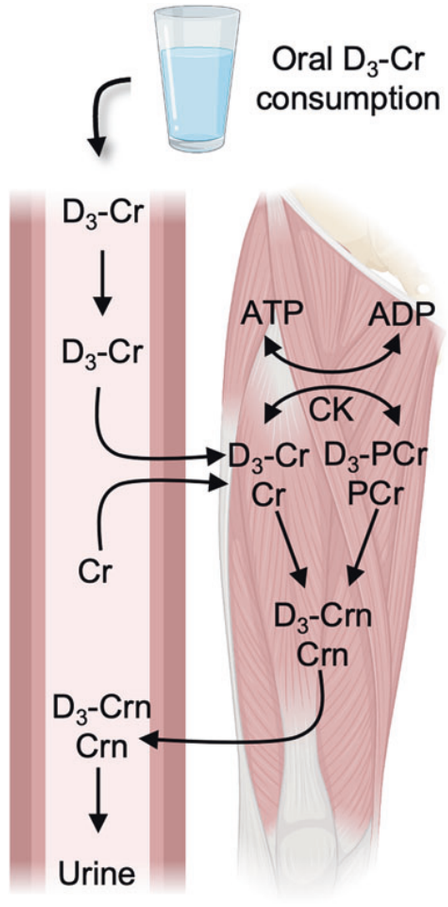



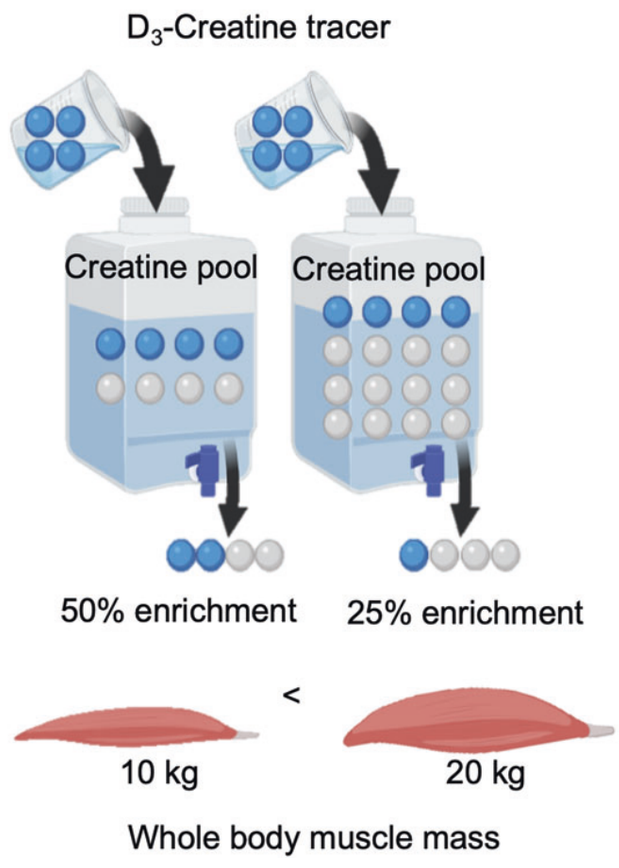

*Kim et al, **EMM**, 2022*

# Molar enrichment



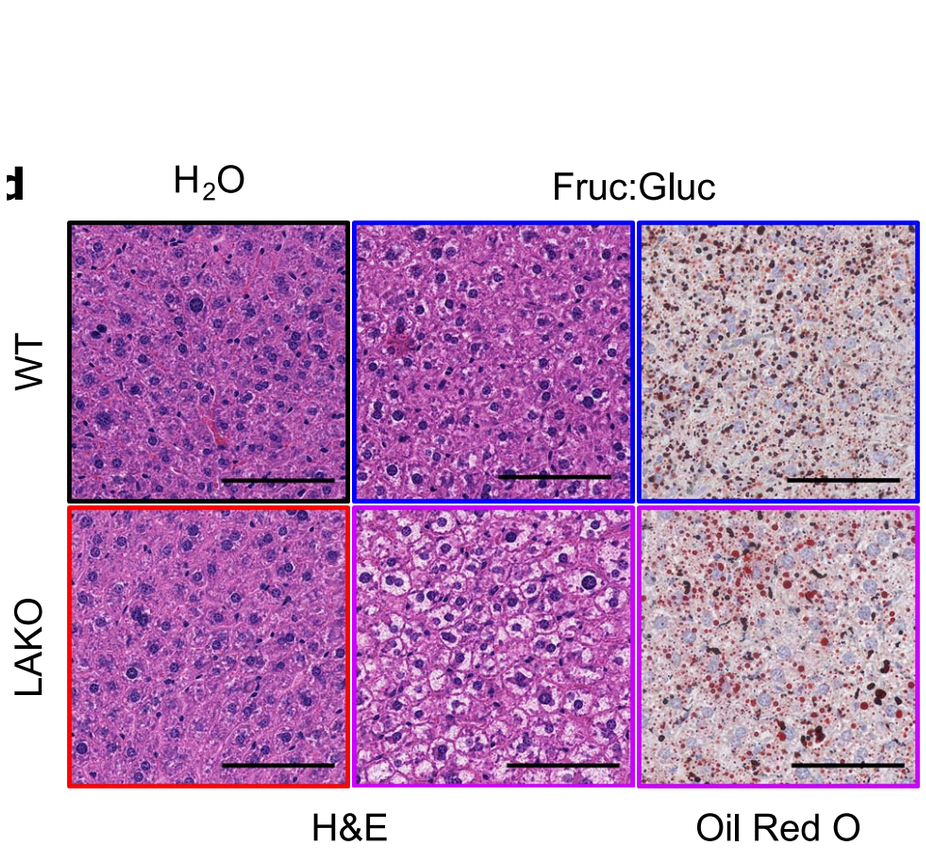



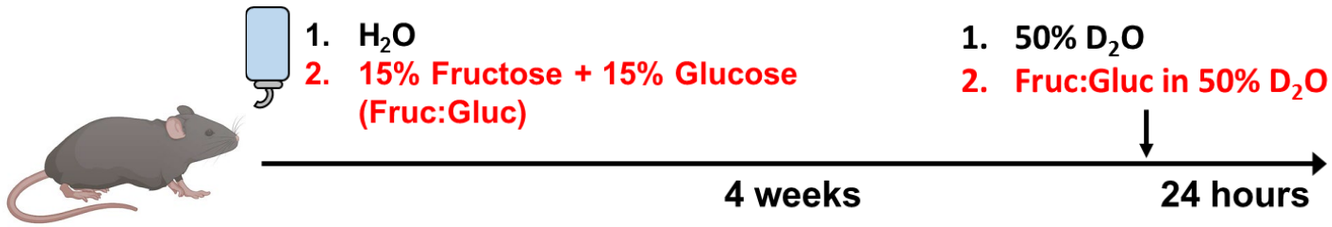



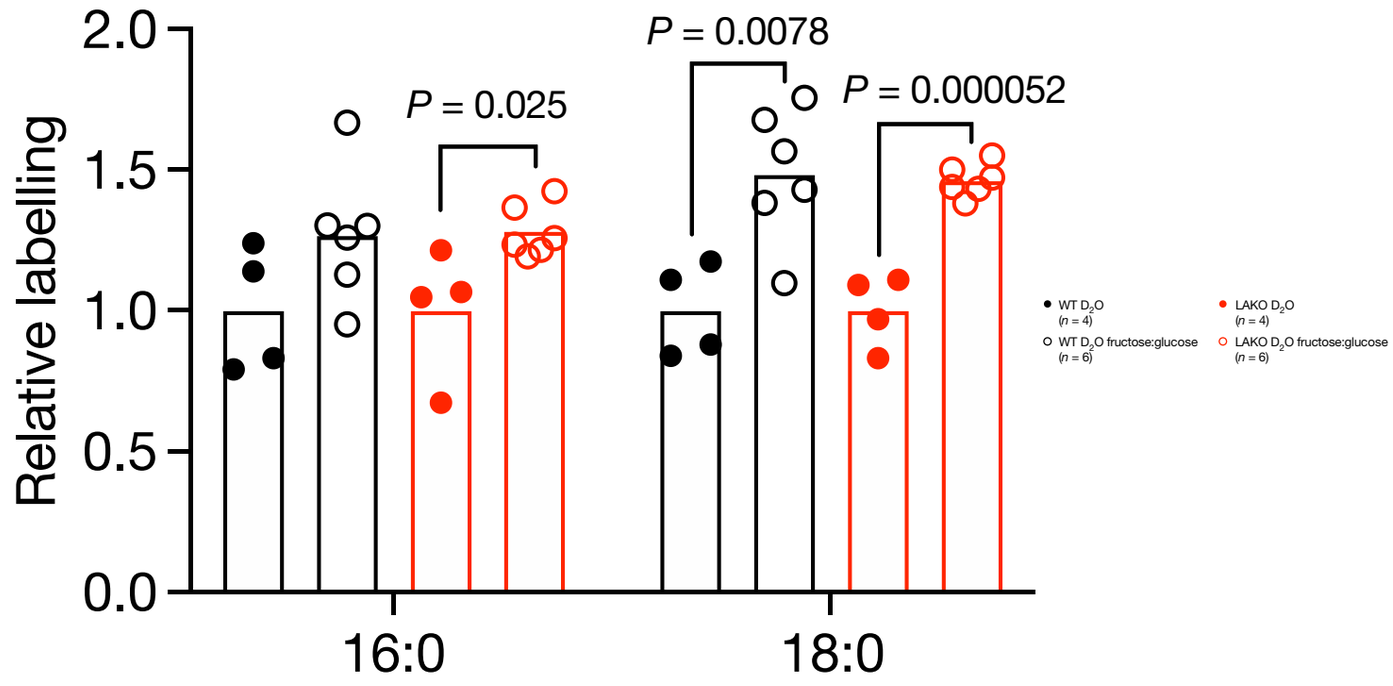

*Kim et al, **EMM**, 2022*

# Deuterium tracing

Using deuterium as tracer is useful to quantify the synthesis rate of macromolecules



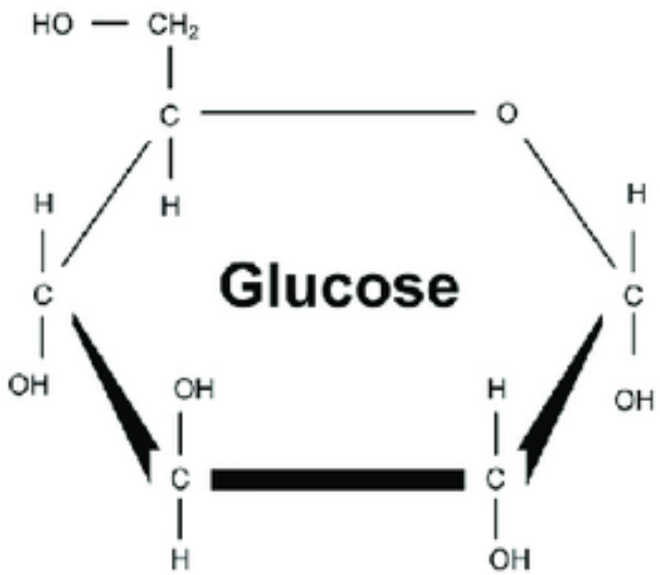



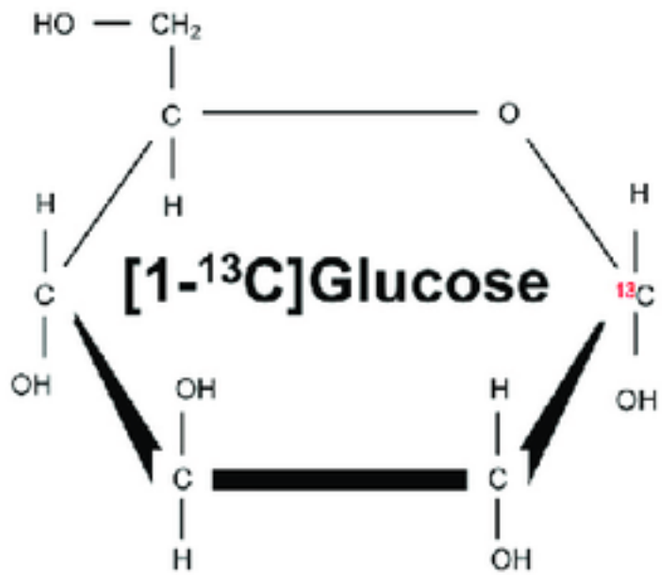



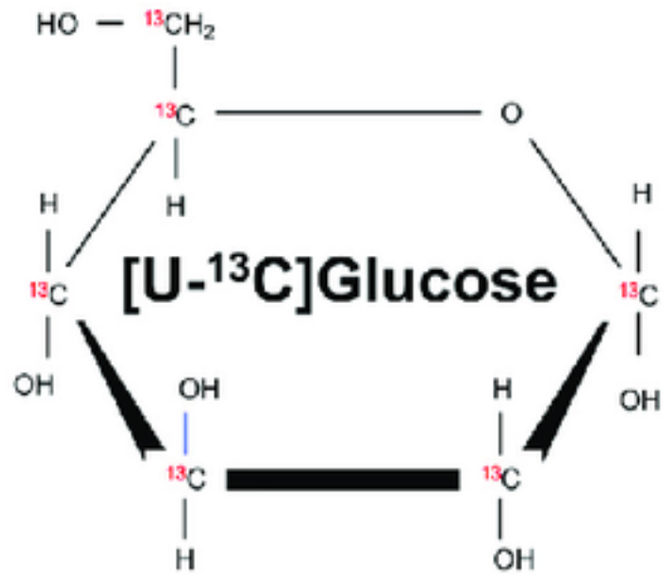

The heavy water labeling of precursors has several potential advantages over the substrate-labeled tracer infusion method. Most notably, heavy water labels a number of different precursors, which enables the simultaneous determination of *FSR*s of a variety of polymers with repeating (mono or multiple) precursor molecules, such as DNA, fatty acids (de novo lipogenesis), and glucose (i.e., gluconeogenesis) in free-living conditions. Water moves rapidly and freely throughout the body, including across cell membranes, and equilibrates with all of the body fluid within ~30 min after administration

