The Flux Capacitor: Using Skyline for efficient processing of LC-MS/MS metabolic flux data

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Work presented here performed at
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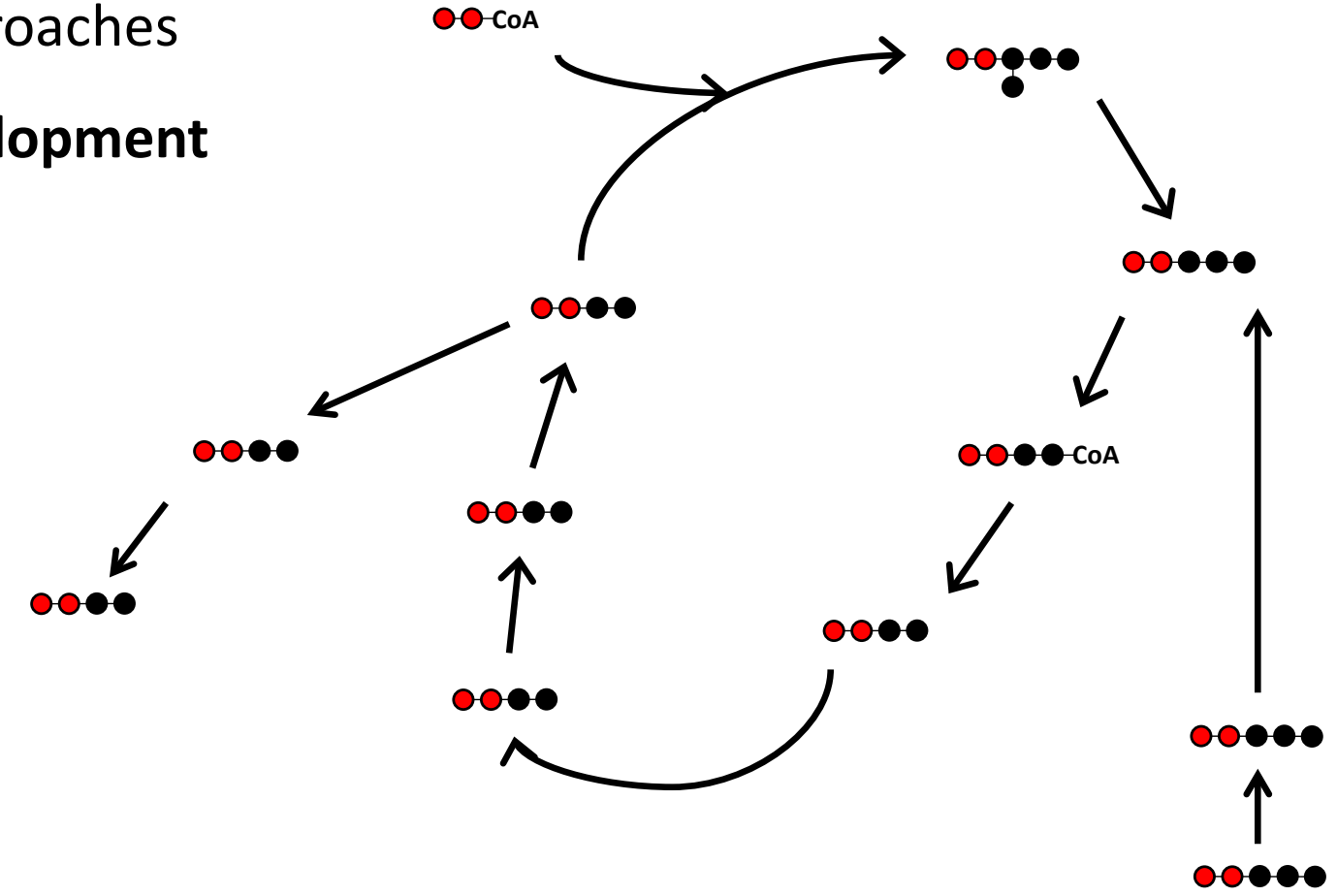
UNIVERSITY of WASHINGTON
SEATTLE

Overview

- **Metabolic flux analysis introduction**
- Advantages over non-flux based approaches
- **Stable isotopic labels and SRM development**
- Methods, LC-MS and cell culture
- **Central carbon metabolism flux data**
- Using skyline for data processing



Metabolic flux analysis

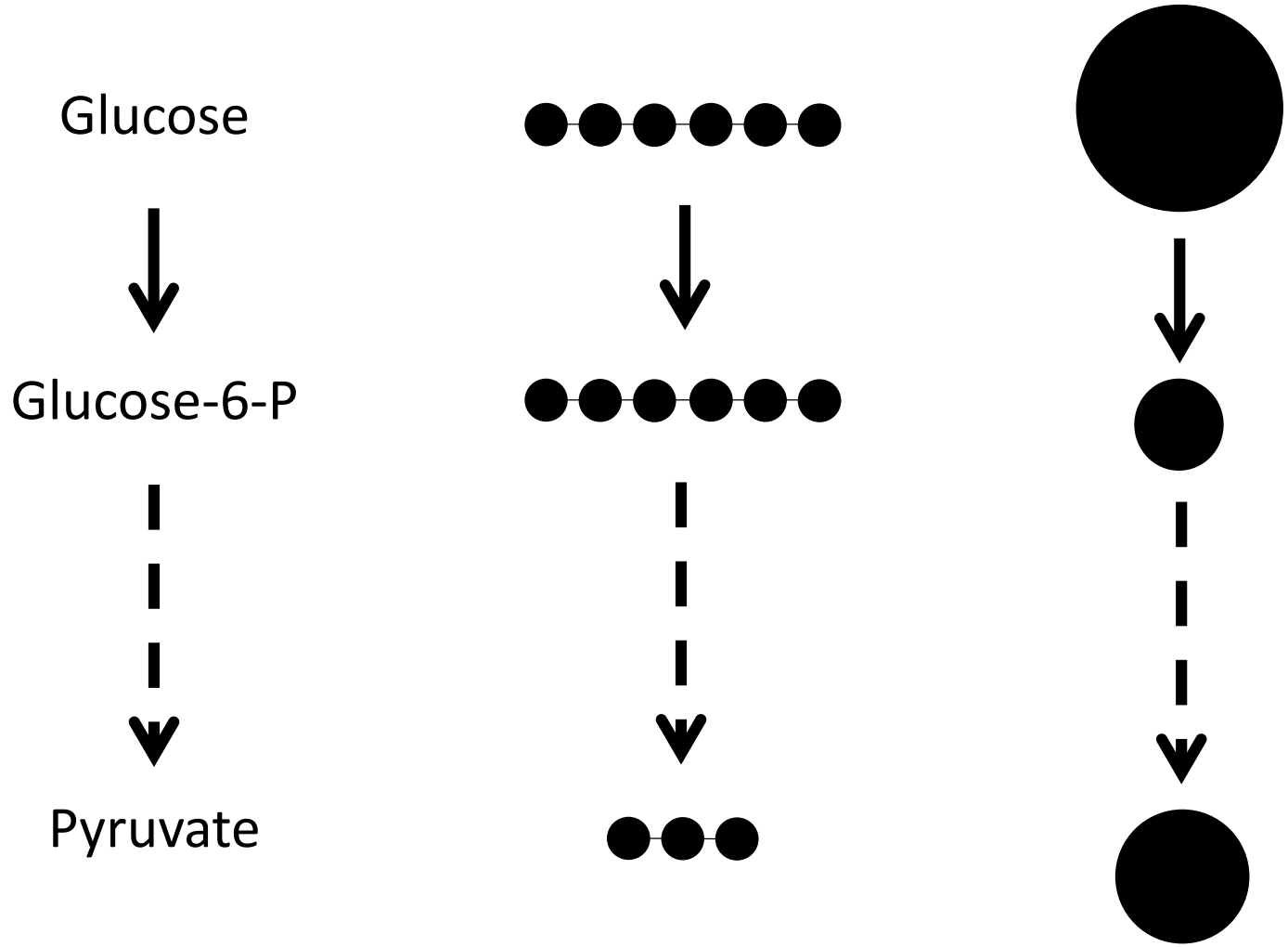
Over the past decade, there has been a comeback in the interest to study metabolism

- It's easier to analyze than in the past (LC-MS)
- Disruptions in metabolism are associated with disease
  (Warburg effect)

Lactate ⟵ Pyruvate ⟶ TCA cycle

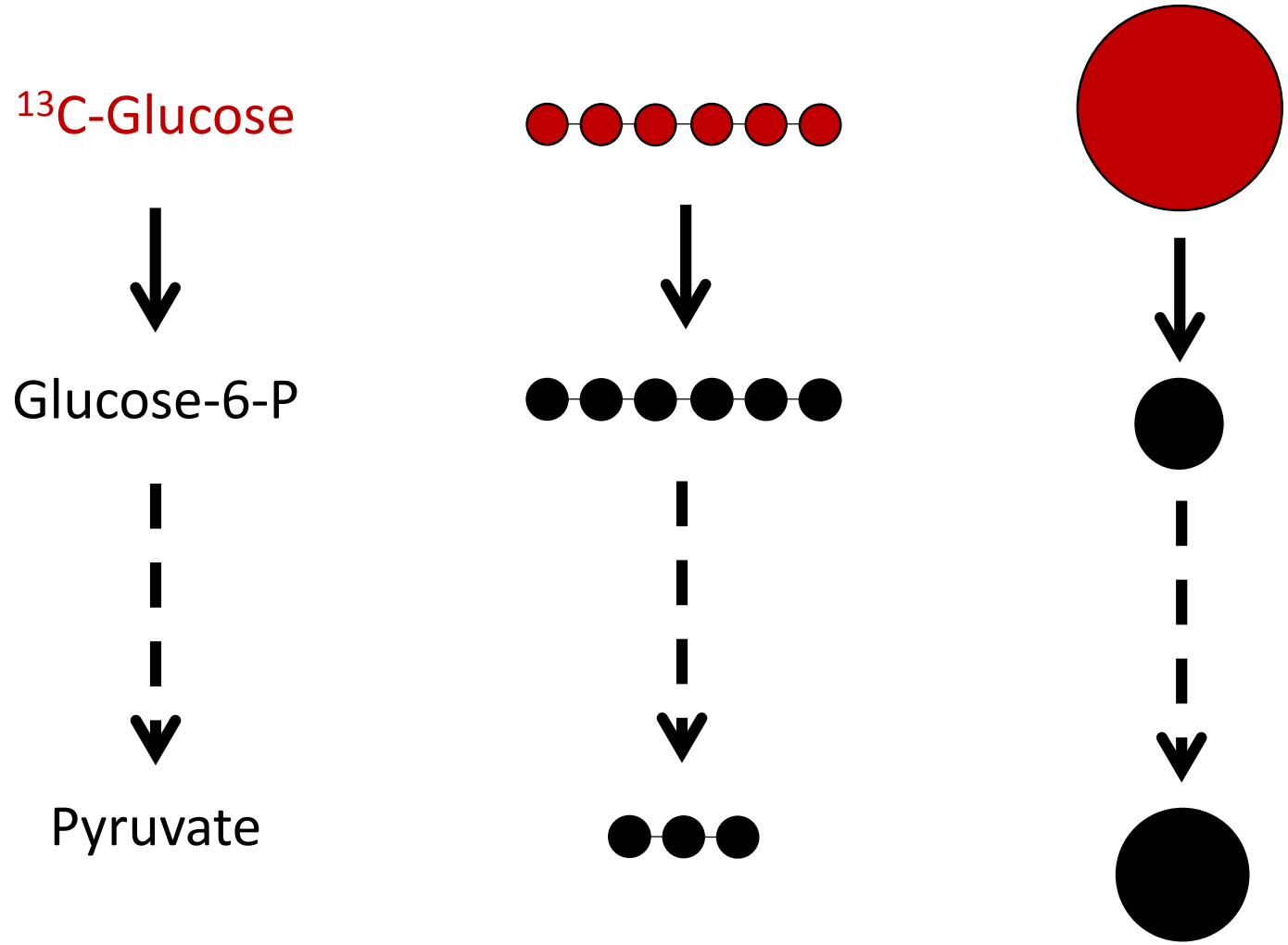
Metabolic flux analysis
Temporally tracking the isotopically labeled atoms of a precursor molecule through downstream metabolic intermediates

Metabolic flux analysis, glycolysis



“Metabolite” “Carbon skeleton” “pool size”

Metabolic flux analysis, glycolysis



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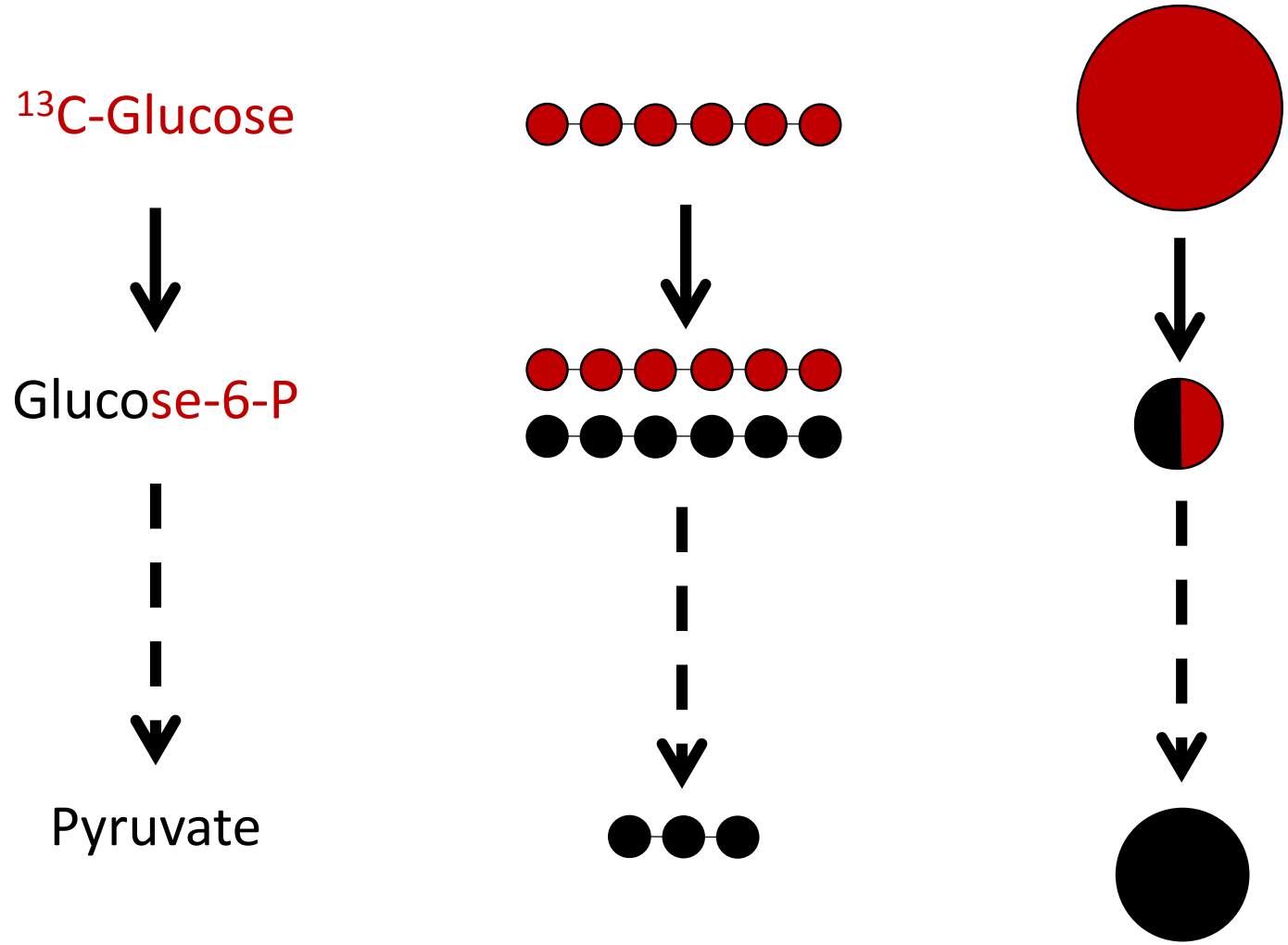

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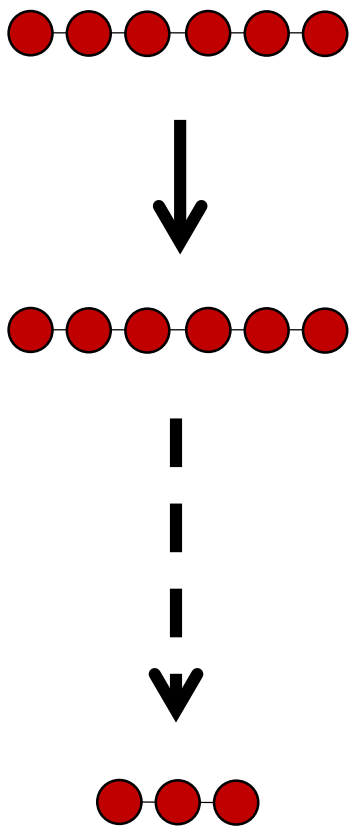
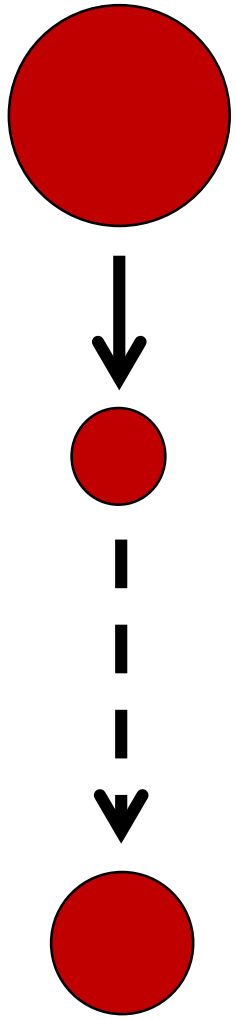
Metabolic flux analysis, glycolysis