# Carbon isotope tracing

[13]-Glucose is often used as a tracer

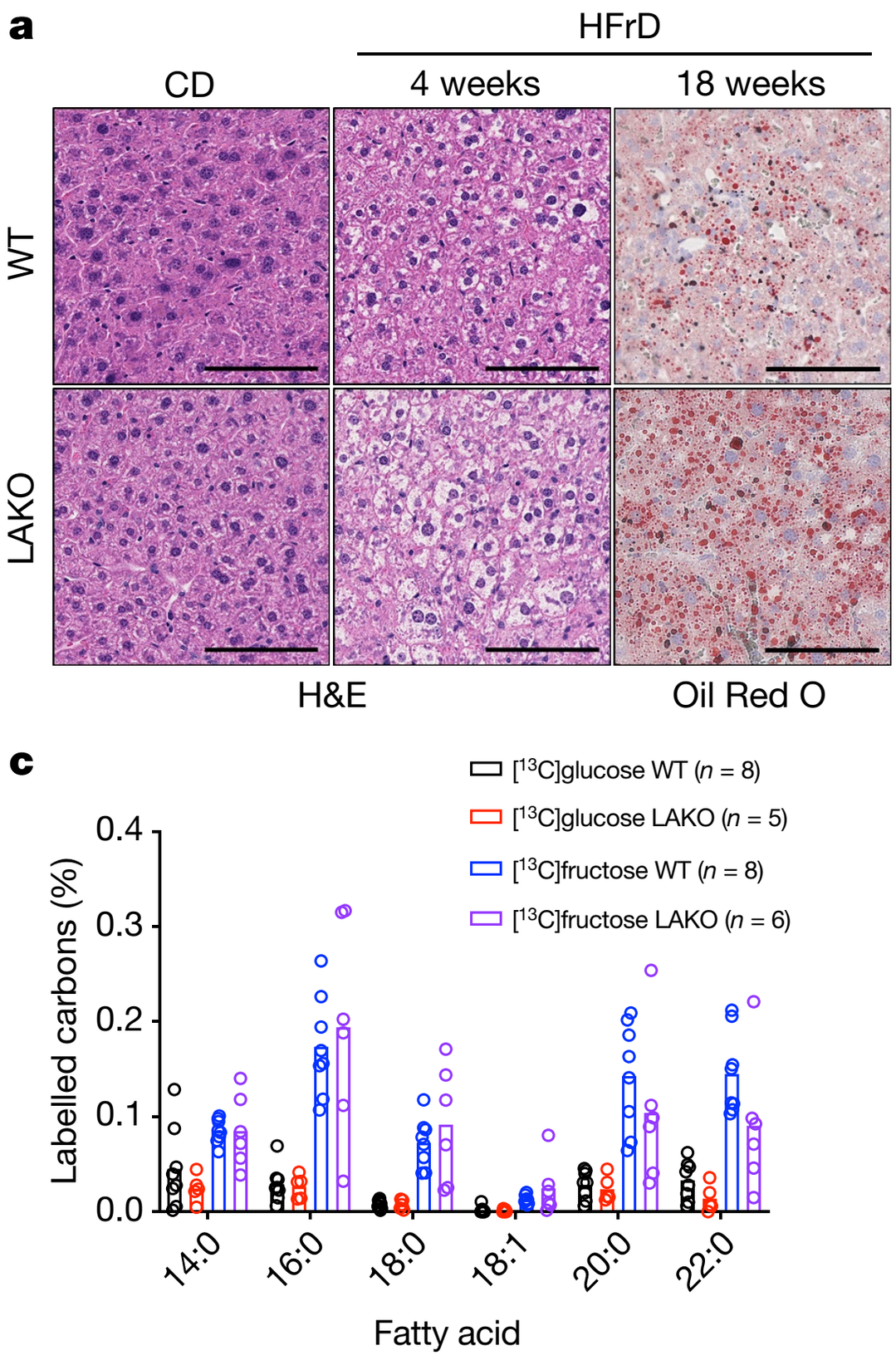



Positionally-labeled

Uniformly-labeled




*Zhao et al,* ***Nature****, 2020*

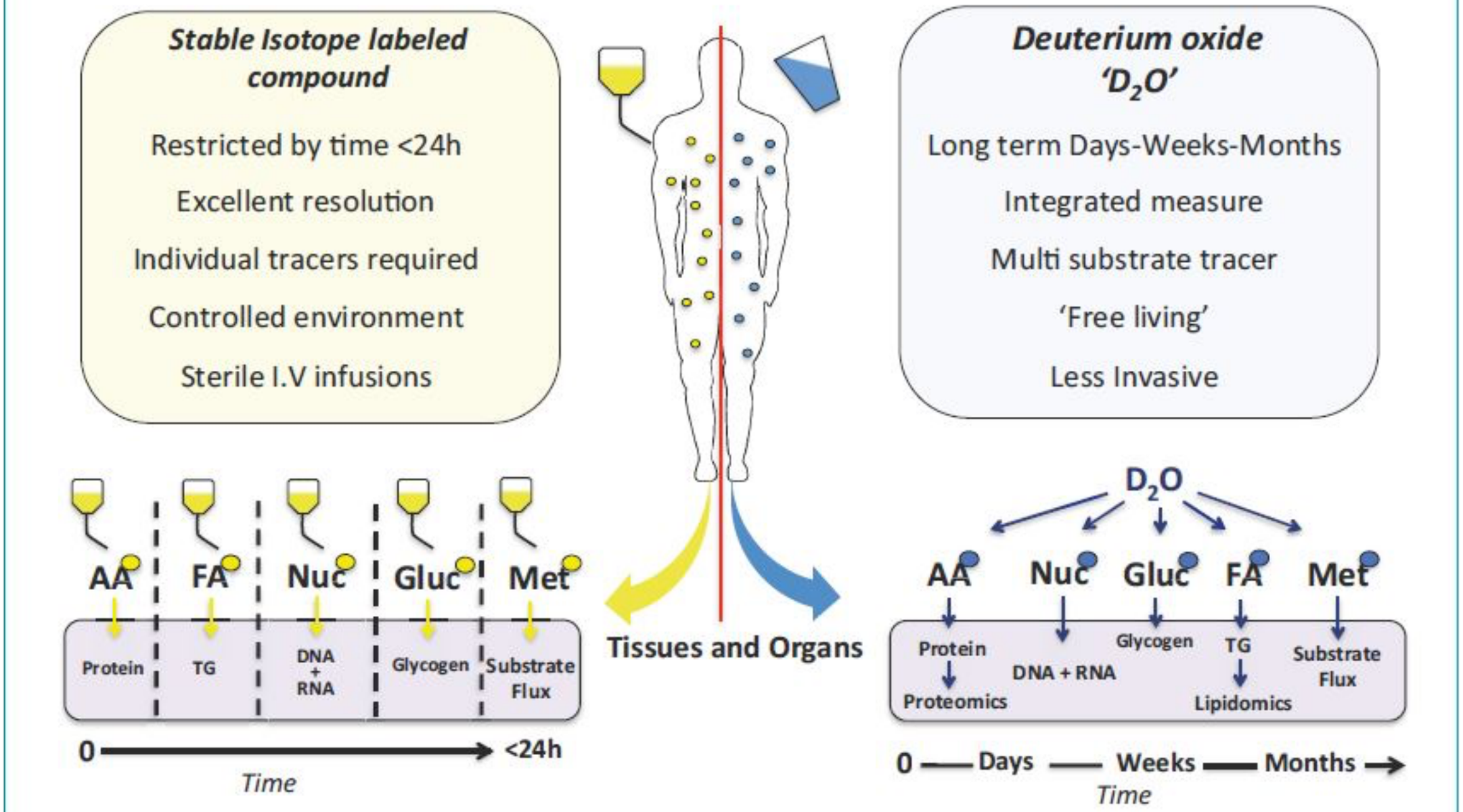
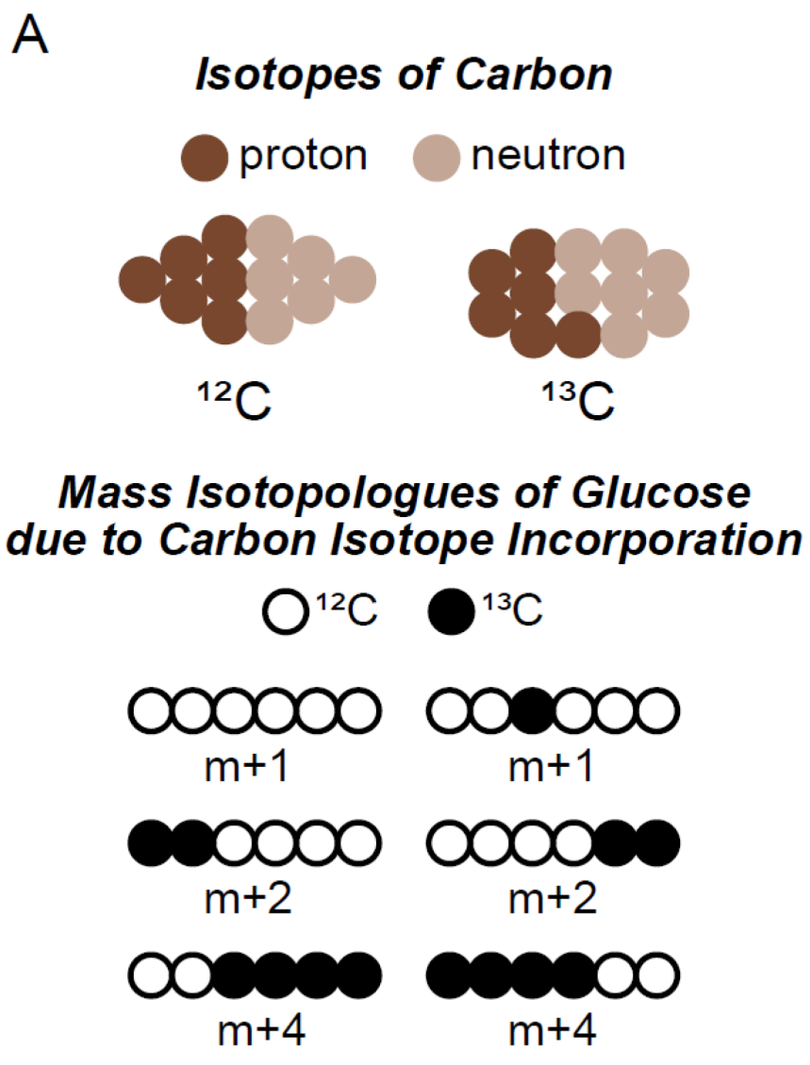


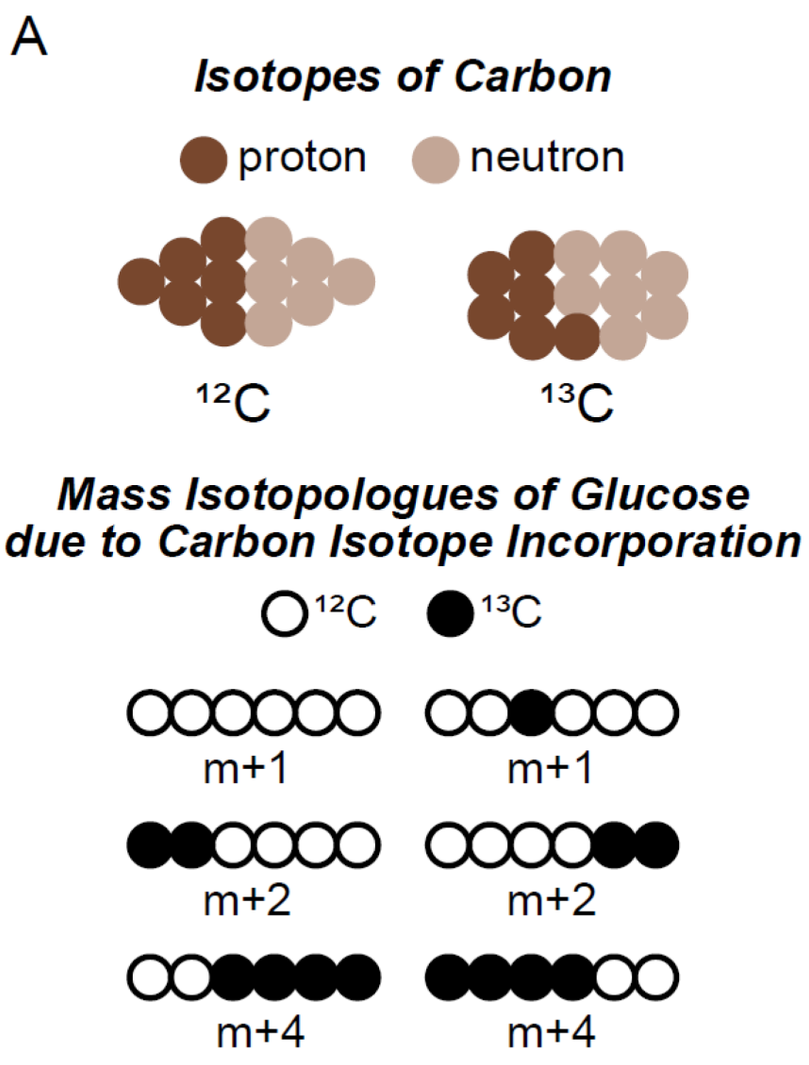

*Figure 3.* Representative diagram of the differences between the uses of substrate specific stable isotope labeled compounds and deuterium oxide '$D_2O$'. **AA** – amino acid. **FA** – fatty acid. **Nuc** – nucleotide. **Gluc** – glucose. **Met** – metabolite. **TG** – triglyceride.

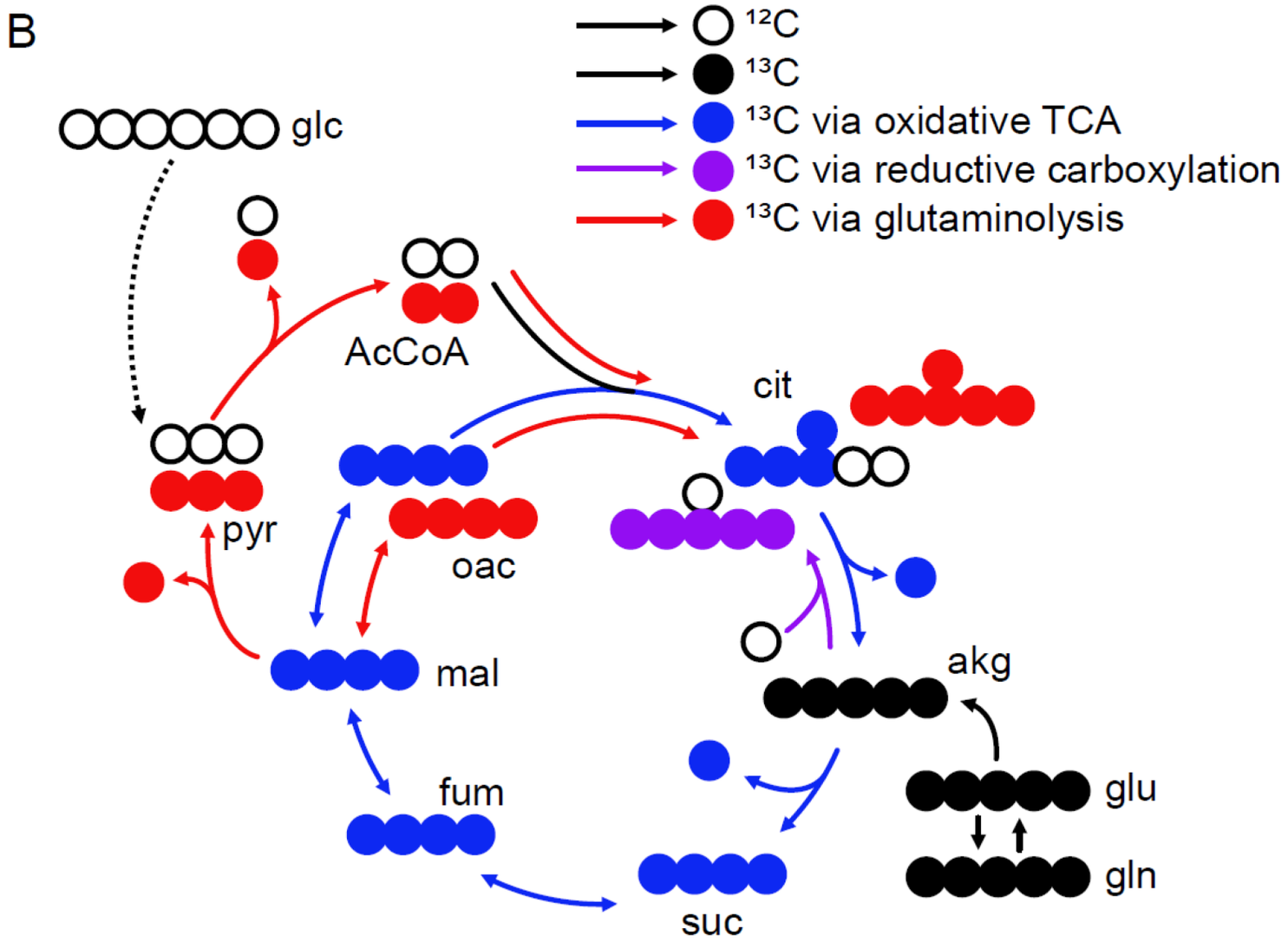
# Carbon isotope tracing




Molar enrichment is an indirect measure of metabolic flux

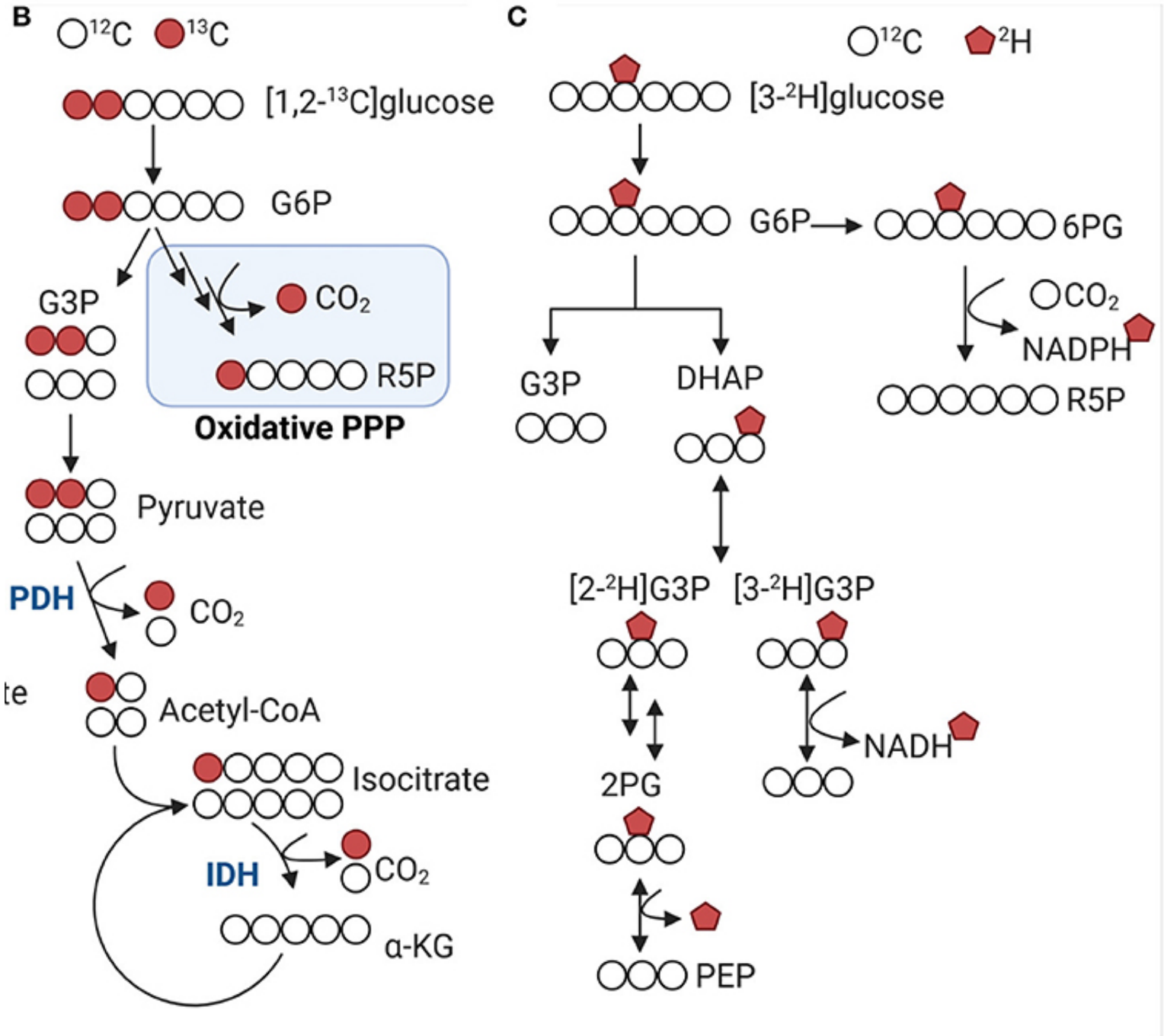
# Carbon isotope tracing




Molar enrichment is an indirect measure of metabolic flux

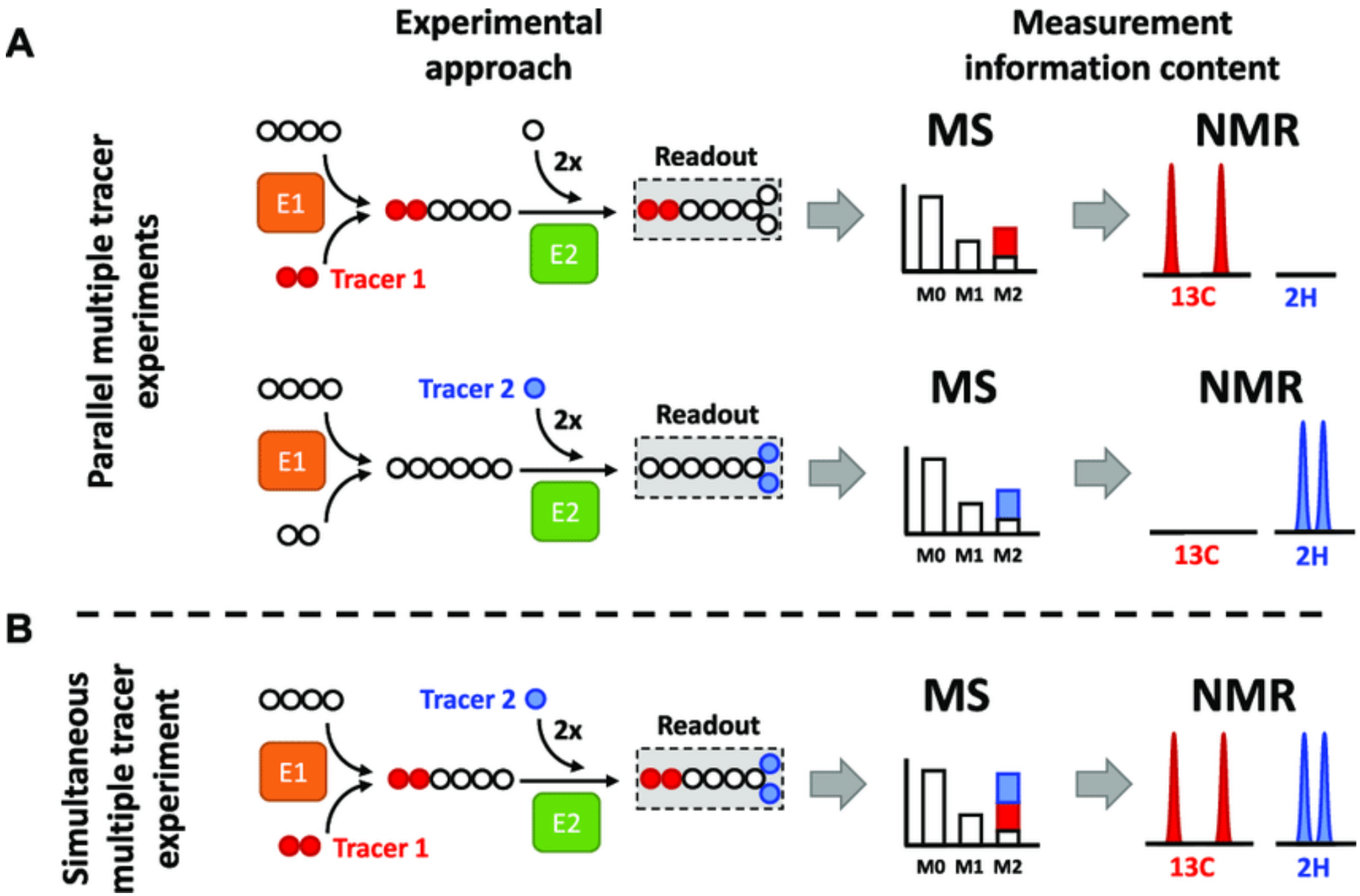
# Carbon isotope tracing




Molar enrichment depends on:

1) Target metabolite
2) Position on nutrient

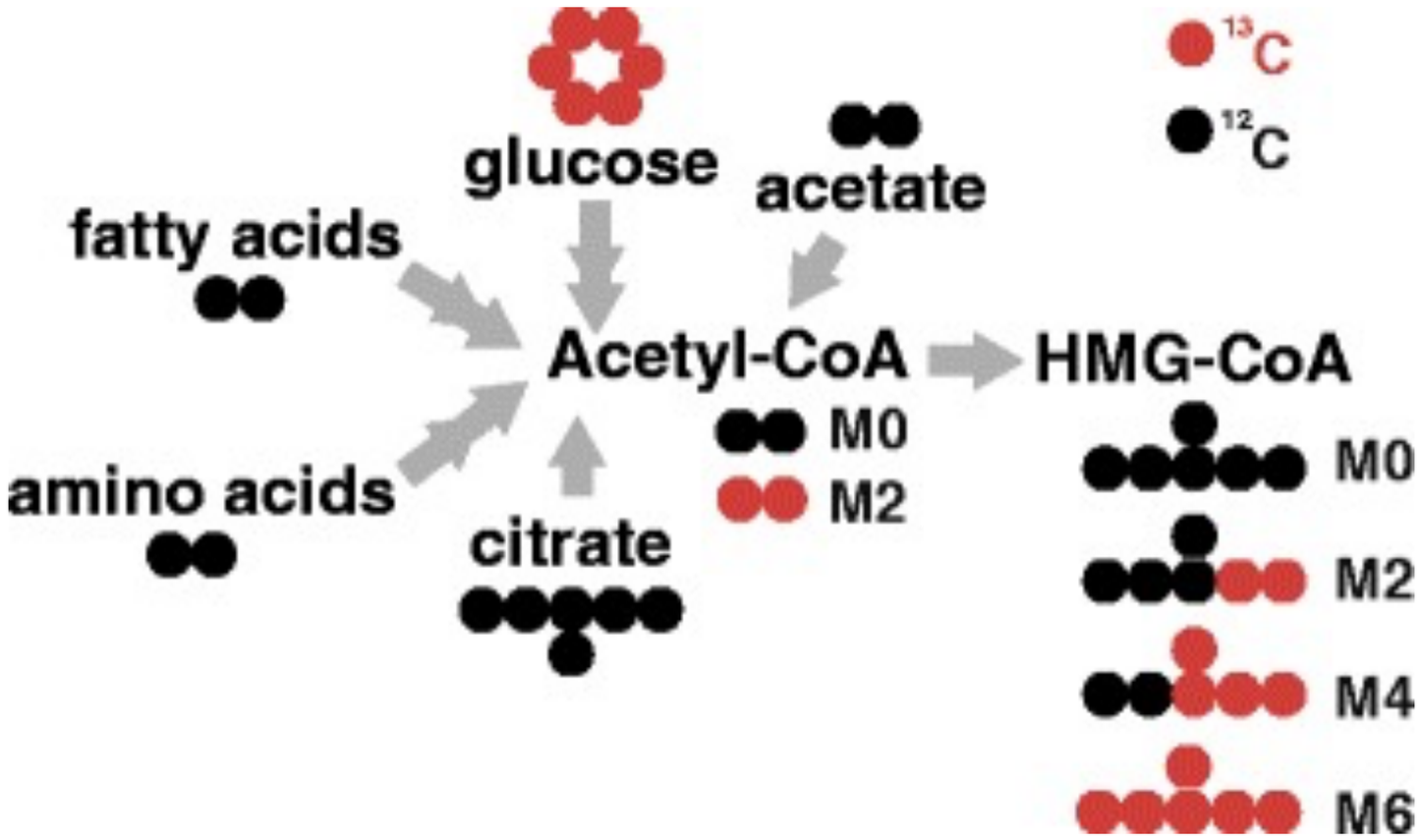
# Carbon isotope tracing




Multiple tracers can be used (in parallel or simultaneously) to infer:

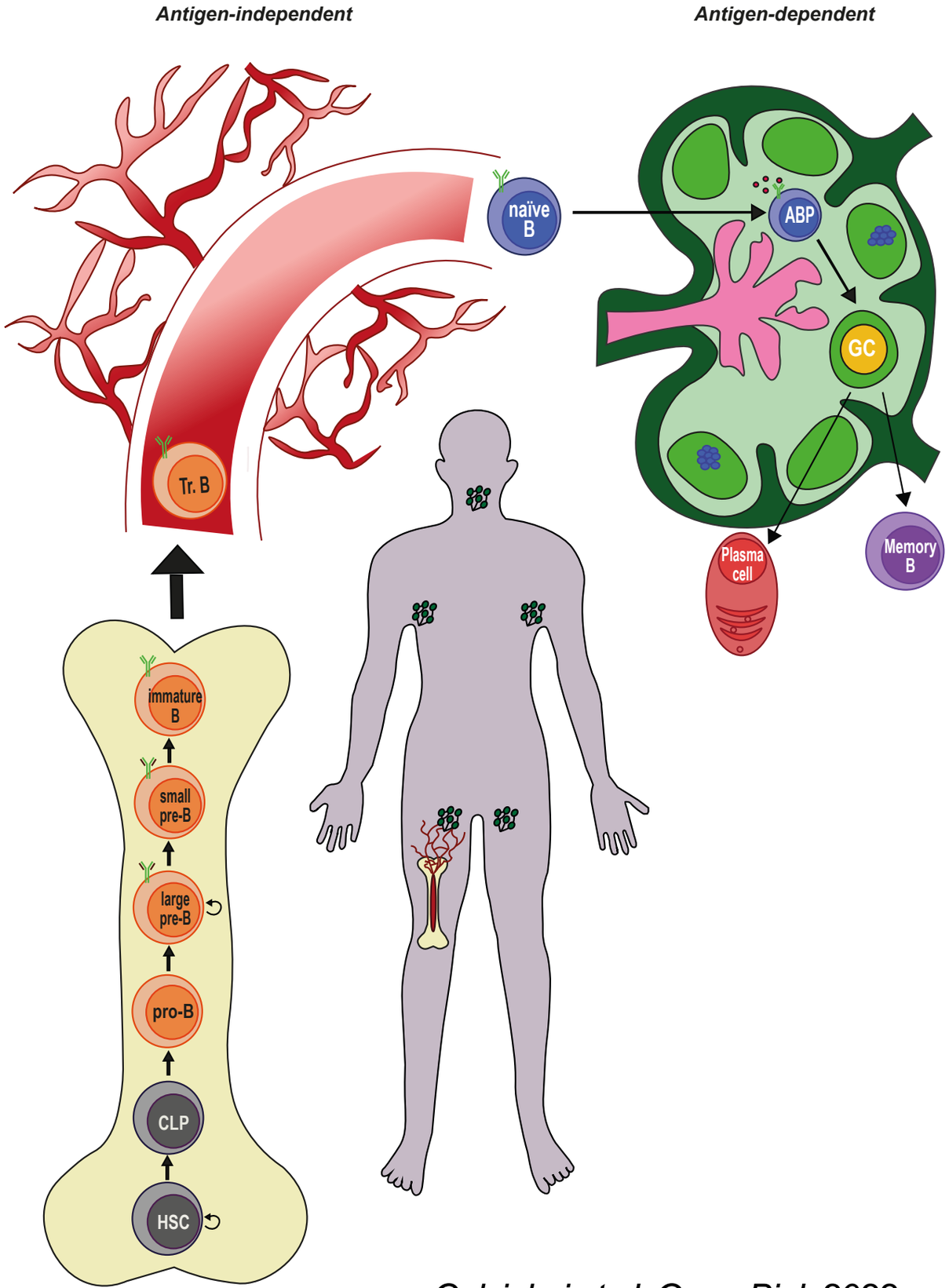
1) Nutrient preference
2) Pathway utilization

# Carbon isotope tracing



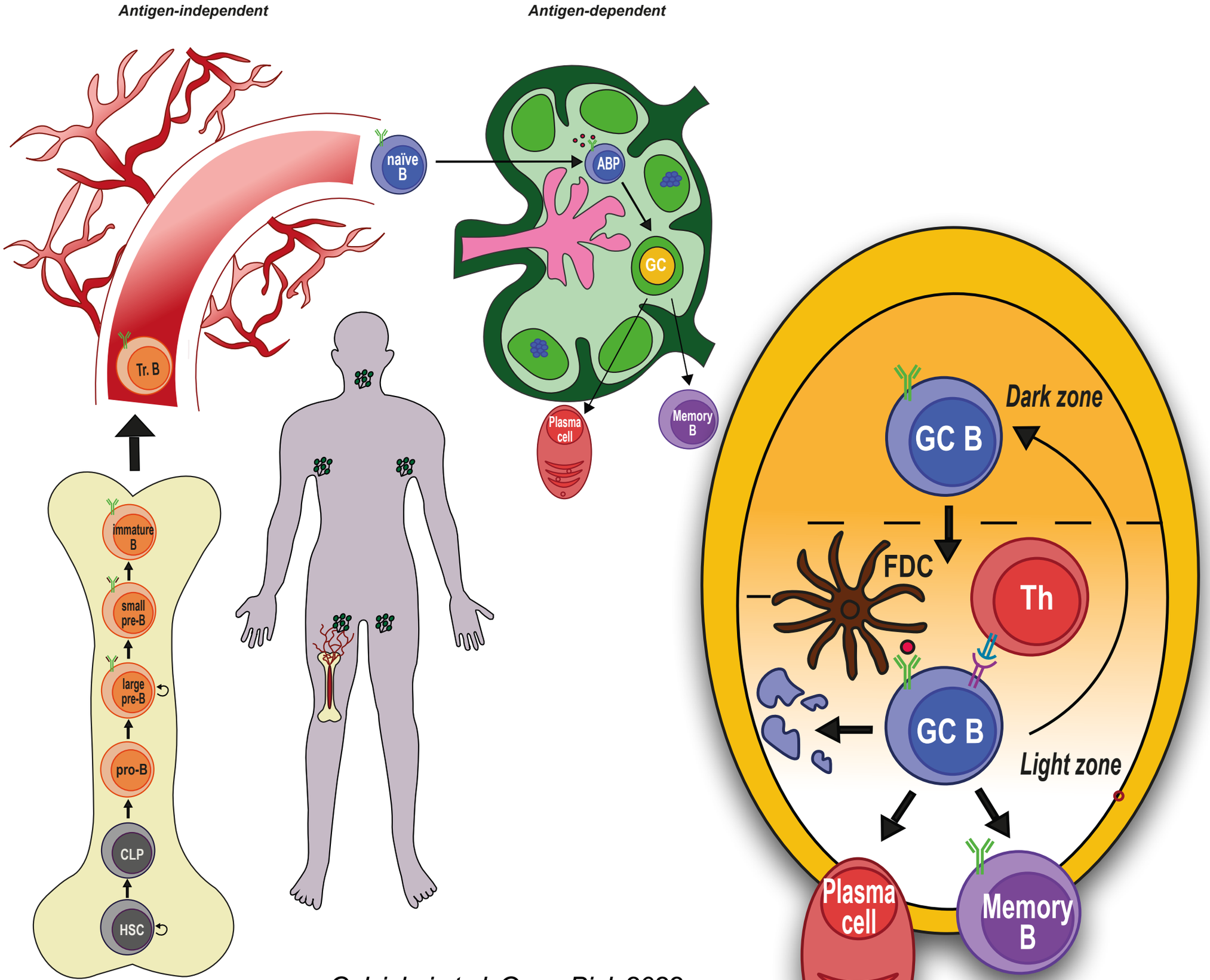

Acetyl-CoA can be generated from multiple carbon sources.
Which one is predominant and in which conditions can be important