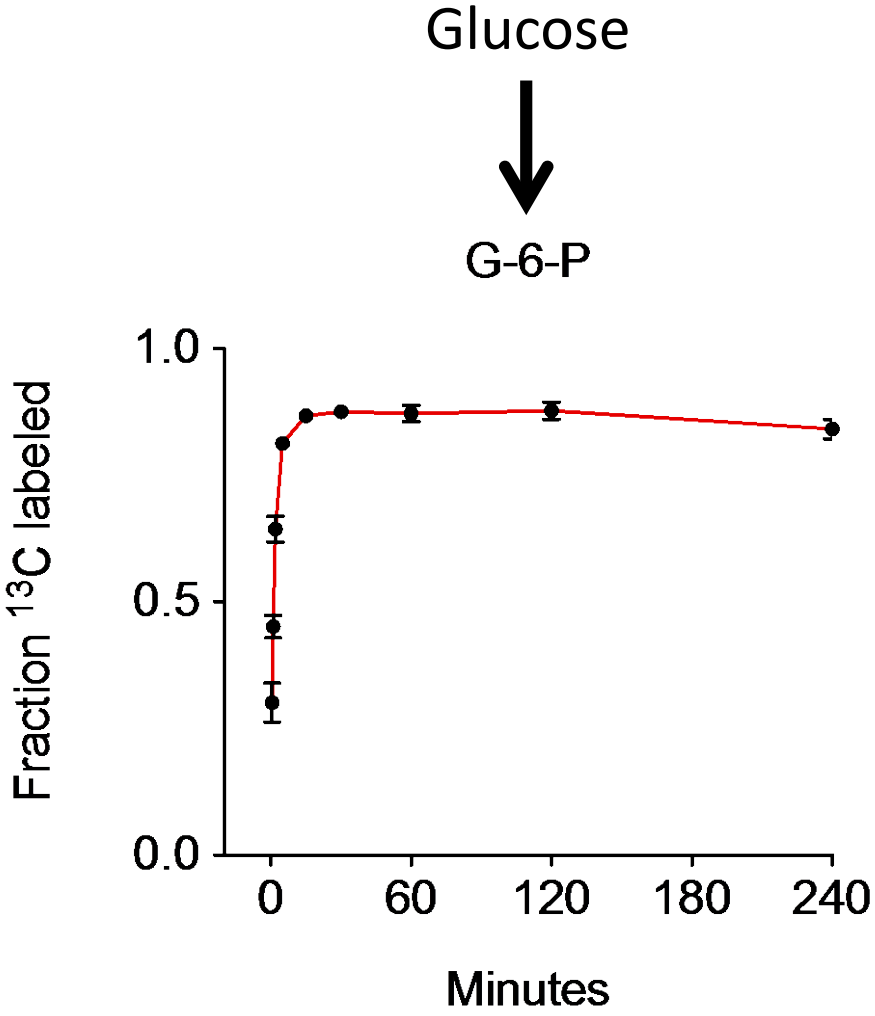
^{13}C-Glucose

Glucose-6-P

Pyruvate







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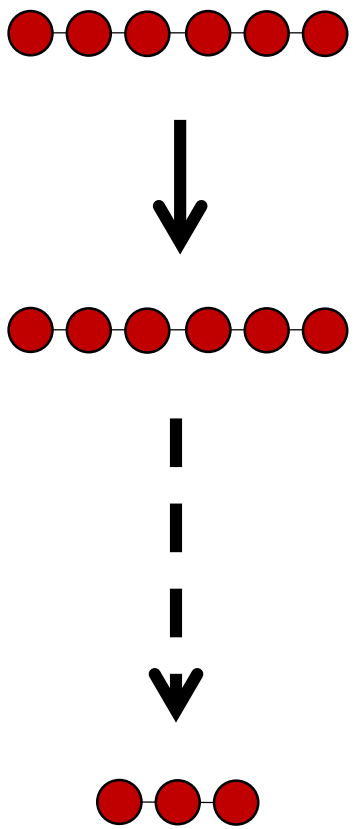
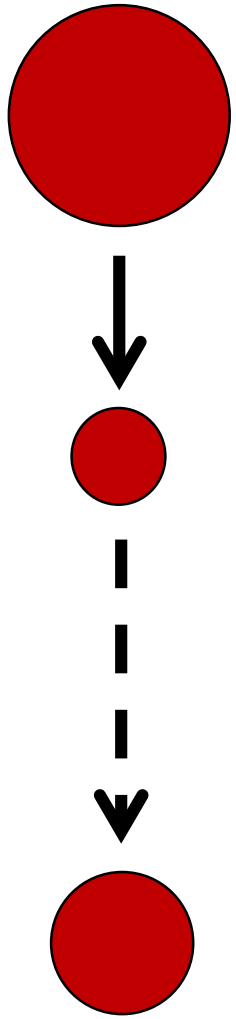
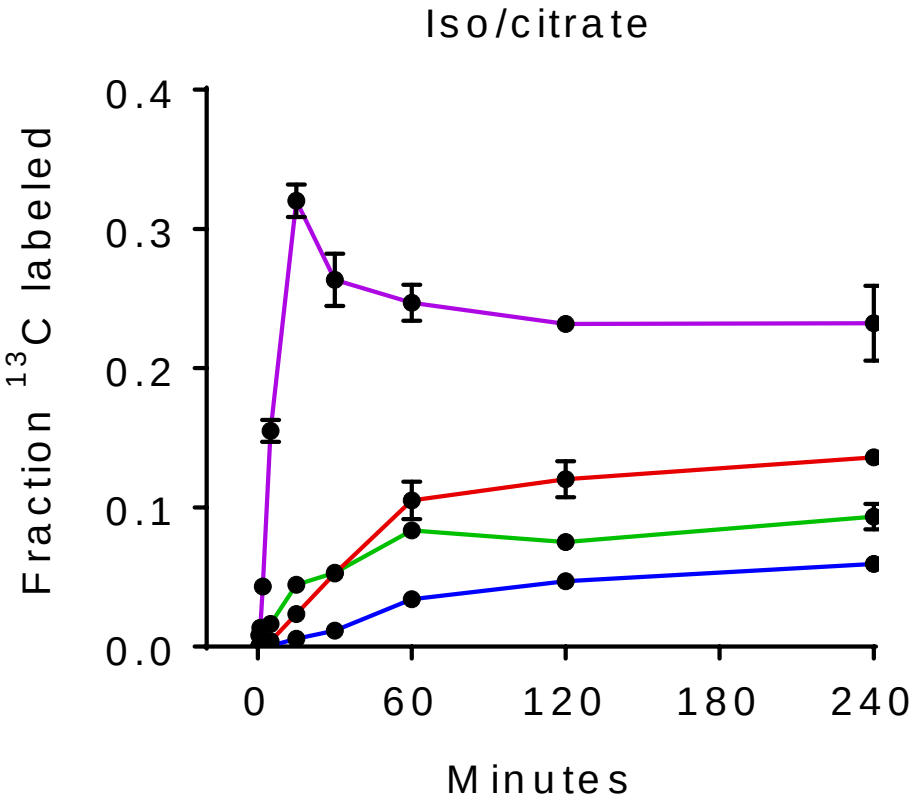






“Metabolite”

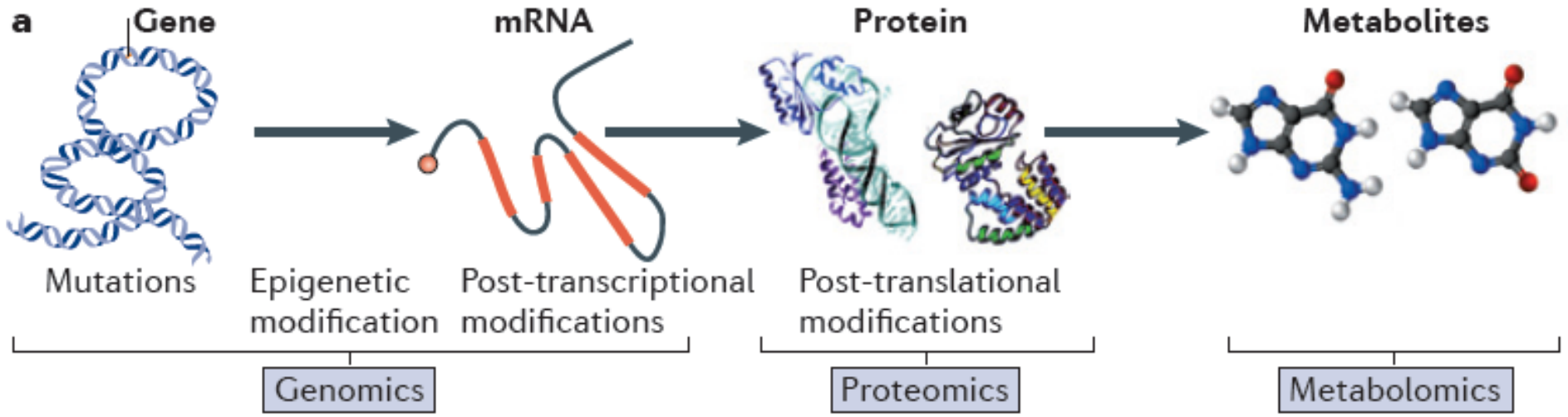
“Carbon skeleton”

“pool size”

Advantages of metabolic flux analysis

Metabolic flux analysis has the potential to answer questions raised by traditional metabolomics studies

Nat Mol Cell Biol (2012) Patti et al



*"..metabolite concentration is not the true functional bottom line of cellular operation.."