# B cell maturation and Germinal Center (GC) reaction



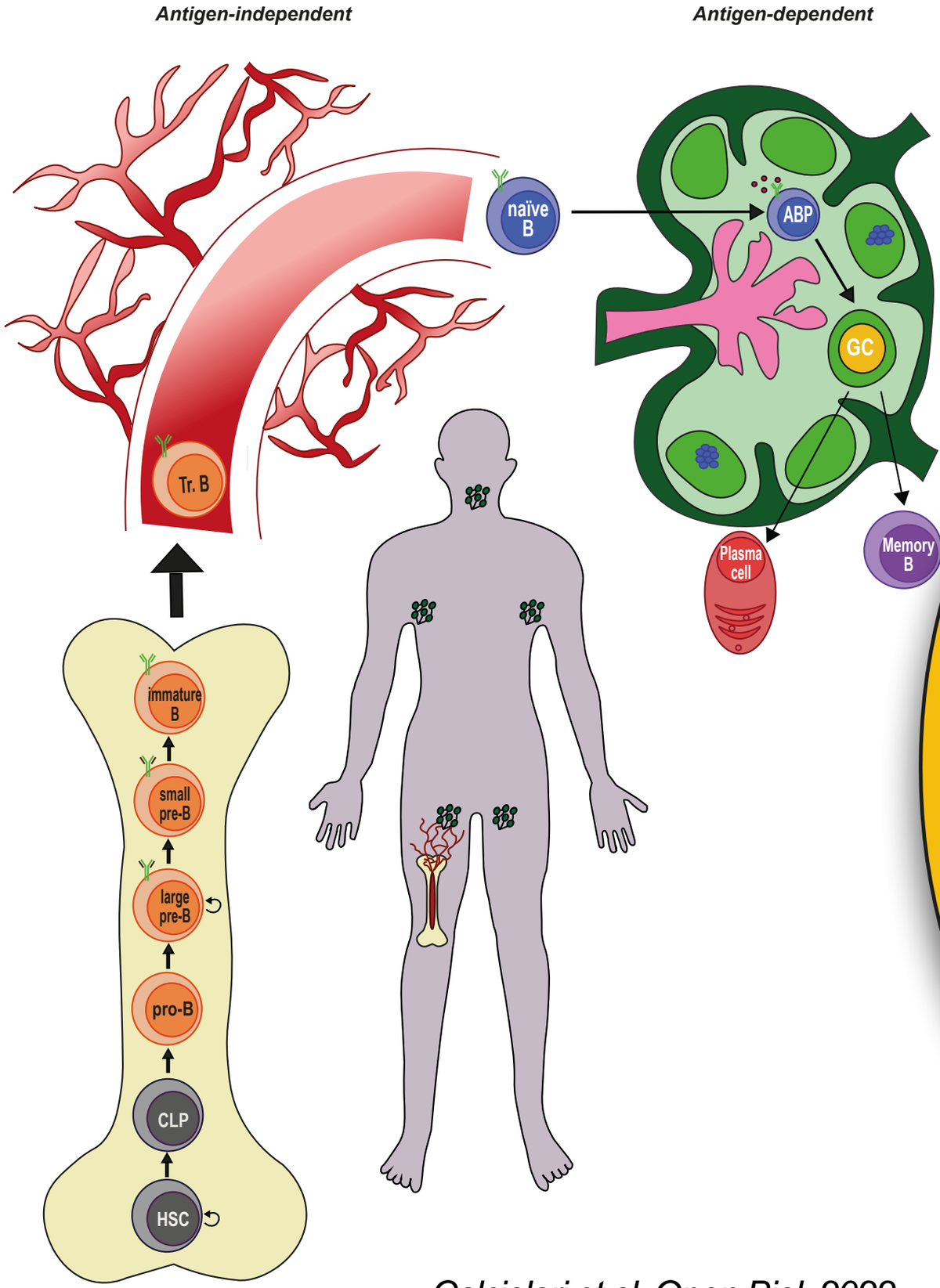

*Calciolari et al, Open Biol, 2022*

# B cell maturation and Germinal Center (GC) reaction

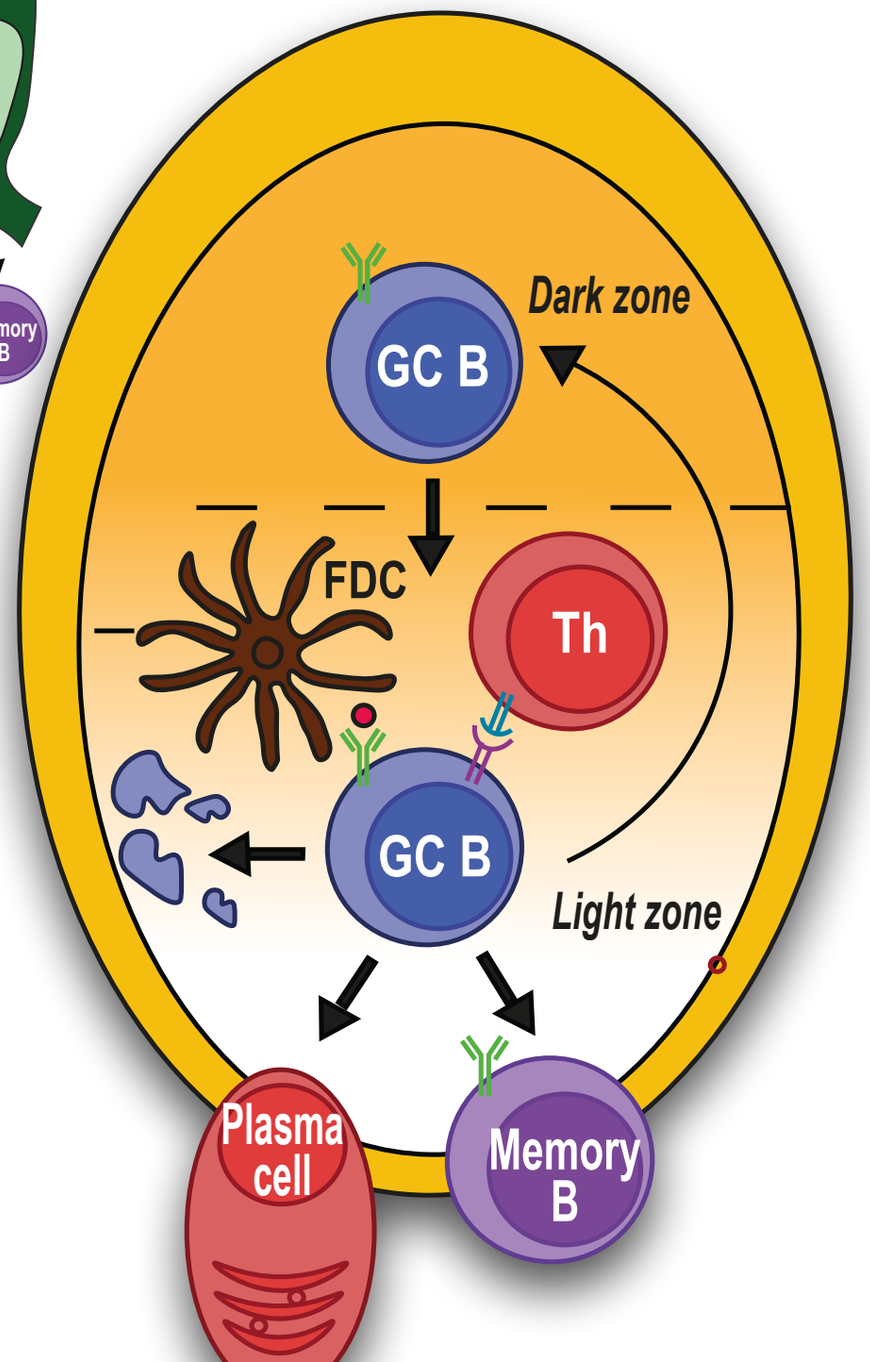



Calciolari et al, Open Biol, 2022

# B cell maturation and Germinal Center (GC) reaction

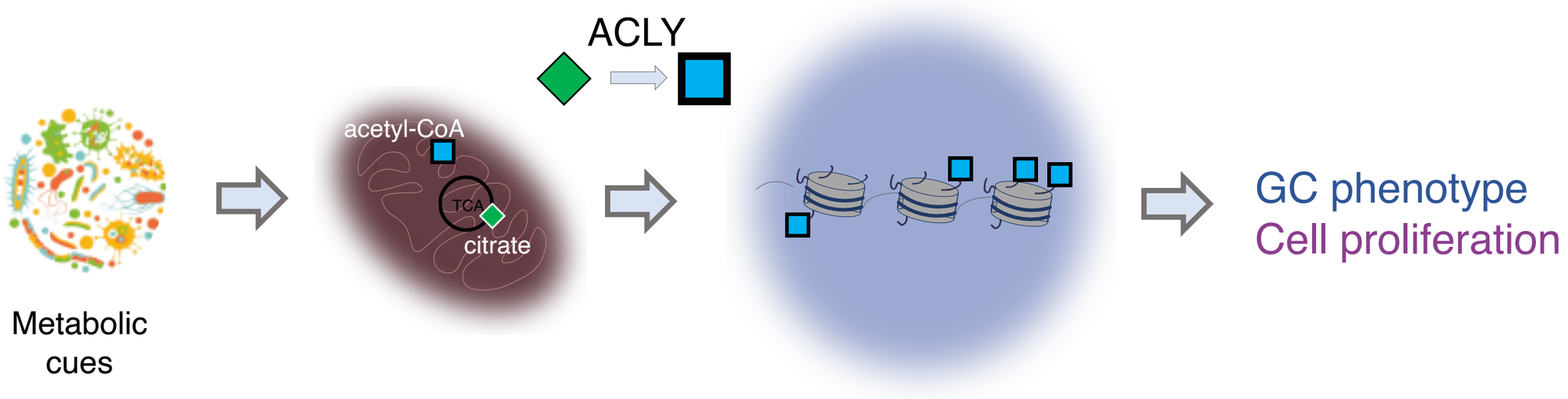



Role of metabolism not elucidated!!!!!

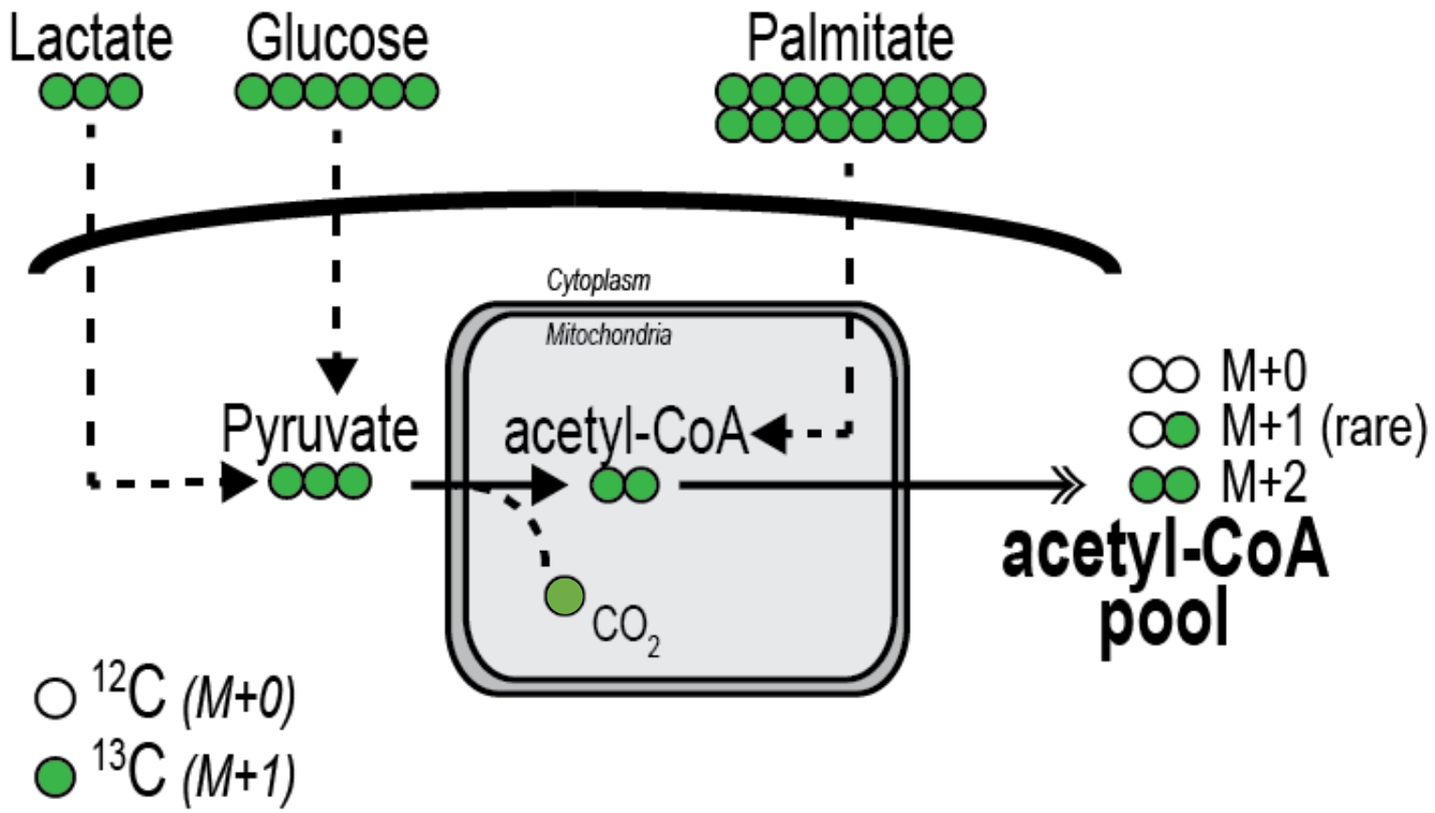



*Calciolari et al, Open Biol, 2022*

# WORKING HYPOTHESIS:
# ACLY INTEGRATES NUTRIENT SENSING INTO THE EPIGENOME



# ISOTOPE TRACING SHOWS ELEVATED DE NOVO PRODUCTION OF ACETYL-COA



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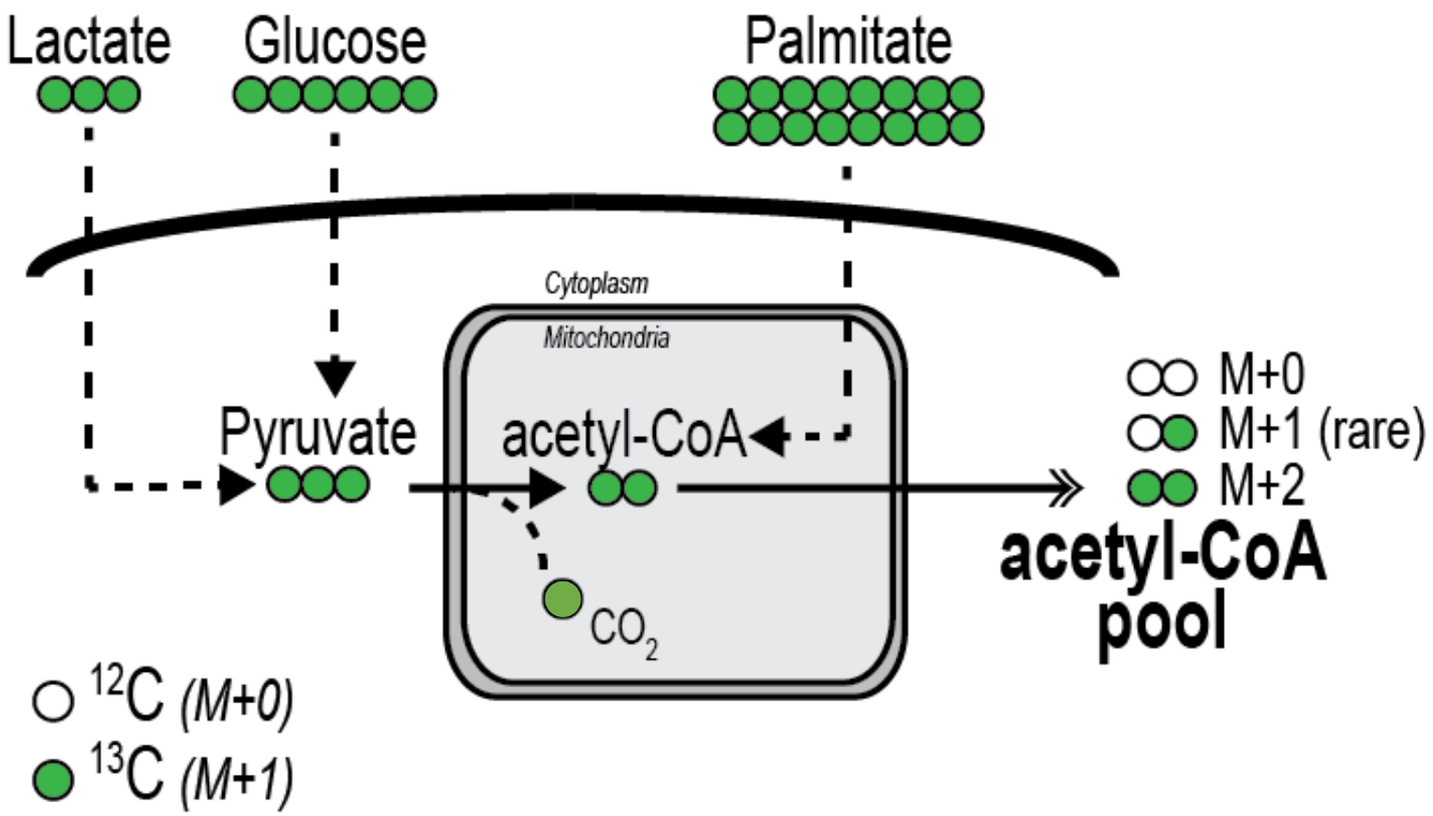
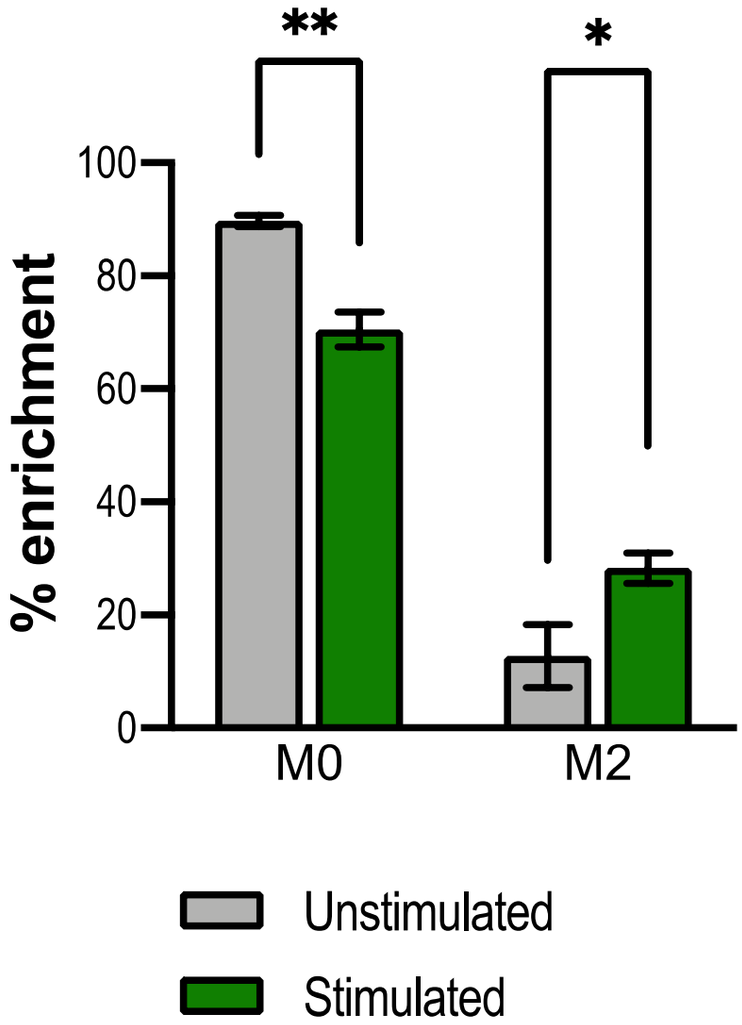
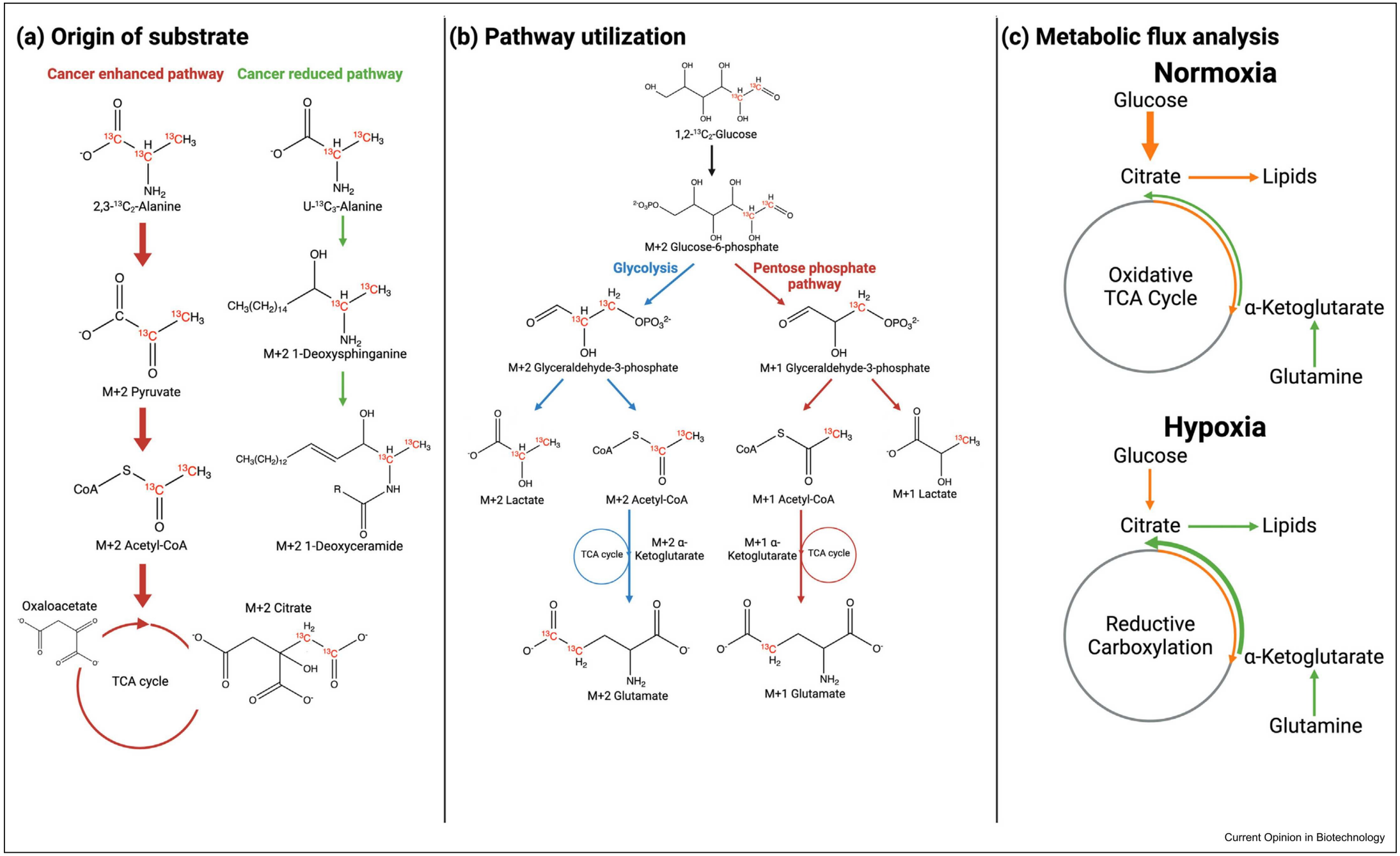
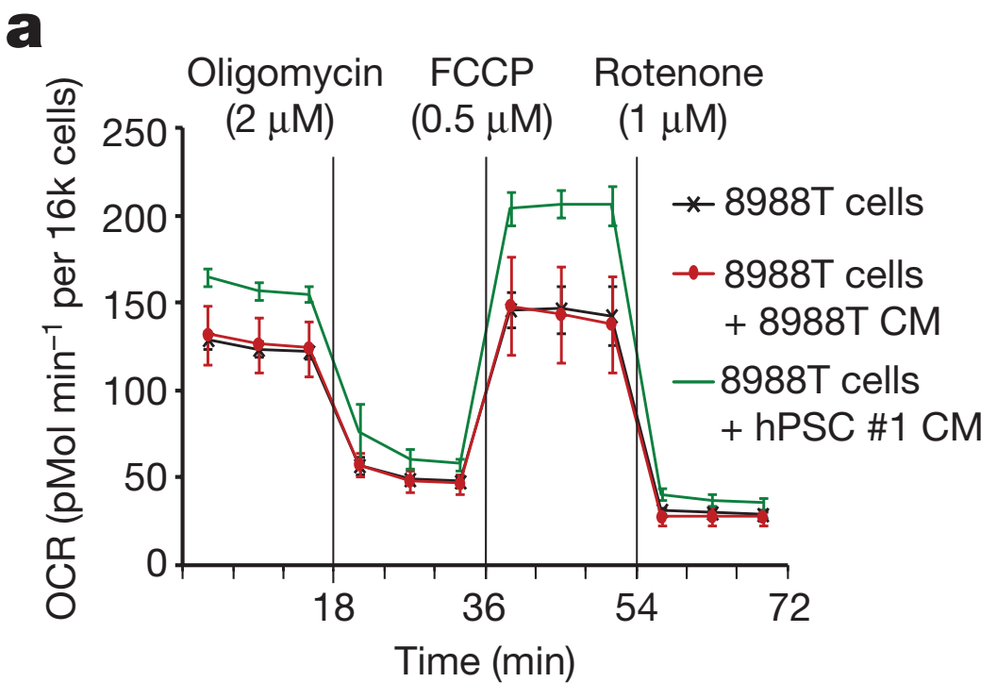