*"Fluxes quantify the integrated network response of gene-protein-metabolite interactions"

*Uwe Sauer, *Mol Sys Bio* 2006

Advantages of metabolic flux analysis

Metabolic flux analysis has the potential to answer questions raised by traditional metabolomics studies

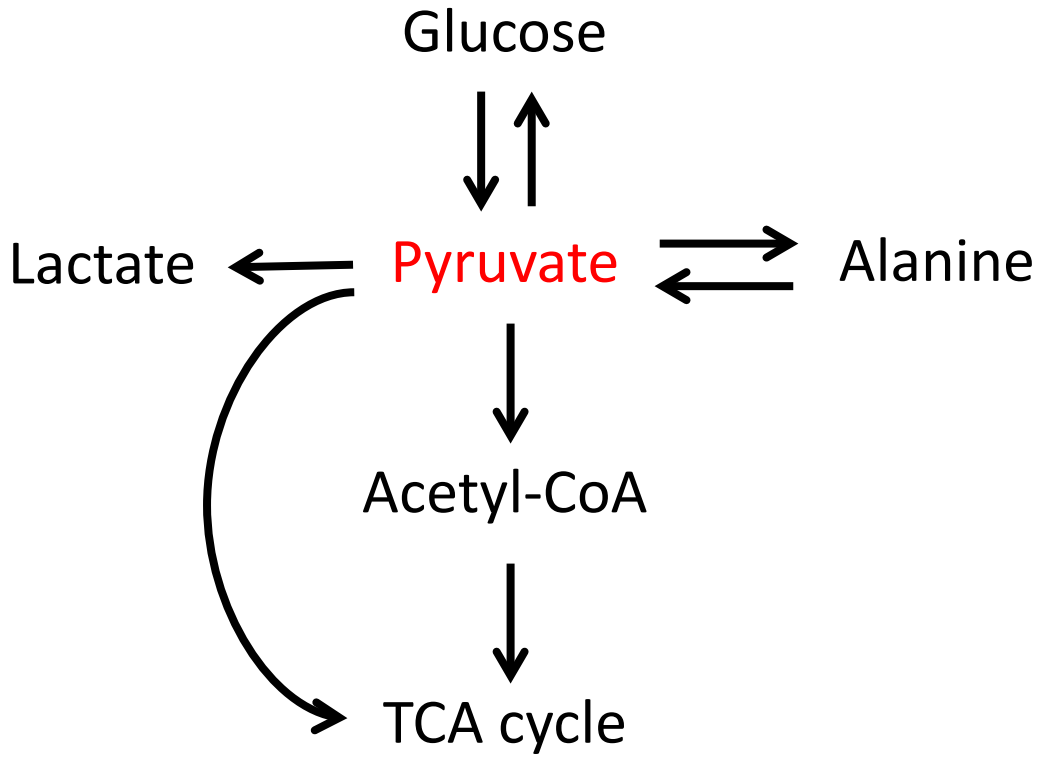
<u>Example</u>

An increase in the concentration of pyruvate is observed during the progression of disease

Is flux into the pyruvate pool increased?

Is flux out of pyruvate pool decreased?

Both?



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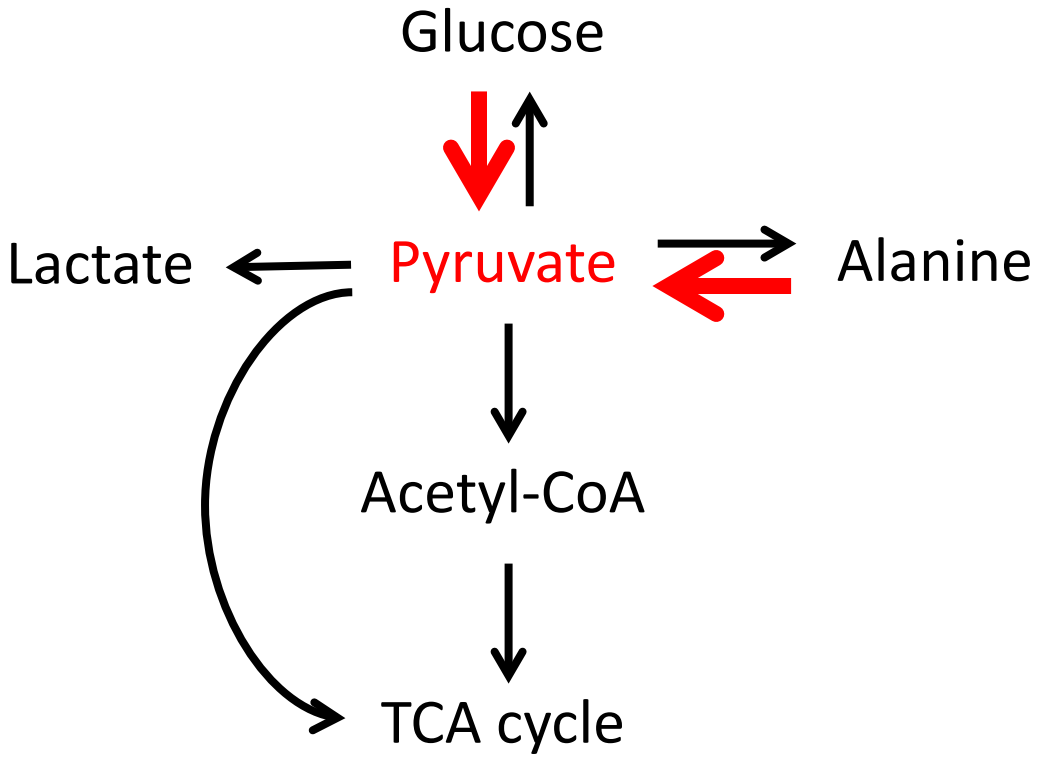

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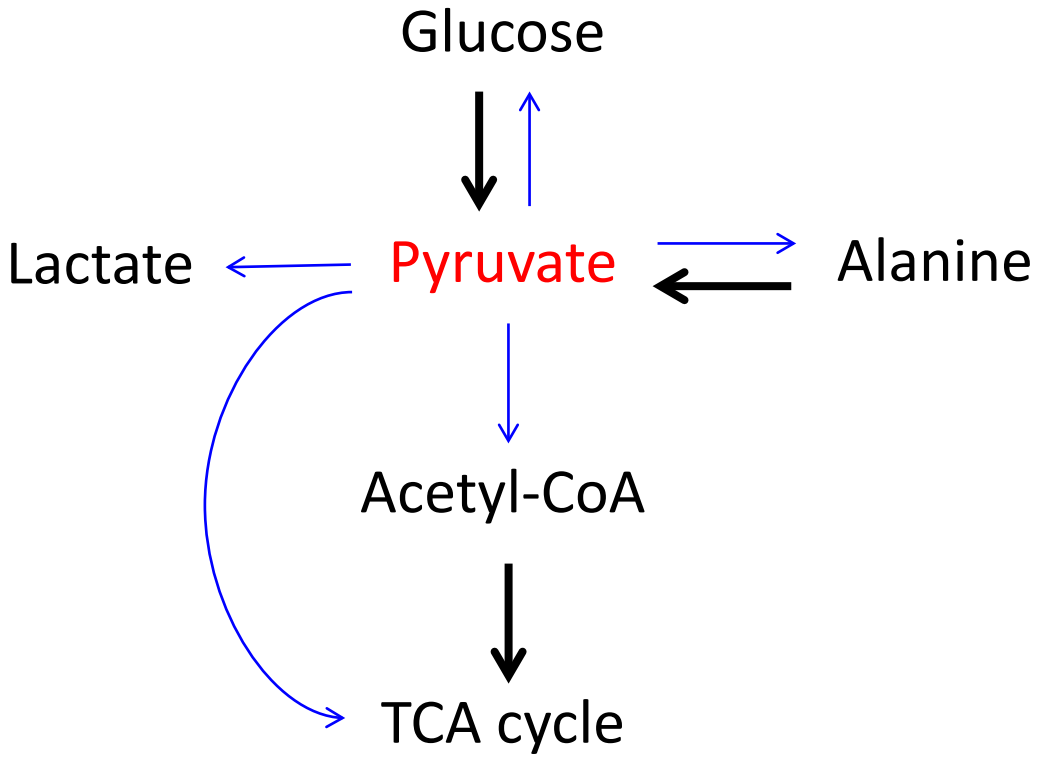

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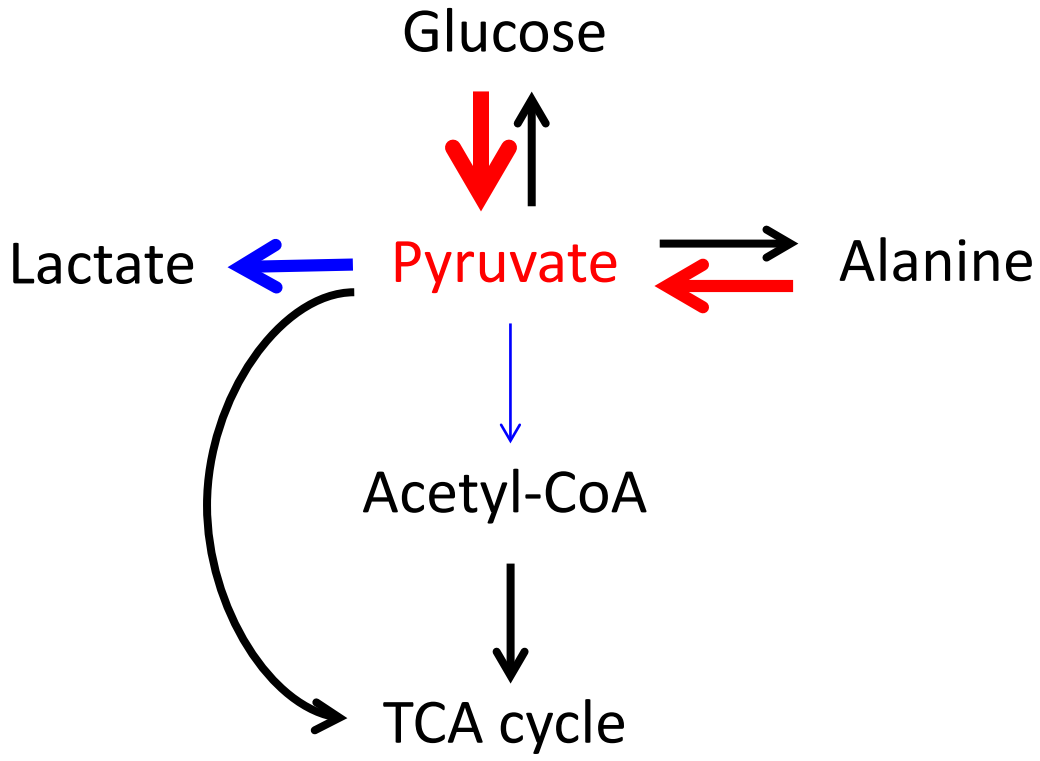

SRM development is guided by:

- Label type
- Molecular formula of parent ion
- Molecular formula of product ion
- Desired information
- Sensitivity

Metabolic flux MS method

27 metabolites
295 SRM transitions

How did we get there?

SRM development is guided by:

- **Label type**
- Molecular formula of parent ion
- Molecular formula of product ion
- Desired information
- Sensitivity

^{13}C-glucose	**Glycolytic activity**
1,2-^{13}C-glucose	**Pentose phosphate pathway**
3,4-^{13}C-glucose	**Pyruvate anaplerosis**
1-^{13}C-glutamine	**TCA cycle reductive carboxylation**
$^{15}NH_3$	**Nitrogen assimilation**
[1-^{2}H]-glucose	**NADP+ reduction**

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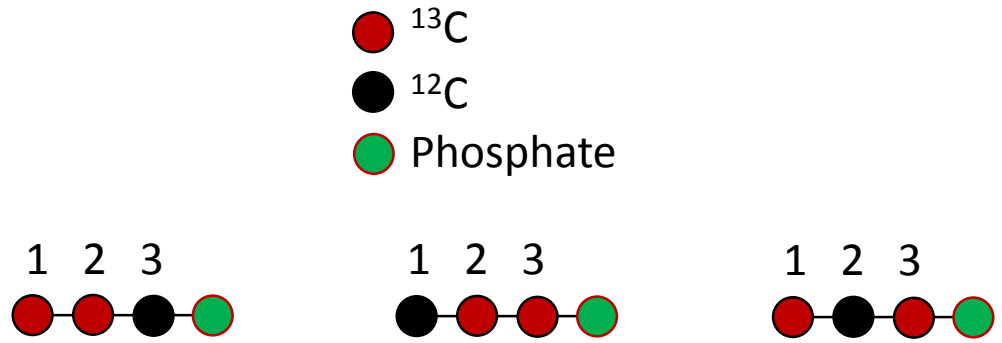
The number of carbons in both the parent and product ions determine the # of SRMs necessary for flux analysis



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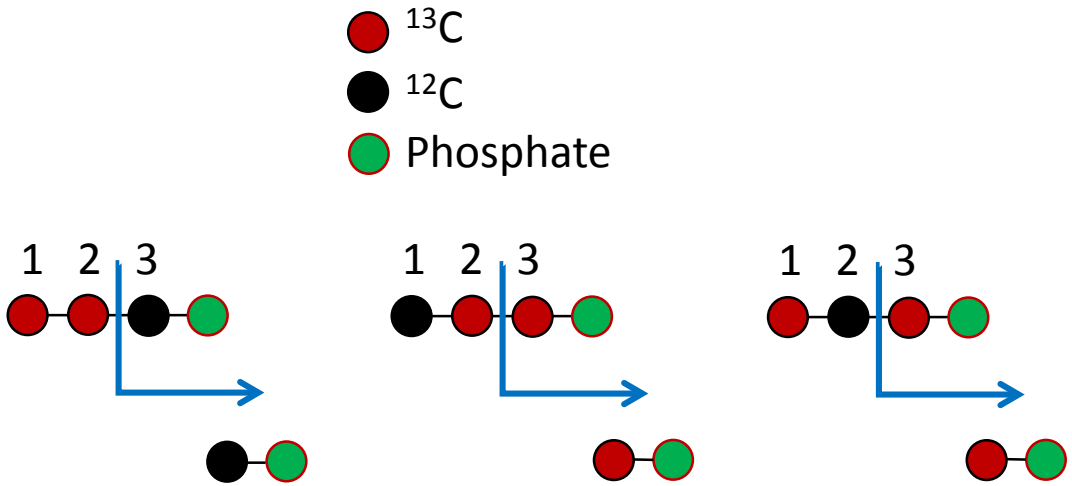

187 → 112
187 → 113

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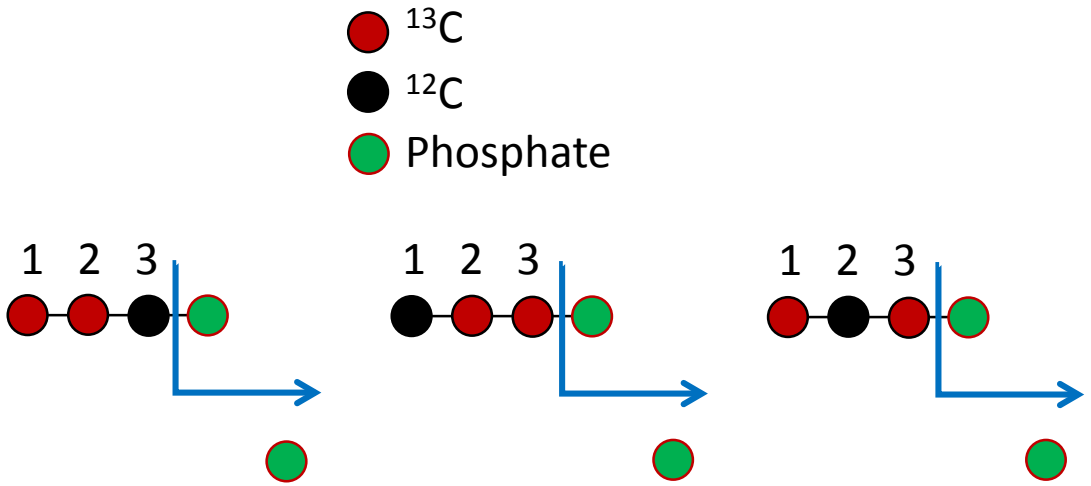
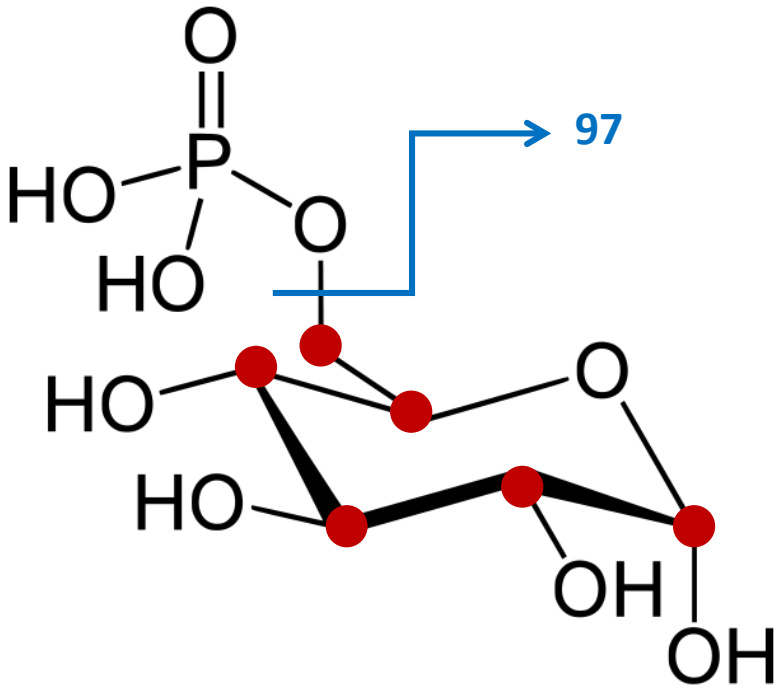


187 → 97

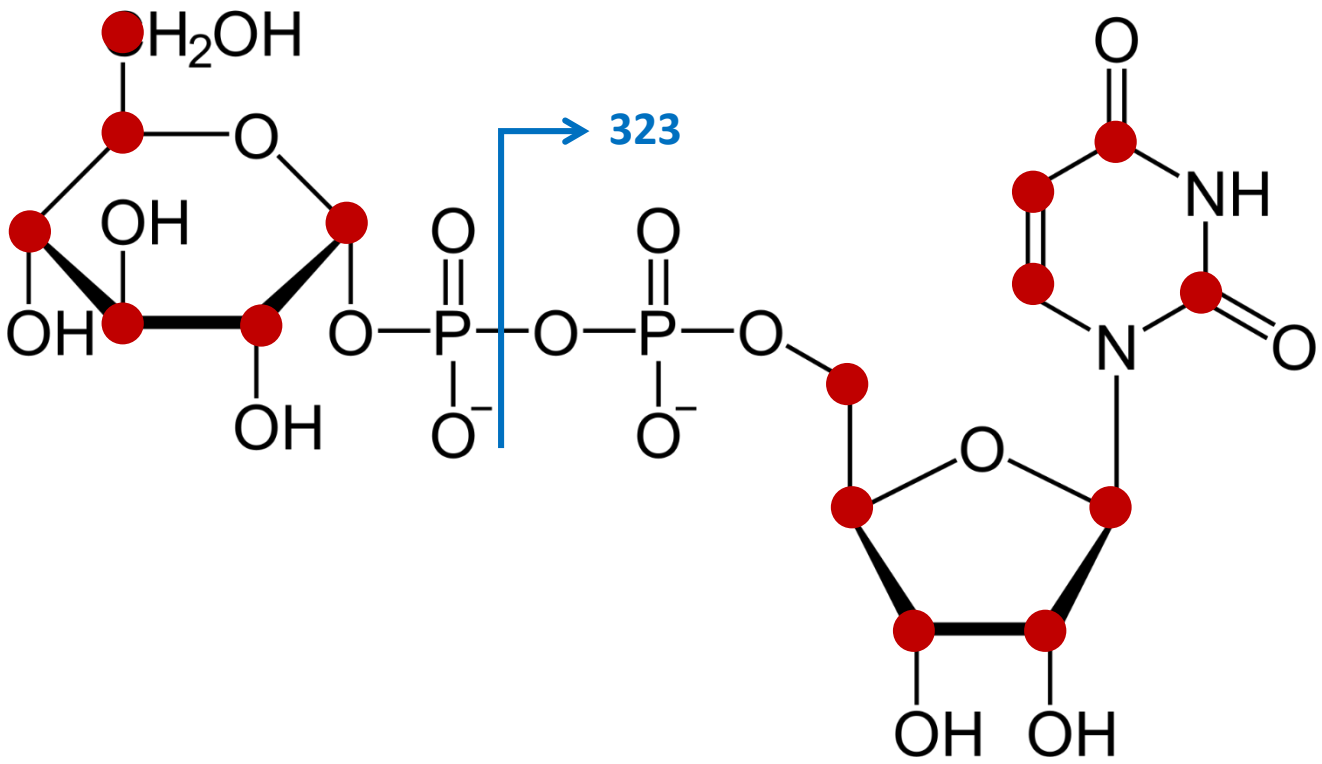
Flux analysis of glucose-6-phosphate



SRM table	pre m/z	prod m/z
hexose_P_0_0	259	97
hexose_P_1_0	260	97
hexose_P_2_0	261	97
hexose_P_3_0	262	97
hexose_P_4_0	263	97
hexose_P_5_0	264	97
hexose_P_6_0	265	97