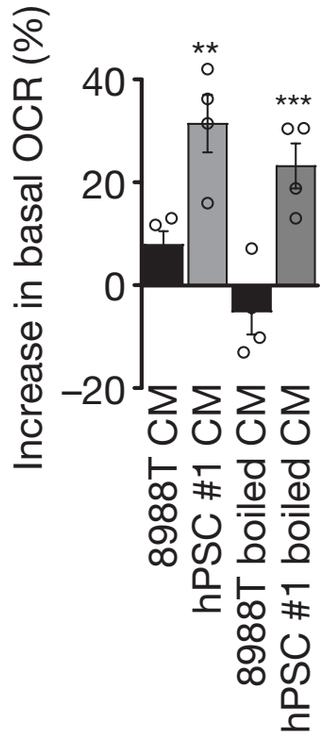



# Carbon isotope tracing

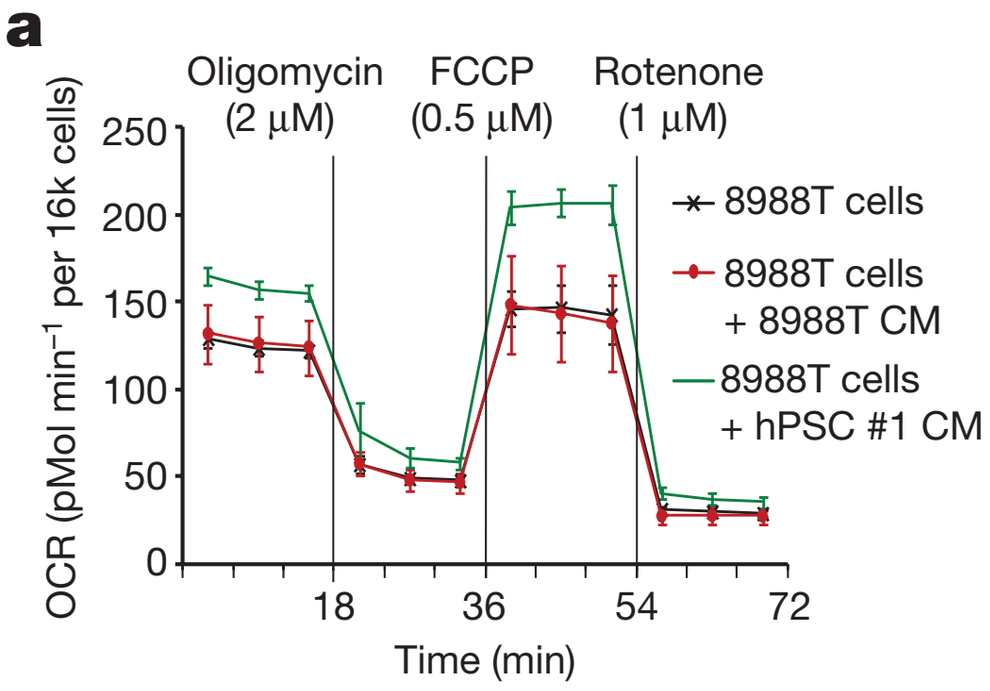


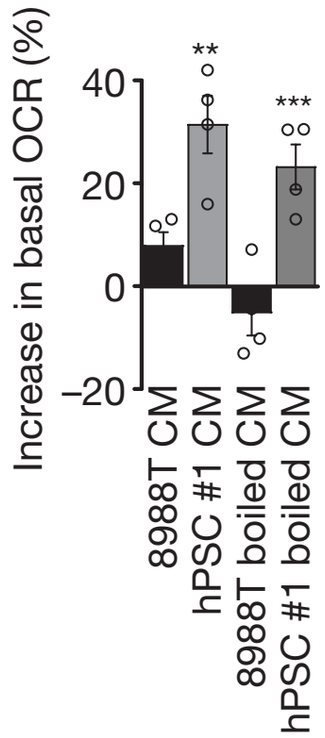

*Dong et al,* ***Curr Opin Biotechnol****, 2022*

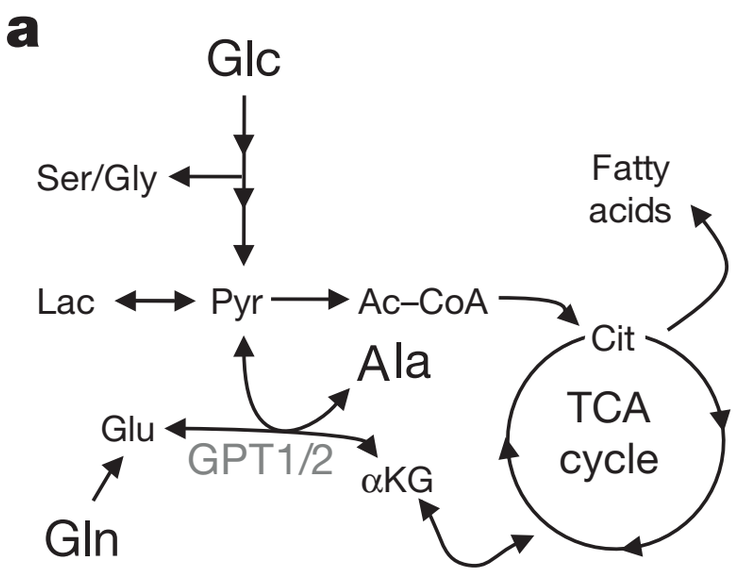


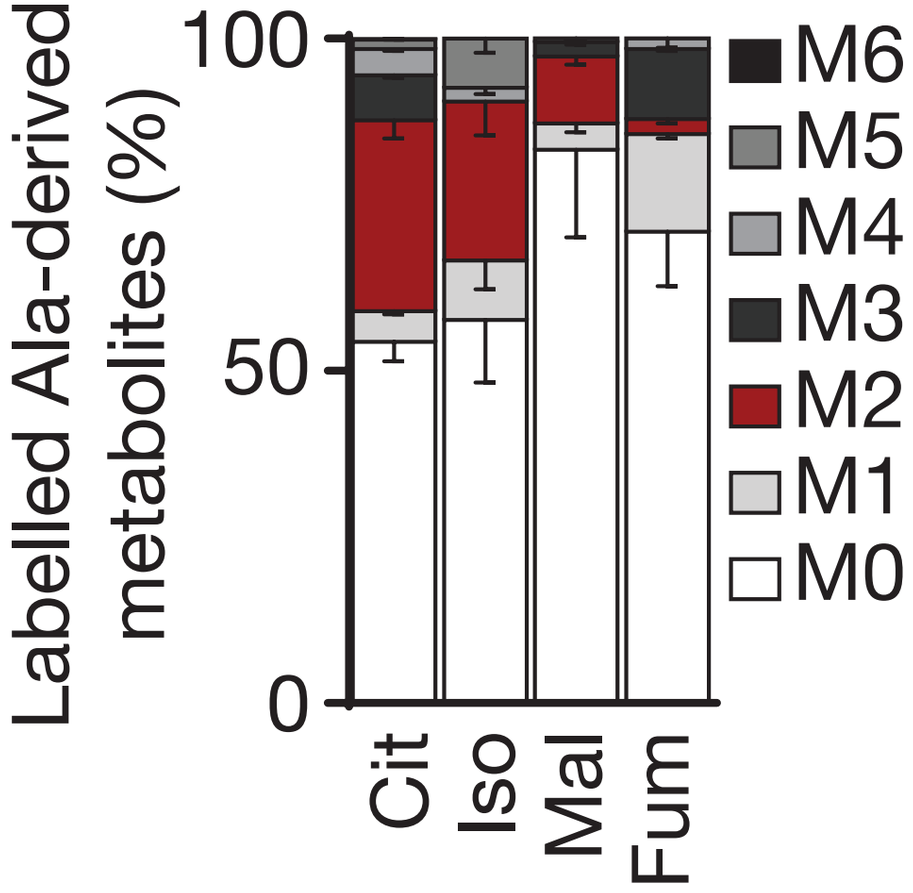




*Sousa et al,* ***Nature****, 2016*

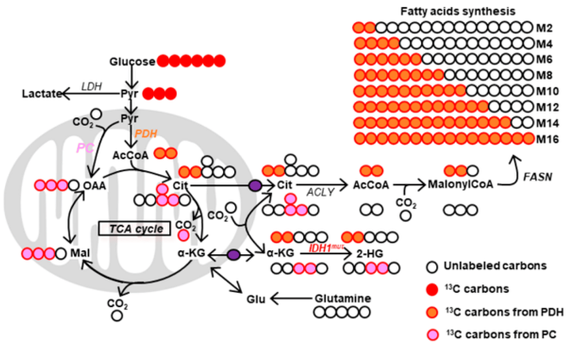


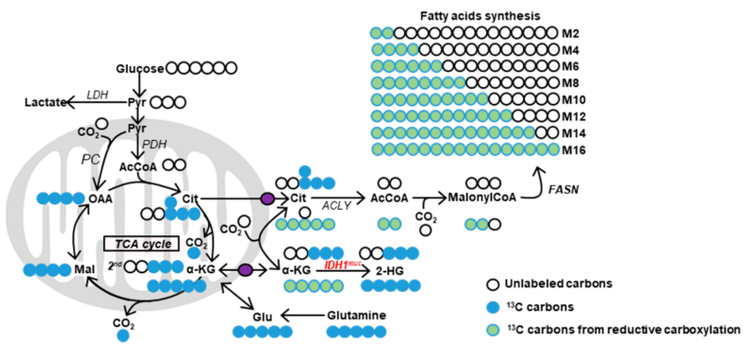



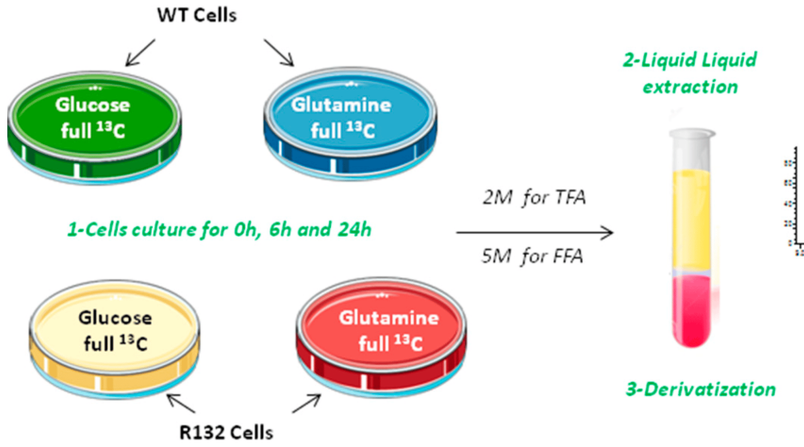

*Sousa et al,* ***Nature****, 2016*


Fatty acids synthesis
M2
M4
M6
M8
M10
M12
M14
M16
Glucose
Lactate
LDH
Pyr
PDH
PC
AcCoA
OAA
Cit
ACLY
MalonylCoA
FASN
TCA cycle
Mal
α-KG
IDH1mut
2-HG
Glu
Glutamine
Unlabeled carbons
13C carbons
13C carbons from PDH
13C carbons from PC

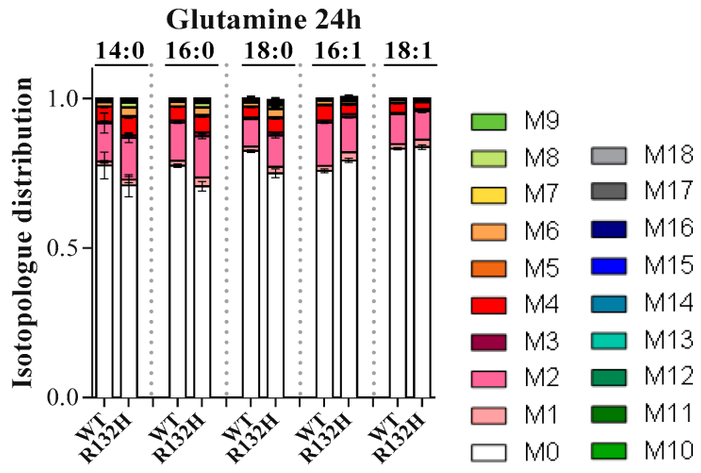
(A)


Fatty acids synthesis
M2
M4
M6
M8
M10
M12
M14
M16
Glucose
Lactate
LDH
Pyr
PDH
PC
AcCoA
OAA
Cit
ACLY
MalonylCoA
FASN
TCA cycle
Mal
α-KG
IDH1mut
2-HG
Glu
Glutamine
Unlabeled carbons
13C carbons
13C carbons from reductive carboxylation

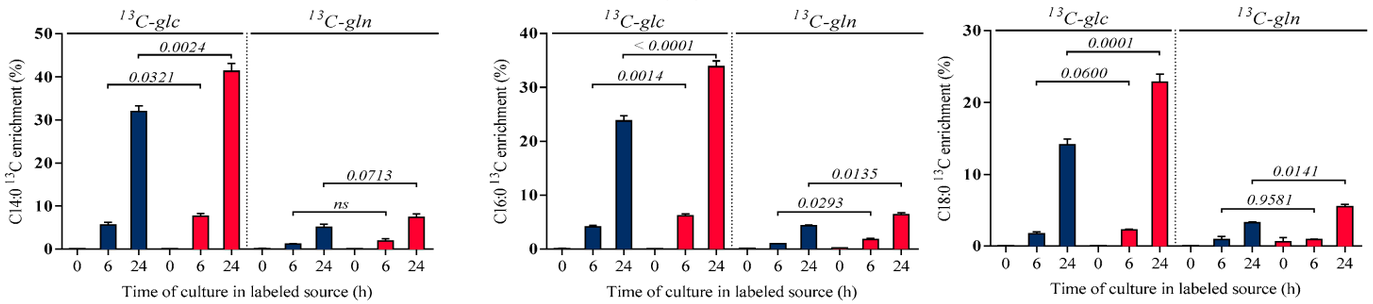
(B)


WT Cells
Glucose full 13C
Glutamine full 13C
1-Cells culture for 0h, 6h and 24h
2M for TFA
5M for FFA
R132 Cells
Glucose full 13C
Glutamine full 13C
2-Liquid Liquid extraction
3-Derivatization
4-GC-MS analysis
5- Data analysis

(C)

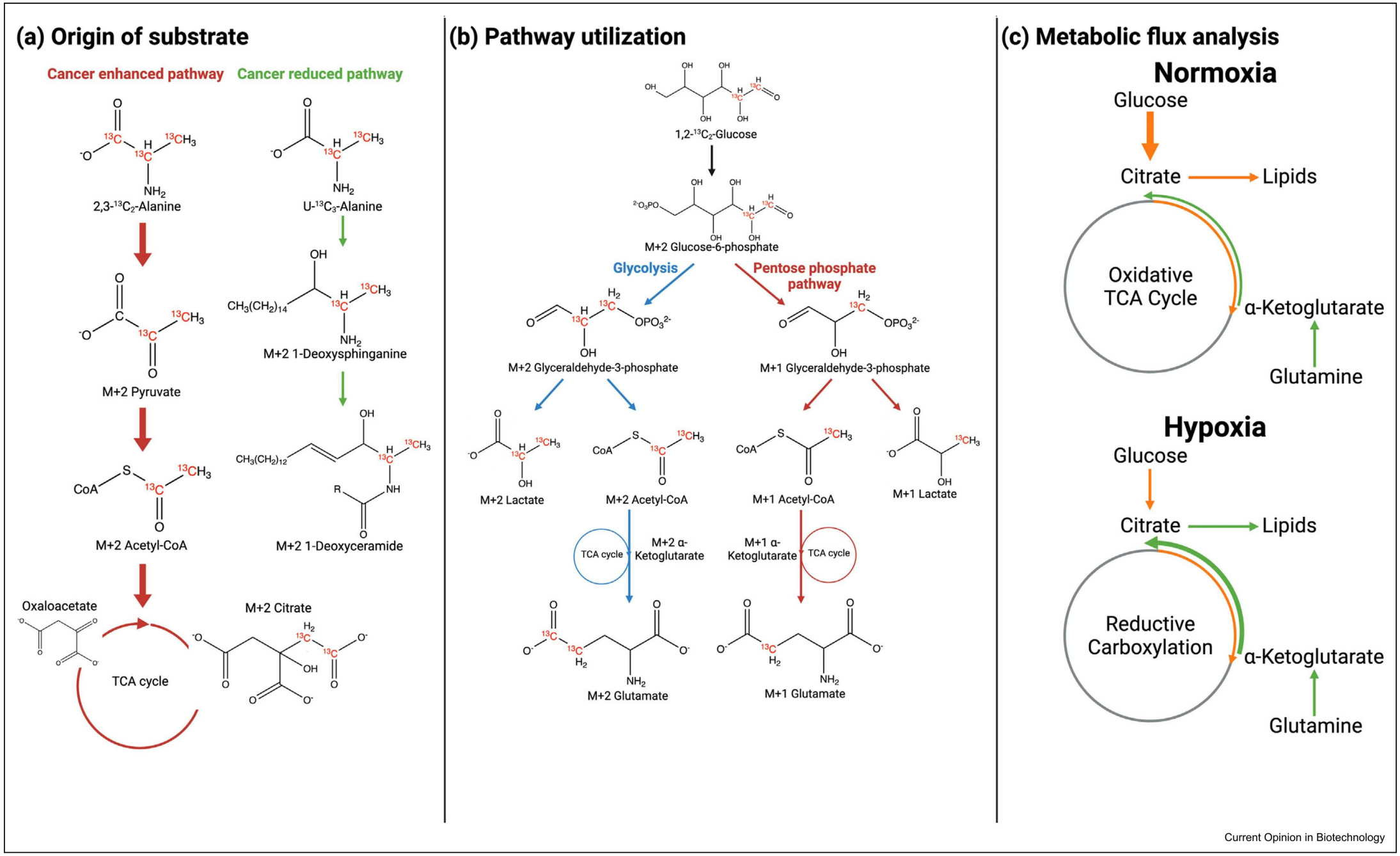

Glutamine 24h
14:0
16:0
18:0
16:1
18:1
Isotopologue distribution
1.0
0.5
0.0
M9
M8
M7
M6
M5
M4
M3
M2
M1
M0
M18
M17
M16
M15
M14
M13
M12
M11
M10
WT
R132H

(A)


13C-glc
13C-gln
C14:0 13C enrichment (%)
0.0024
0.0321
0.0713
ns
C16:0 13C enrichment (%)
< 0.0001
0.0014
0.0135
0.0293
C18:0 13C enrichment (%)
0.0001
0.0600
0.0141
0.9581
C16:1 13C enrichment (%)
0.2956
0.0001
0.0256
0.0274
C18:1 13C enrichment (%)
0.0025
0.0002
0.1425
0.0118
Time of culture in labeled source (h)

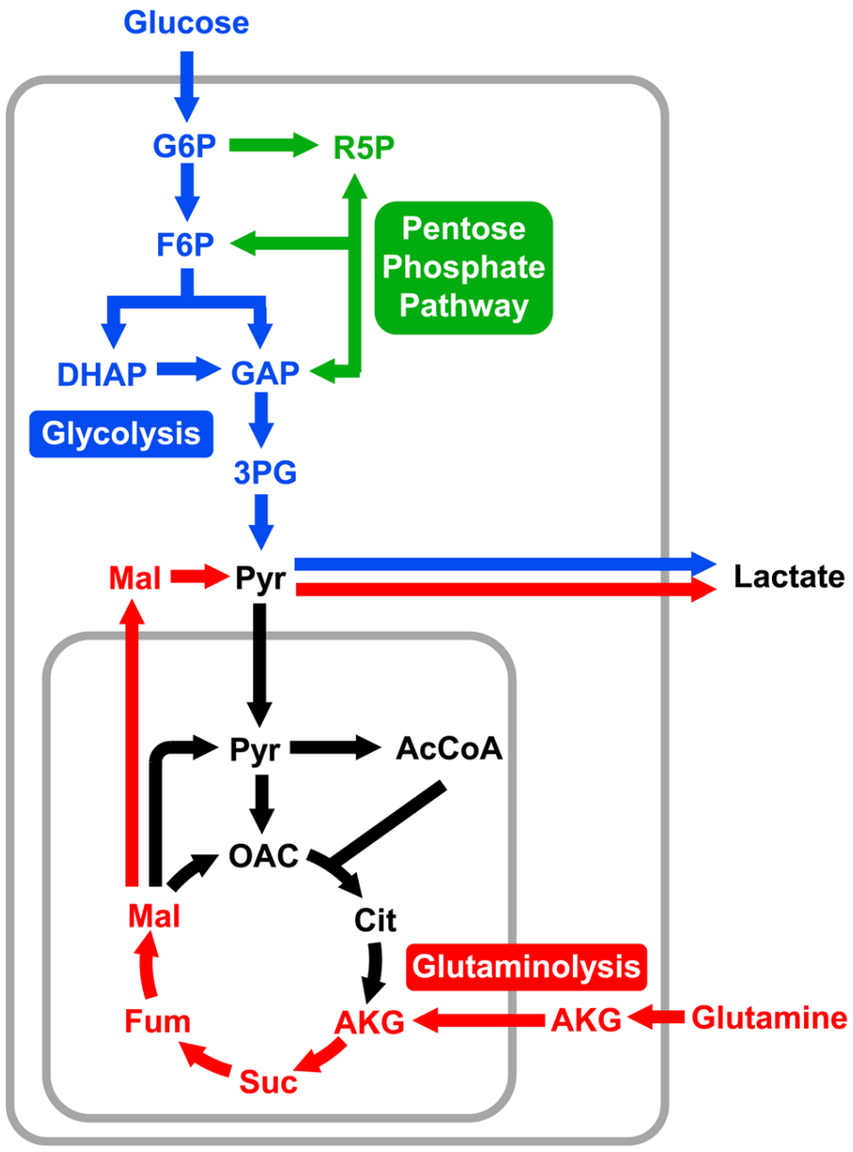
(B)

# Carbon isotope tracing

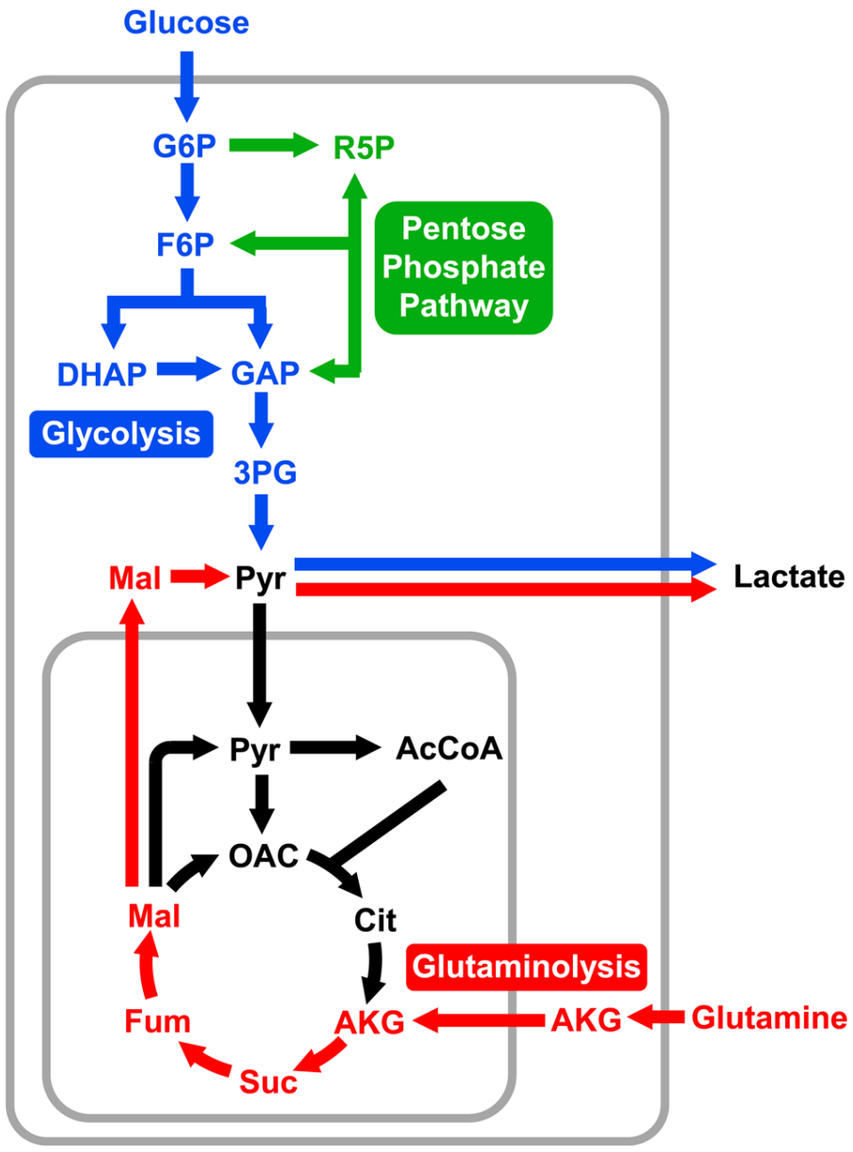


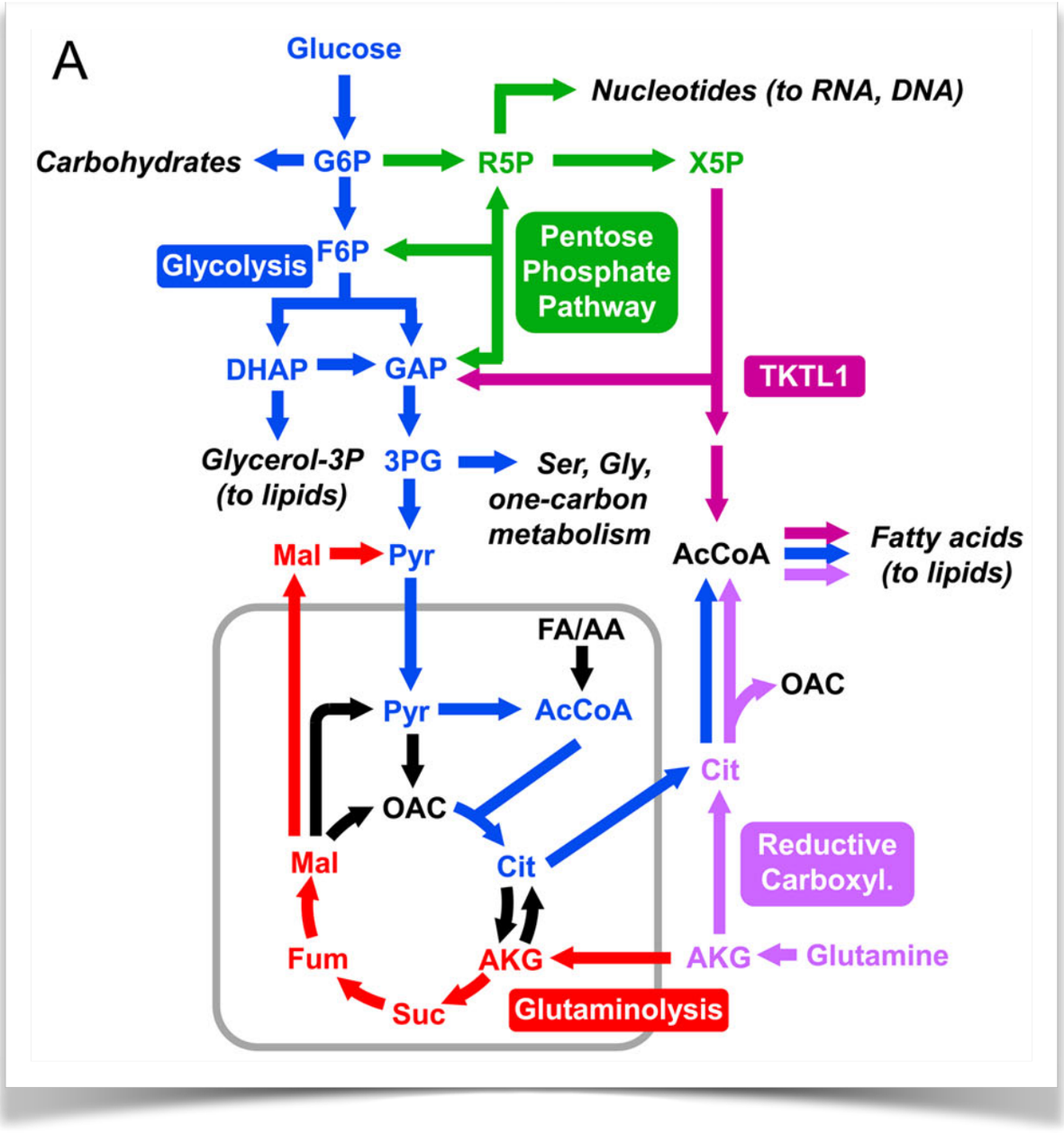

*Dong et al,* ***Curr Opin Biotechnol****, 2022*

# Tracing can reveal nutrient utilization pathways



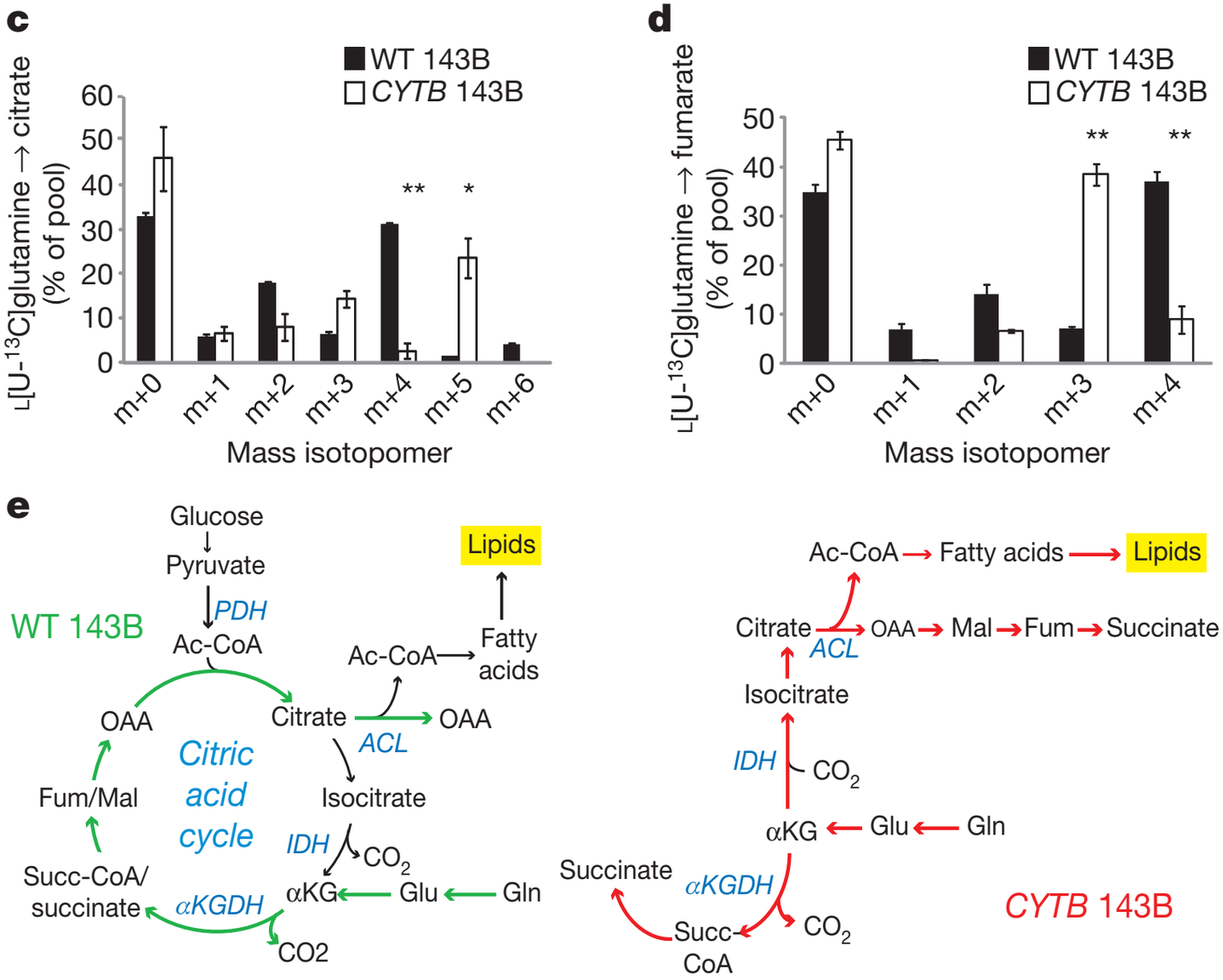
# Tracing can reveal nutrient utilization pathways



# Reductive carboxylation supports growth in tumour cells with defective mitochondria

Andrew R. Mullen[1], William W. Wheaton[2,3], Eunsook S. Jin[4,5], Pei-Hsuan Chen[1], Lucas B. Sullivan[2,3], Tzuling Cheng[1], Youfeng Yang[6], W. Marston Linehan[6], Navdeep S. Chandel[2,3] & Ralph J. DeBerardinis[1,7,8]