Molecular weight = 260
Parent M-H = 259
Product M- = 97

Flux analysis of UDP-glucose



Molecular weight = 566

Parent M-H = 565

Product M- = 323

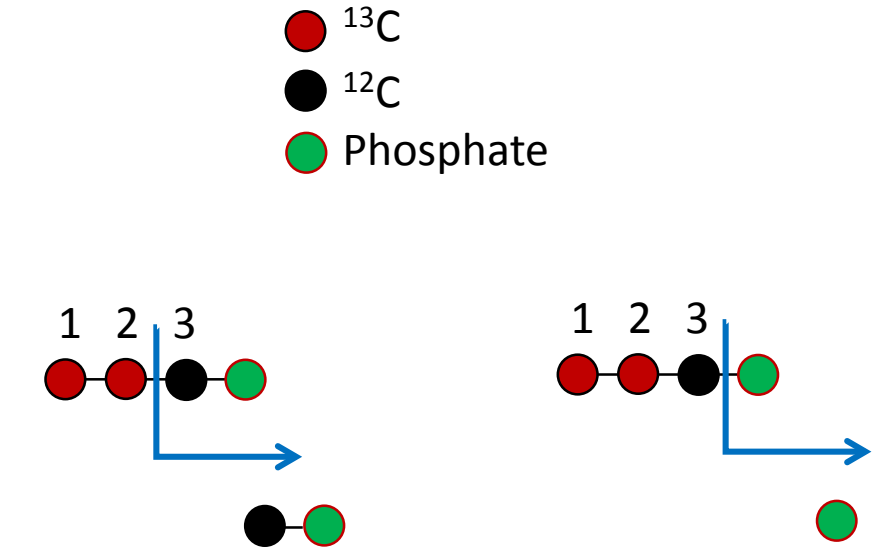
70 SRMs for UDP-glucose

SRM table	pre m/z	prod m/z
UDP_glucose_0_0	565	323
UDP_glucose_1_0	566	323
UDP_glucose_1_1	566	324
UDP_glucose_2_0	567	323
UDP_glucose_2_1	567	324
UDP_glucose_2_2	567	325
UDP_glucose_3_0	568	323
UDP_glucose_3_1	568	324
UDP_glucose_3_2	568	325
UDP_glucose_3_3	568	326
UDP_glucose_4_0	569	323
UDP_glucose_4_1	569	324
UDP_glucose_4_2	569	325
UDP_glucose_4_3	569	326
UDP_glucose_4_4	569	327
UDP_glucose_5_0	570	323
UDP_glucose_5_1	570	324
UDP_glucose_5_2	570	325
UDP_glucose_5_3	570	326
UDP_glucose_5_4	570	327
UDP_glucose_5_5	570	328
UDP_glucose_6_0	571	323
UDP_glucose_6_1	571	324
UDP_glucose_6_2	571	325
UDP_glucose_6_3	571	326
UDP_glucose_6_4	571	327
UDP_glucose_6_5	571	328
UDP_glucose_6_6	571	329
UDP_glucose_7_1	572	324
UDP_glucose_7_2	572	325
UDP_glucose_7_3	572	326
UDP_glucose_7_4	572	327
UDP_glucose_7_5	572	328
UDP_glucose_7_6	572	329
UDP_glucose_7_7	572	330
UDP_glucose_8_2	573	325
UDP_glucose_8_3	573	326
UDP_glucose_8_4	573	327

SRM development is guided by:

- Label type
- Molecular formula of parent ion
- Molecular formula of product ion
- **Desired information**
- Sensitivity

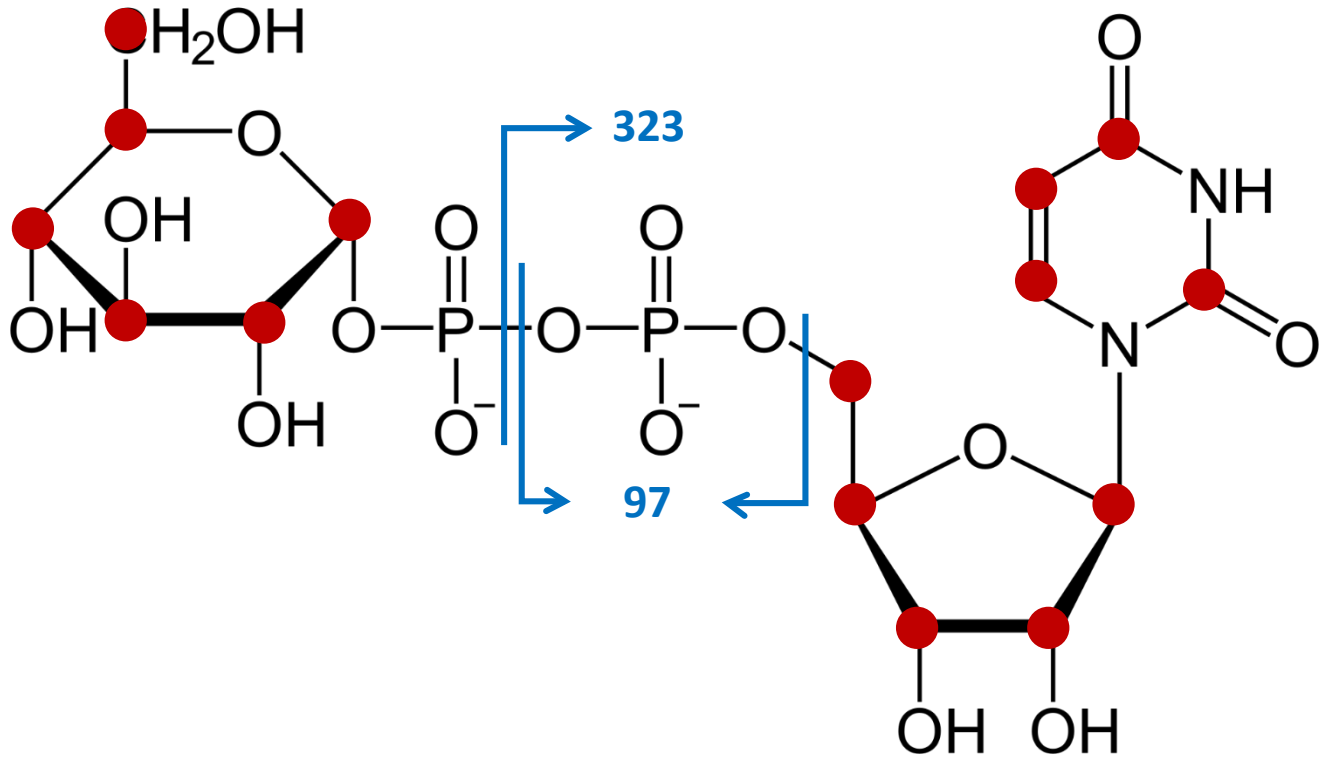
Positional labeling information can be acquired with selective fragmentation



SRM development is guided by:

- Label type
- Molecular formula of parent ion
- Molecular formula of product ion
- Desired information
- **Sensitivity**

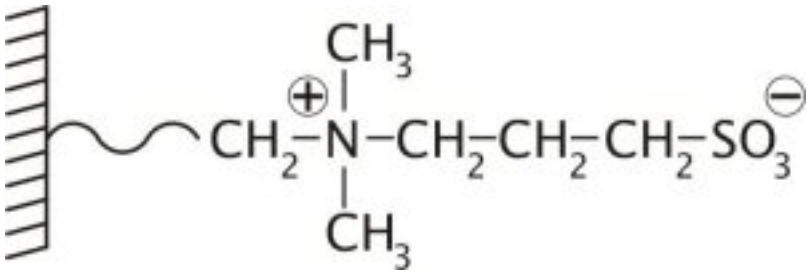
Strike a balance between # of SRMs required for a given fragment and required sensitivity



Method overview

LC-MS

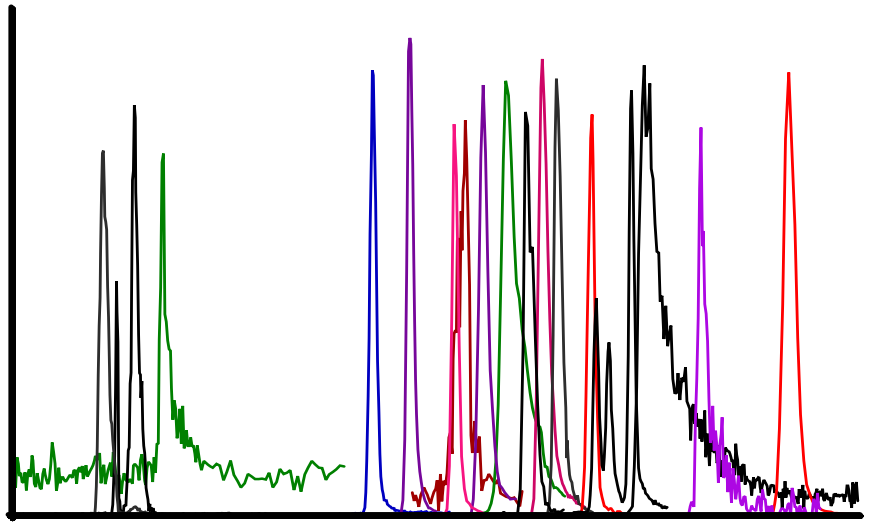
Waters Acquity UPLC
Zwitterionic HILIC column
Ammonium bicarbonate and ACN
270 µL per min
3 µL injection
50° C column
37 min run time