*Mullen et al,* ***Nature****, 2012*

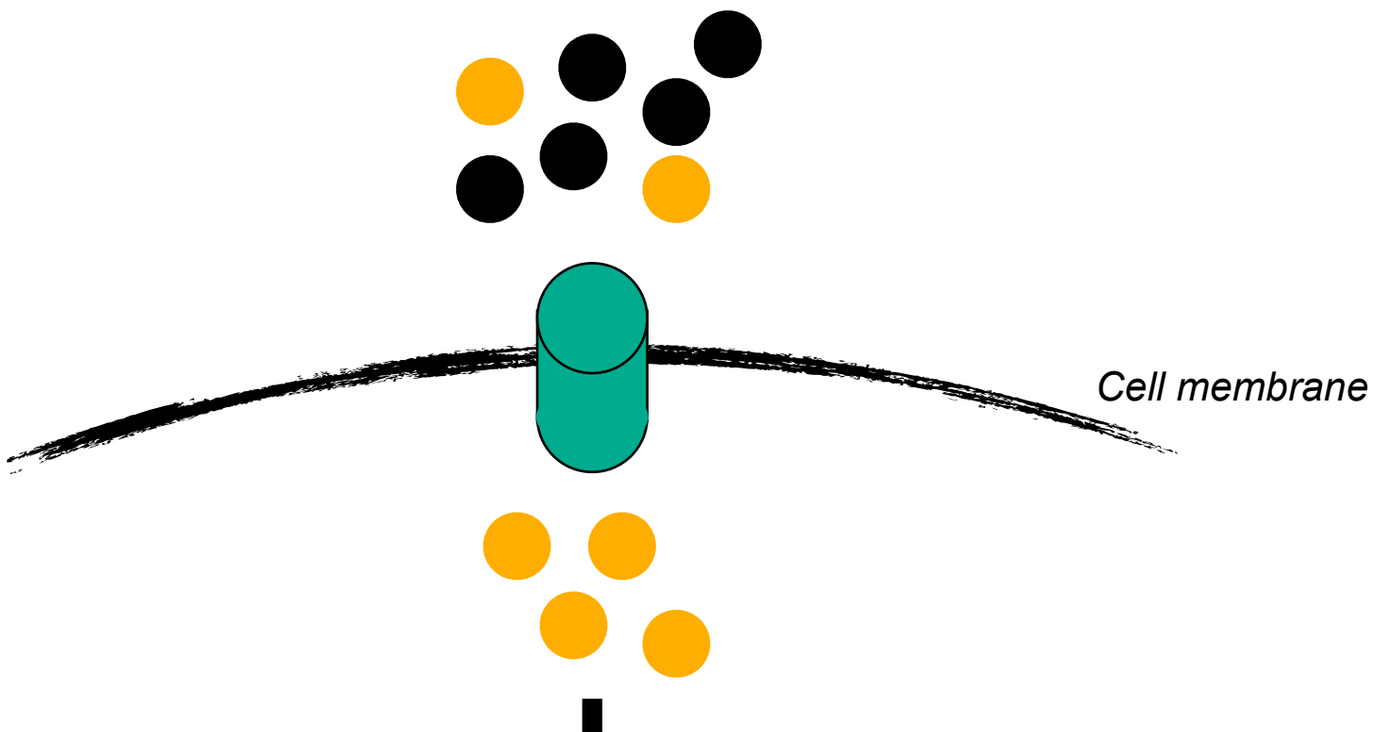
**Table 2. Isotopic Tracers for Measuring Pathway Activities**

| Application | Tracer | Metabolite readouts | Interpretation |
|---|---|---|---|
| Pentose phosphate pathway (PPP) | | | |
| PPP overflow | [1,2-$^{13}$C]glucose | Lactate M+1, M+2 | Flux through the combined oxidative and non-oxidative PPP generates M+1 lactate from [1,2-$^{13}$C]glucose, while glycolysis generates only M+2 lactate (Lee et al., 1998). LacM+1 / LacM+2 reflects ratio of PPP overflow to glycolysis. |
| Source of ribose (oxidative versus non-oxidative branch of PPP) | [1,2-$^{13}$C]glucose | Ribose phosphate M+1, M+2 | The oxPPP make M+1 ribose phosphate; the non-oxPPP makes M+2. Ratio of M+1/M+2 depends on the gross flux (net flux + exchange flux) of each branch: Reversibility of the non-oxPPP can make M+2 even if all net ribose production is by oxPPP. |
| Glycolysis, TCA and gluconeogenesis | | | |
| Glycolytic rate | [U-$^{13}$C]glucose | FBP<br>Dihydroxyacetone phosphate<br>3-phosphoglycerate | Higher flux yields faster labeling. Labeling results should be confirmed by glucose uptake and lactate excretion measurements. |
| Reversibility of glycolysis | 50%: 50% mix of [U-$^{12}$C]: [U-$^{13}$C] glucose | Glucose-6-phosphate M+3<br>FBP M+3 | Feeding a mixture of labeled and unlabeled glucose results in unlabeled and M+3 triose phosphates. Reversibility of aldolase produces M+3 FBP. Fructose bisphosphatase activity yields M+3 glucose-6-phosphate (Park et al., 2016). |
| Gluconeogenesis | [U-$^{13}$C]lactate<br>[U-$^{13}$C]glutamine | Glucose M+2, M+3<br>Glucose-6-phosphate M+2, M+3<br>3-phosphoglycerate M+2, M+3 | Lactate and glutamine are major TCA feedstocks. Flux from TCA to glycolysis catalyzed by PEPCK results in triose phosphate labeling. Fructose bisphosphatase activity then makes labeled hexose phosphates. |
| Pyruvate carboxylase contribution to TCA | [3-$^{13}$C]glucose<br>[1-$^{13}$C]pyruvate | Aspartate M+1<br>Malate M+1 | C1 of pyruvate comes from glucose C3/C4. Pyruvate C1 is lost in making acetyl-CoA, but can enter TCA via pyruvate carboxylase which makes M+1 oxaloacetate and thus M+1 aspartate and M+1 malate (Sellers et al., 2015). |
| Reductive carboxylation ("backwards" TCA flux) | [U-$^{13}$C]glutamine<br>[1-$^{13}$C]glutamine | Citrate M+5, Malate M+3 or<br>Citrate M+1, Malate M+1 | Reductive carboxylation of α-ketoglutarate (derived from labeled glutamine) produces M+5 citrate from [U-$^{13}$C]glutamine and M+1 citrate from [1-$^{13}$C]glutamine, and subsequent ATP citrate lyase produces M+3 or M+1 malate, respectively (Yoo et al., 2008) |
| TCA carbon sources | [U-$^{13}$C]nutrients | Succinate<br>Malate<br>Citrate<br>α-ketoglutarate | Carbon enrichment (number of $^{13}$C atoms versus total carbon atoms) reflects carbon contribution from the nutrient; useful *in vivo* with correction for circulating nutrient enrichment (Davidson et al., 2016; Faubert et al., 2017; Hui et al., 2017) |

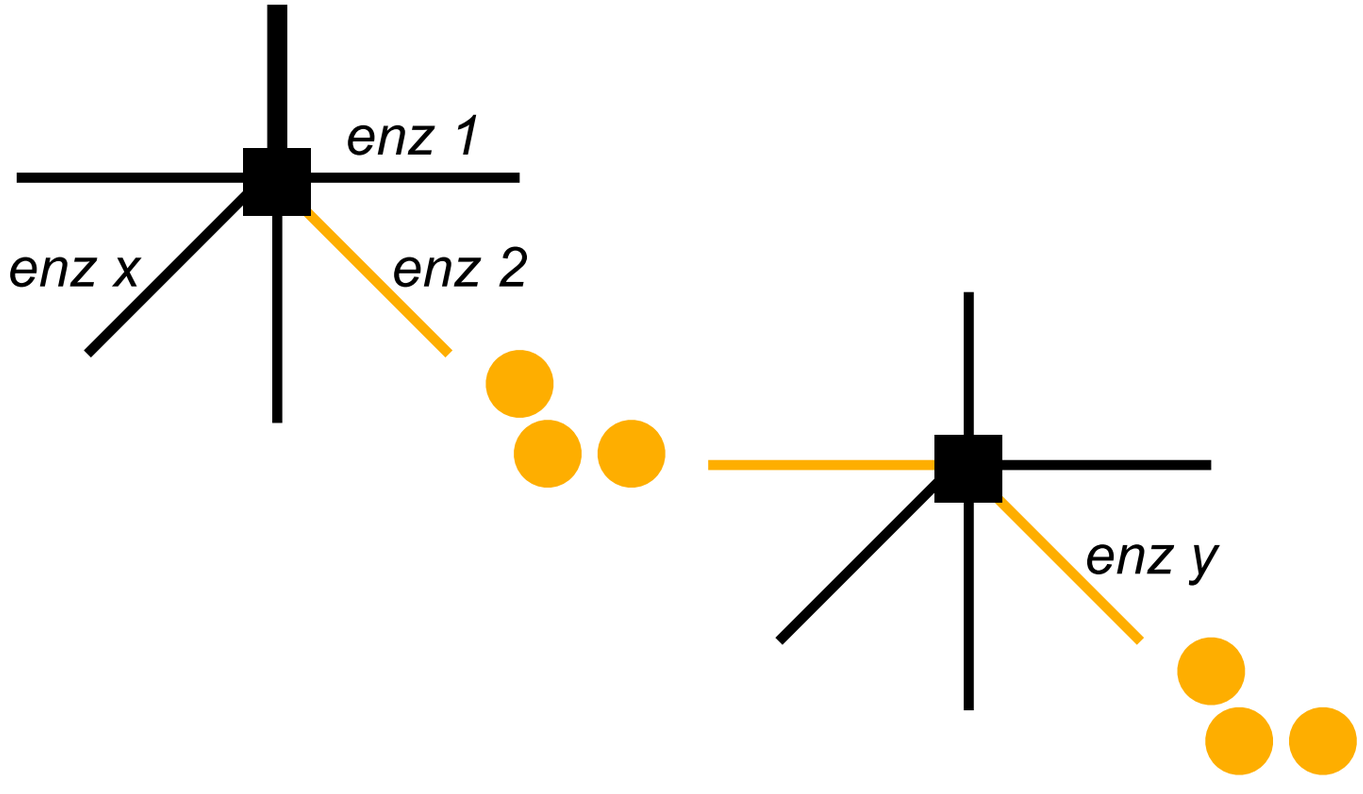
*Jang et al, **Cell**, 2020*

**Nutrient availability**

**Nutrient uptake**



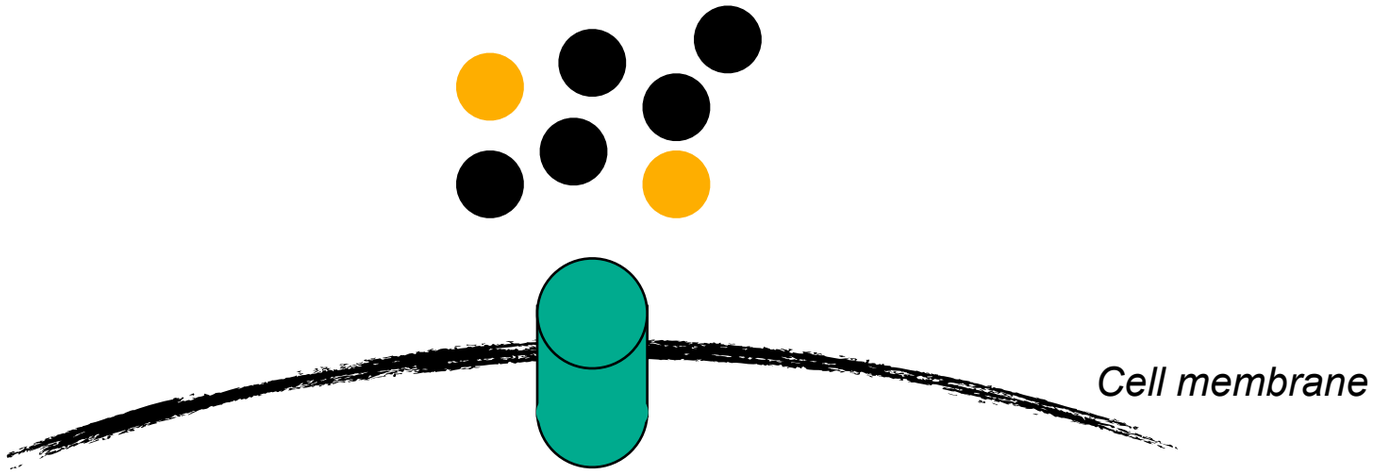

**Nutrient channeling (wiring)**



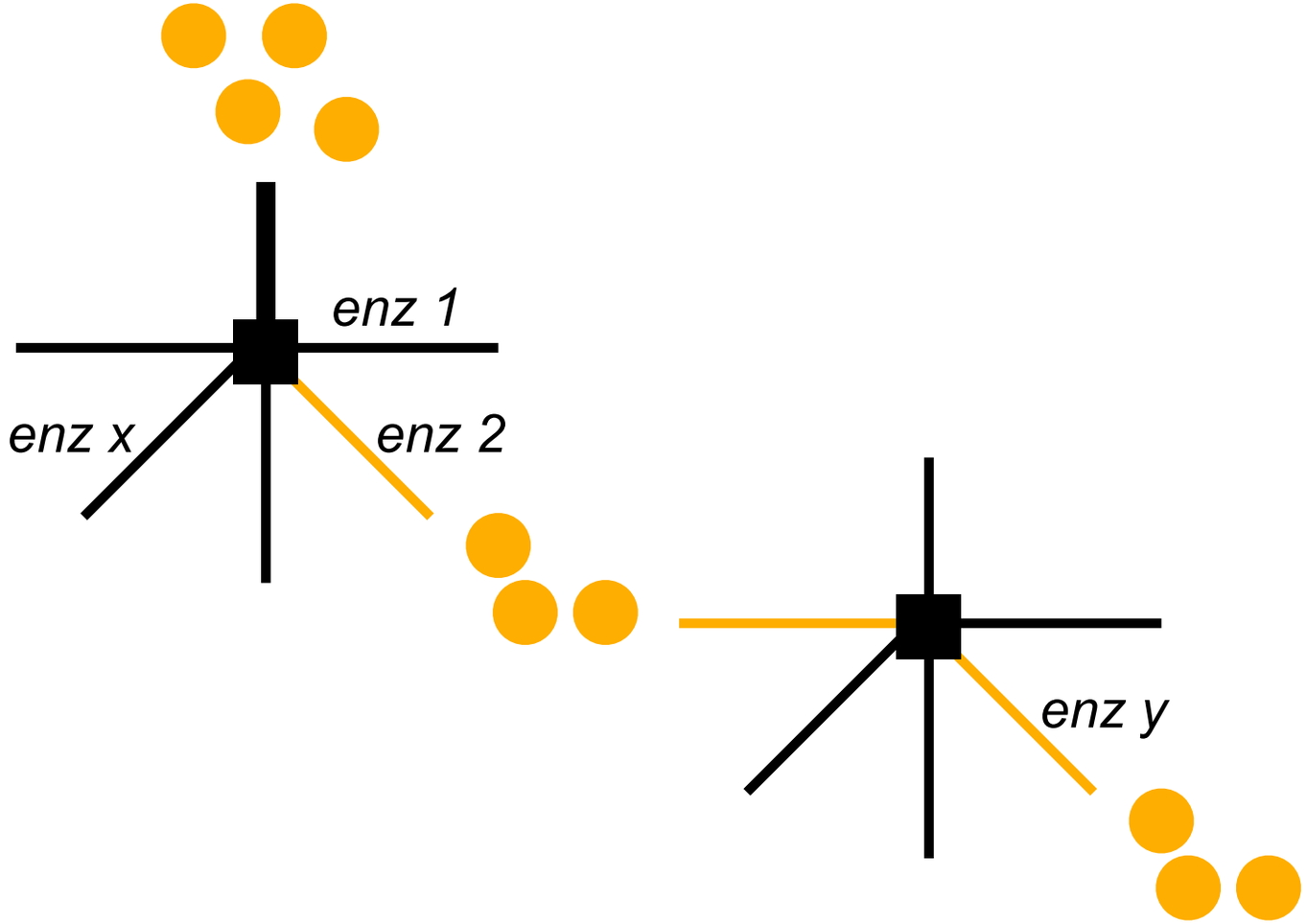
# Multi-level regulation of intracellular metabolism