Waters TQ-S triple quad MS
27 metabolites
295 SRMs
Dwell times between 3 and 10 ms
Interscan delay of 3 ms
Polarity switching

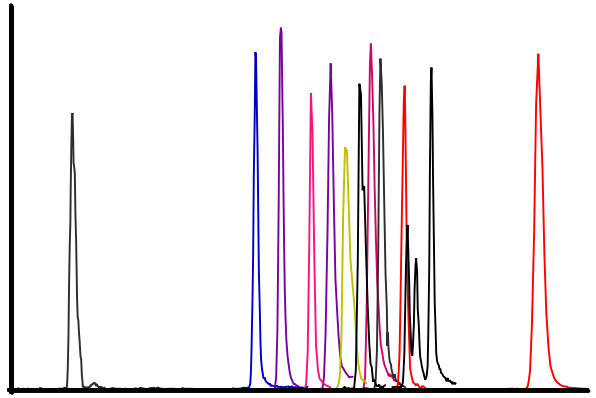
Method overview

LC-MS



Minutes

Good peak shape



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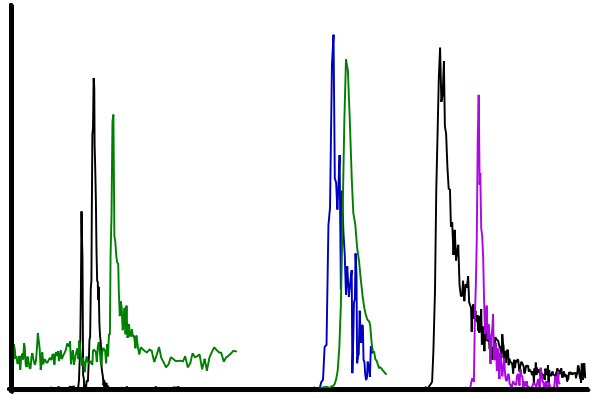
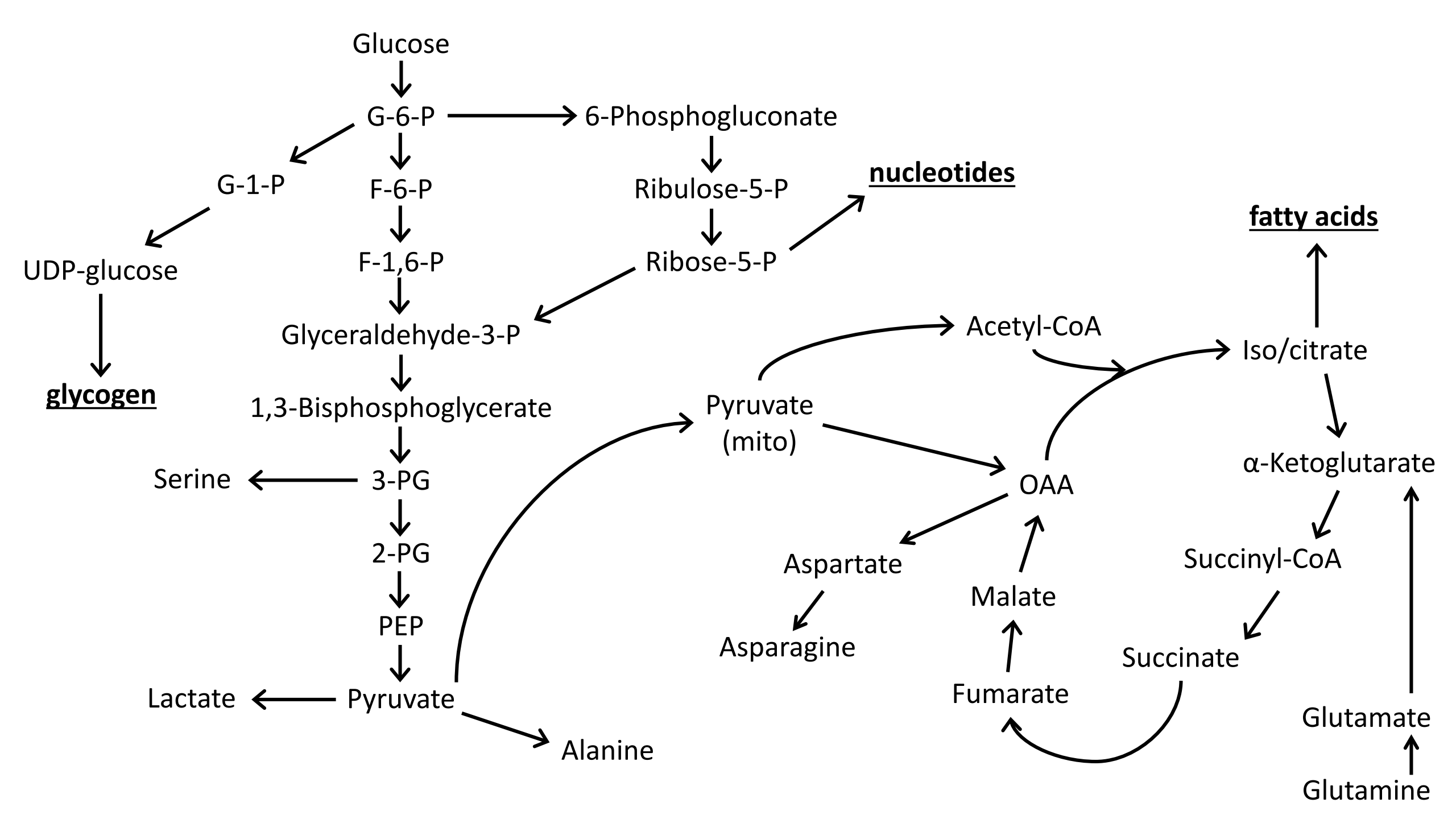


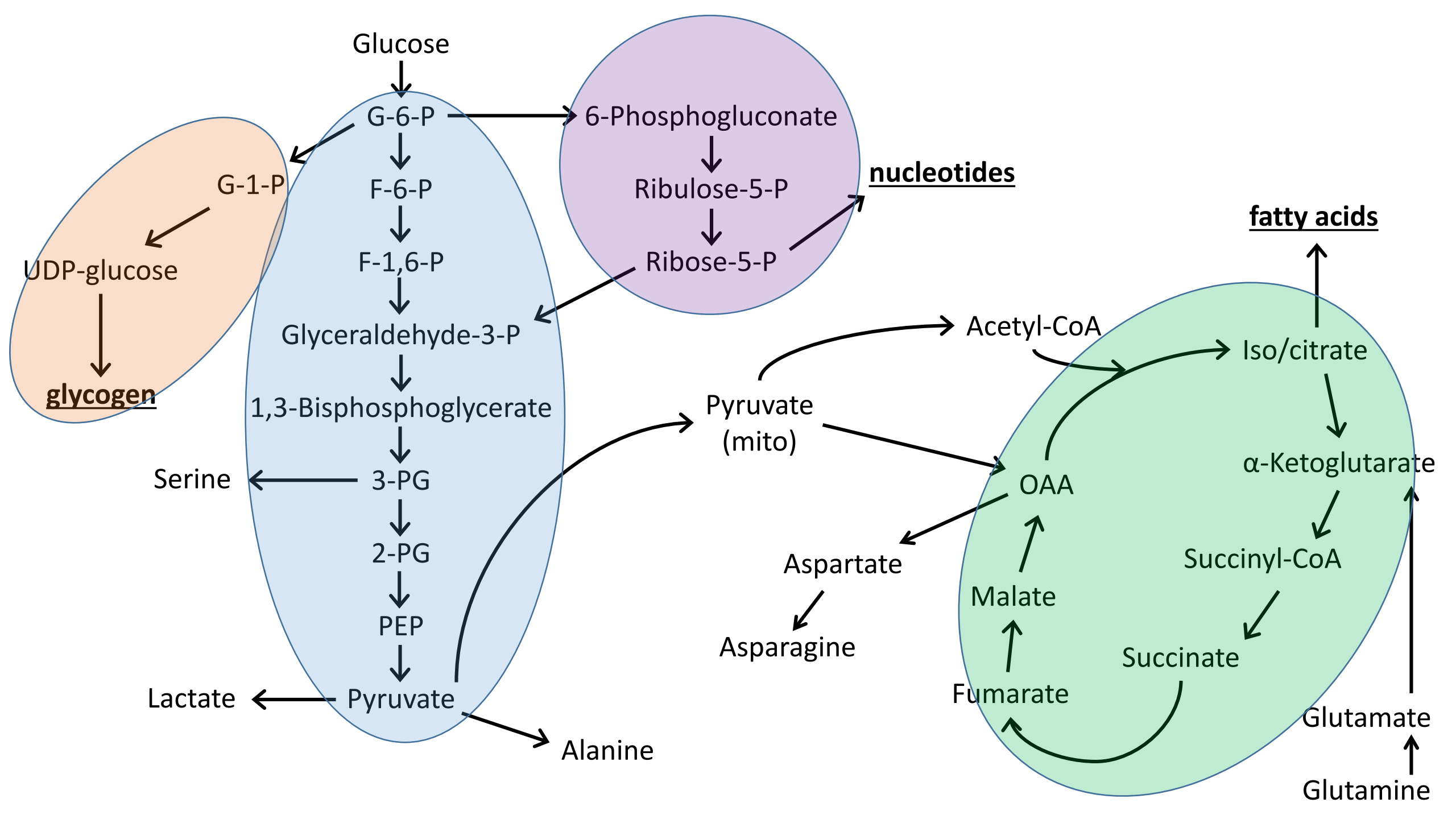
Method overview

Cell culture

Human huh7 liver cells

- Cells are grown up in normal serum containing medium
- 24 hr prior to switch to media with ^{13}C-glucose, cells are acclimated to serum free media
- 2 hr prior to switch to media with ^{13}C-glucose, media is refreshed to remove waste products
- After replacing media with ^{13}C-glucose media, cells are extracted as early as 30 seconds