**Nutrient availability**

**Nutrient uptake**




**Nutrient channeling (wiring)**



# Measuring nutrient availability

## Systemic metabolism

Study absorption mechanisms

Assess what liver does

Circulating levels of nutrients

## Tissue metabolism

Study local nutrient availability

Assess “tug war” between cell types

## Cell metabolism

Quantify metabolite abundance

Assess nutrient uptake

Assess carbon usage

Profile compartmentalization

Evaluate energy storage

# Major challenges of analytical methods

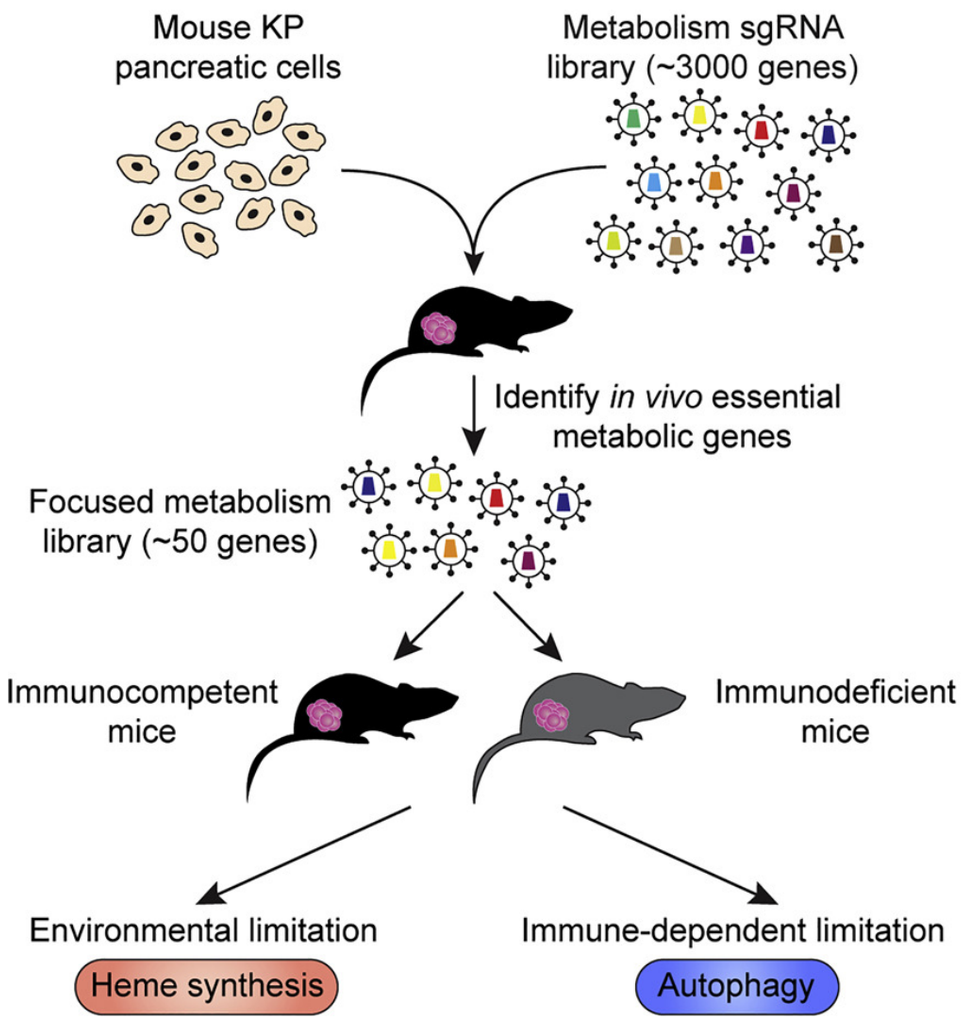
## Compartmentalization

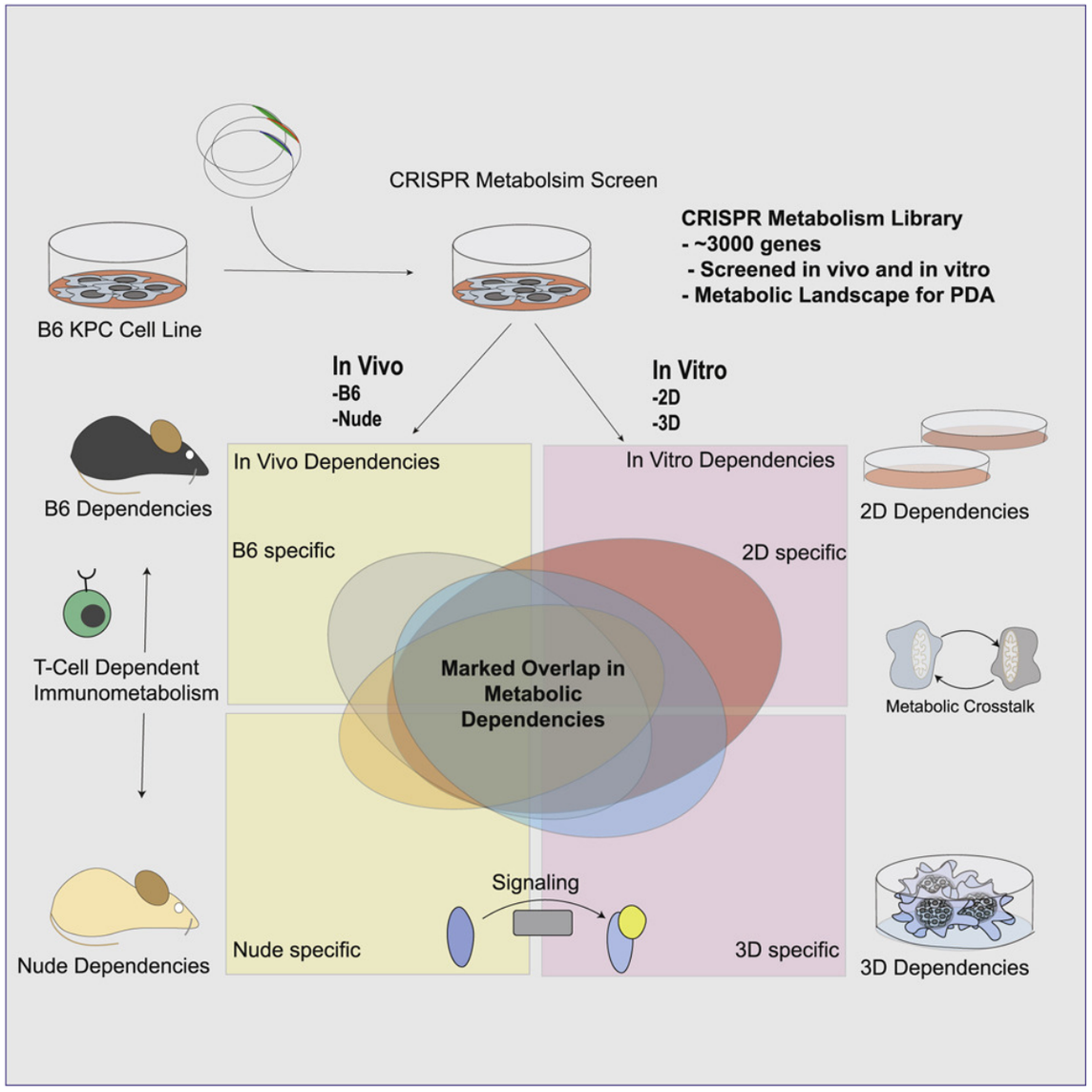
Analyses on whole cell extracts do not capture the availability in each cellular compartment. Confined abundance extremely important for enzymatic activity.

## *In vivo* vs *in vitro/ex vivo*

Analyses on purified cells do not capture the complexity of in vivo systems, which however are extremely dynamic, diverse and difficult to study.

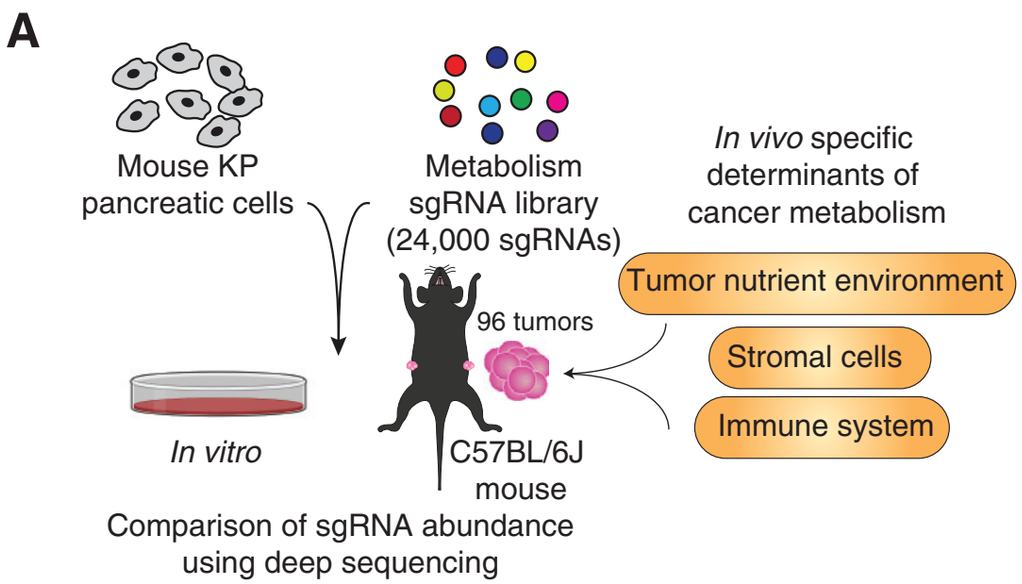
The application of functional genomics allows to test the importance of metabolic enzymes for a certain biological readout (e.g.: tumor growth)

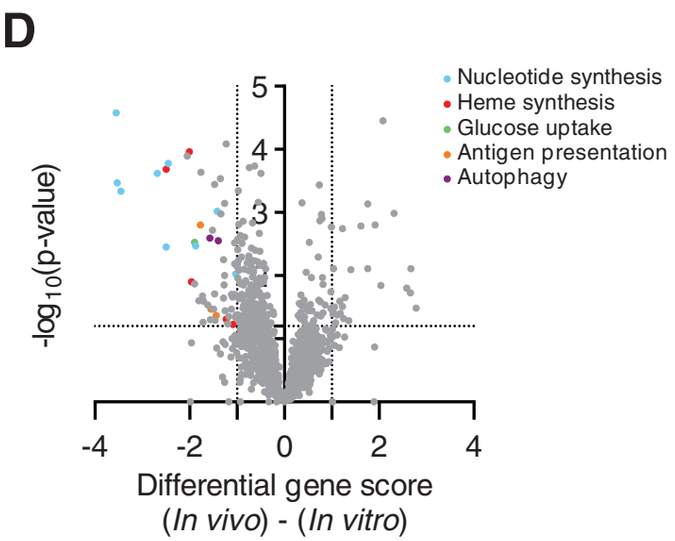


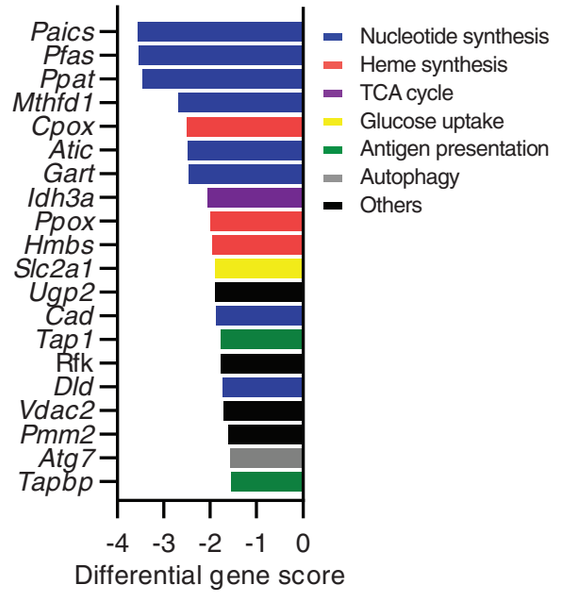



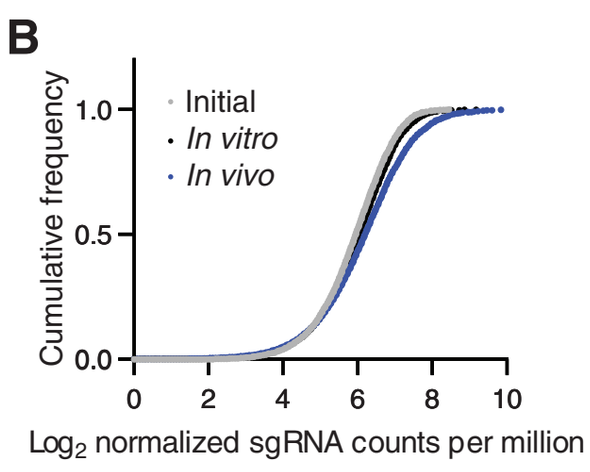

*Biancur et al,* ***Cell Metab****, 2021*

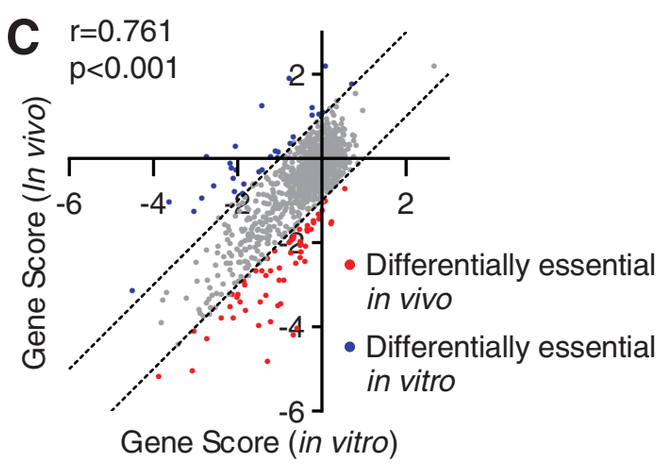
*Zhu et al,* ***Cell Metab****, 2021*

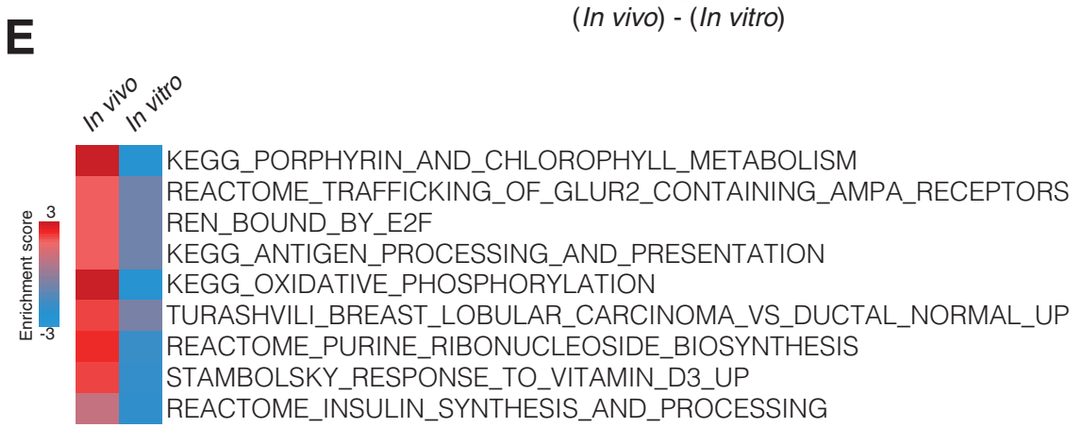


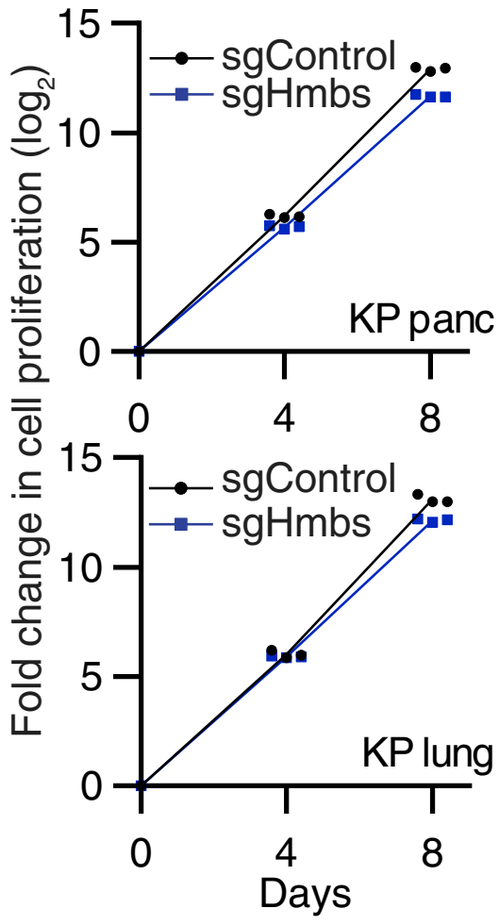



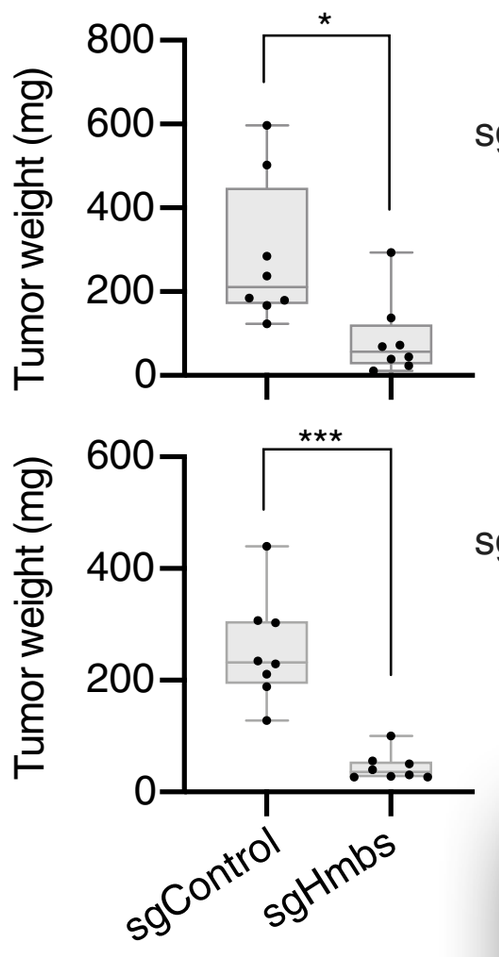



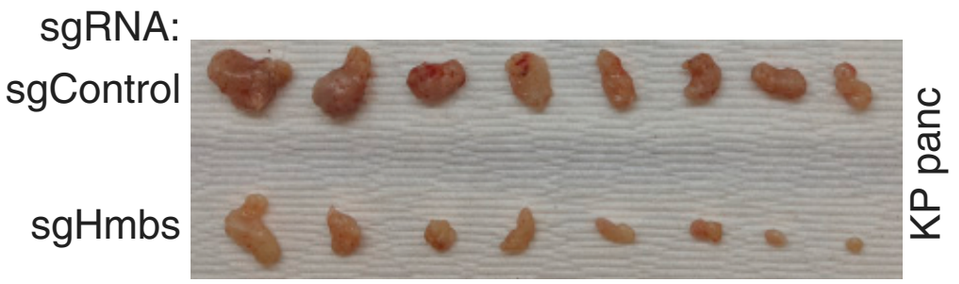

*Zhu et al,* ***Cell Metab****, 2021*

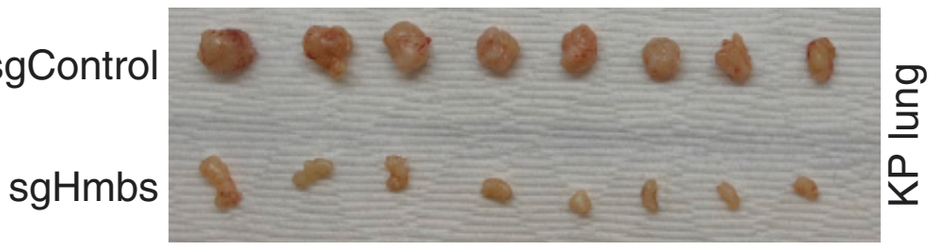
**B**

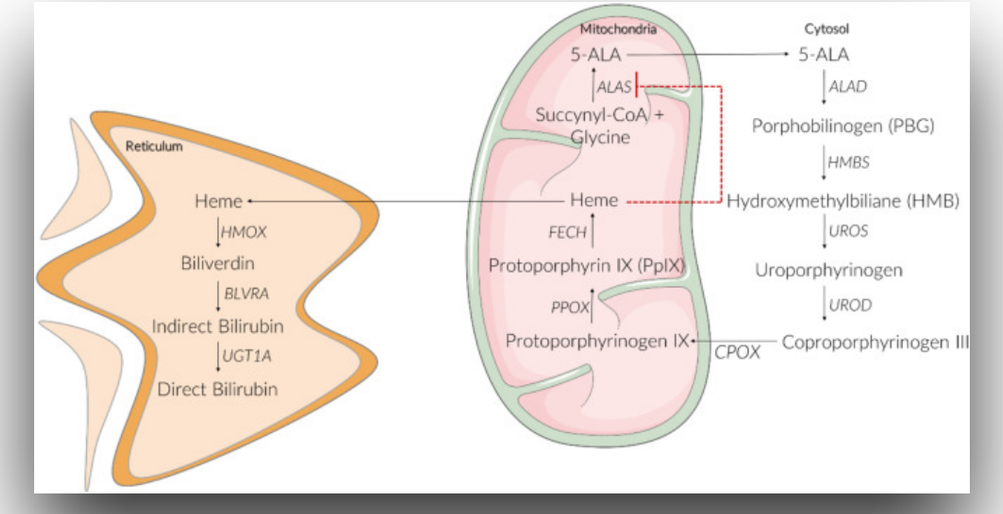



**C**




*Zhu et al,* ***Cell Metab****, 2021*