Glucose
G-6-P
6-Phosphogluconate
G-1-P
F-6-P
Ribulose-5-P
nucleotides
fatty acids
UDP-glucose
F-1,6-P
Ribose-5-P
glycogen
Glyceraldehyde-3-P
Acetyl-CoA
Iso/citrate
1,3-Bisphosphoglycerate
Pyruvate
(mito)
Serine
3-PG
OAA
α-Ketoglutarate
2-PG
Aspartate
Succinyl-CoA
PEP
Malate
Asparagine
Succinate
Lactate
Pyruvate
Fumarate
Glutamate
Alanine
Glutamine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1,3-Bisphosphoglycerate
Serine
3-PG
2-PG
PEP
Lactate
Pyruvate
Alanine
Pyruvate (mito)
Acetyl-CoA
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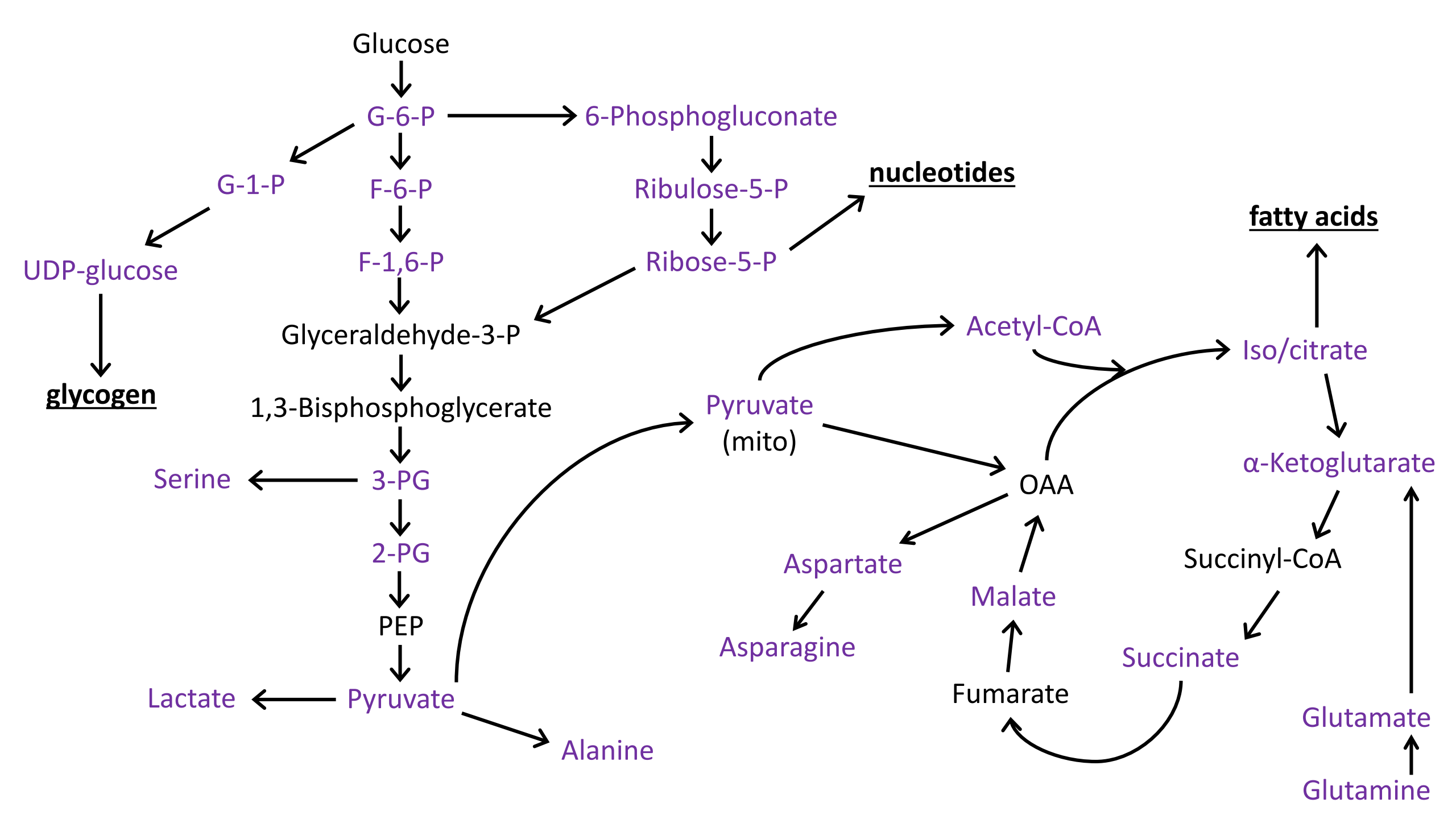
Iso/citrate
α-Ketoglutarate
OAA
Aspartate
Asparagine
Succinyl-CoA
Malate
Succinate
Fumarate
Glutamate
Glutamine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Malate
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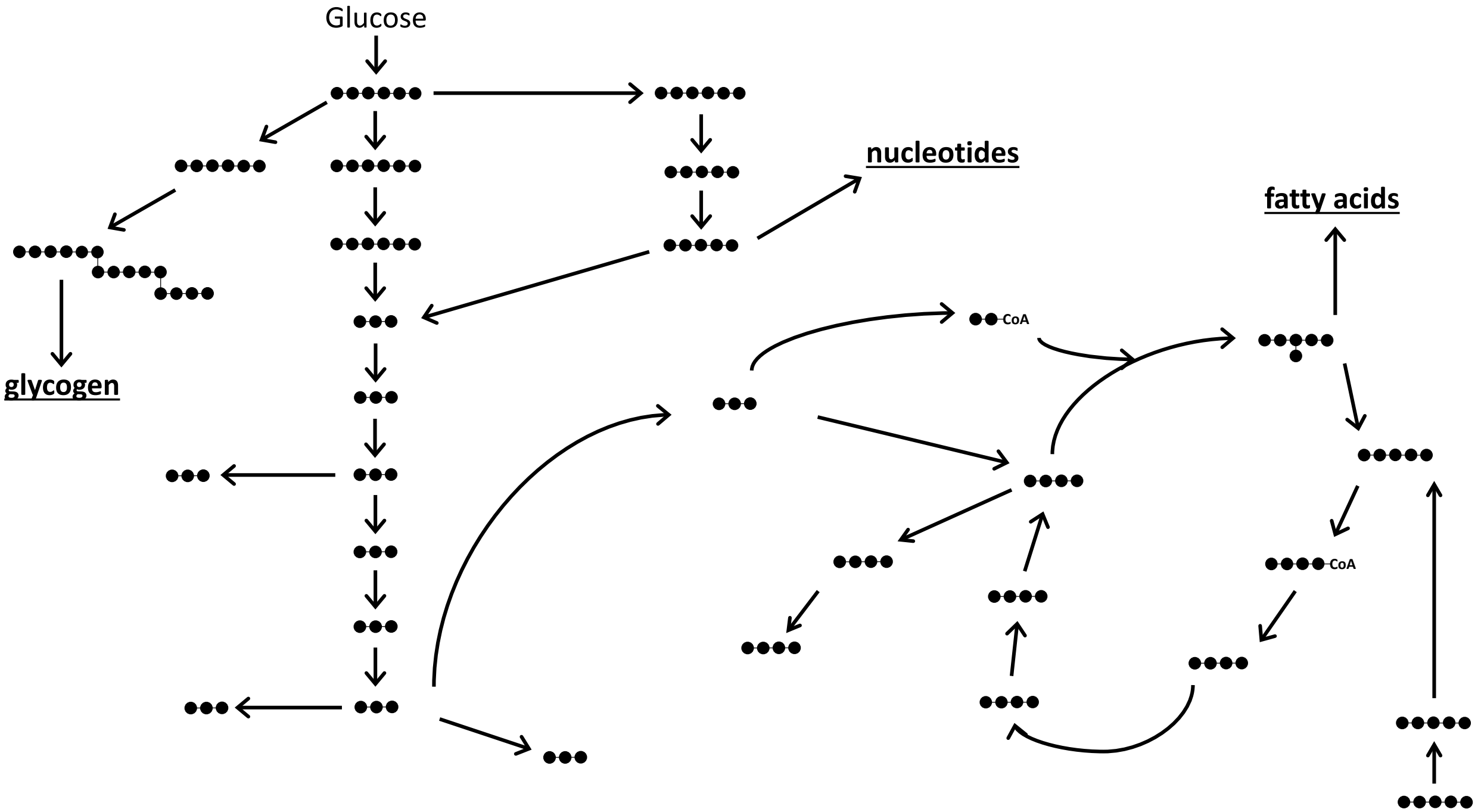
Succinate
Lactate
Pyruvate
Fumarate
Glutamate
Alanine
Glutamine


Glucose
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glycogen
CoA
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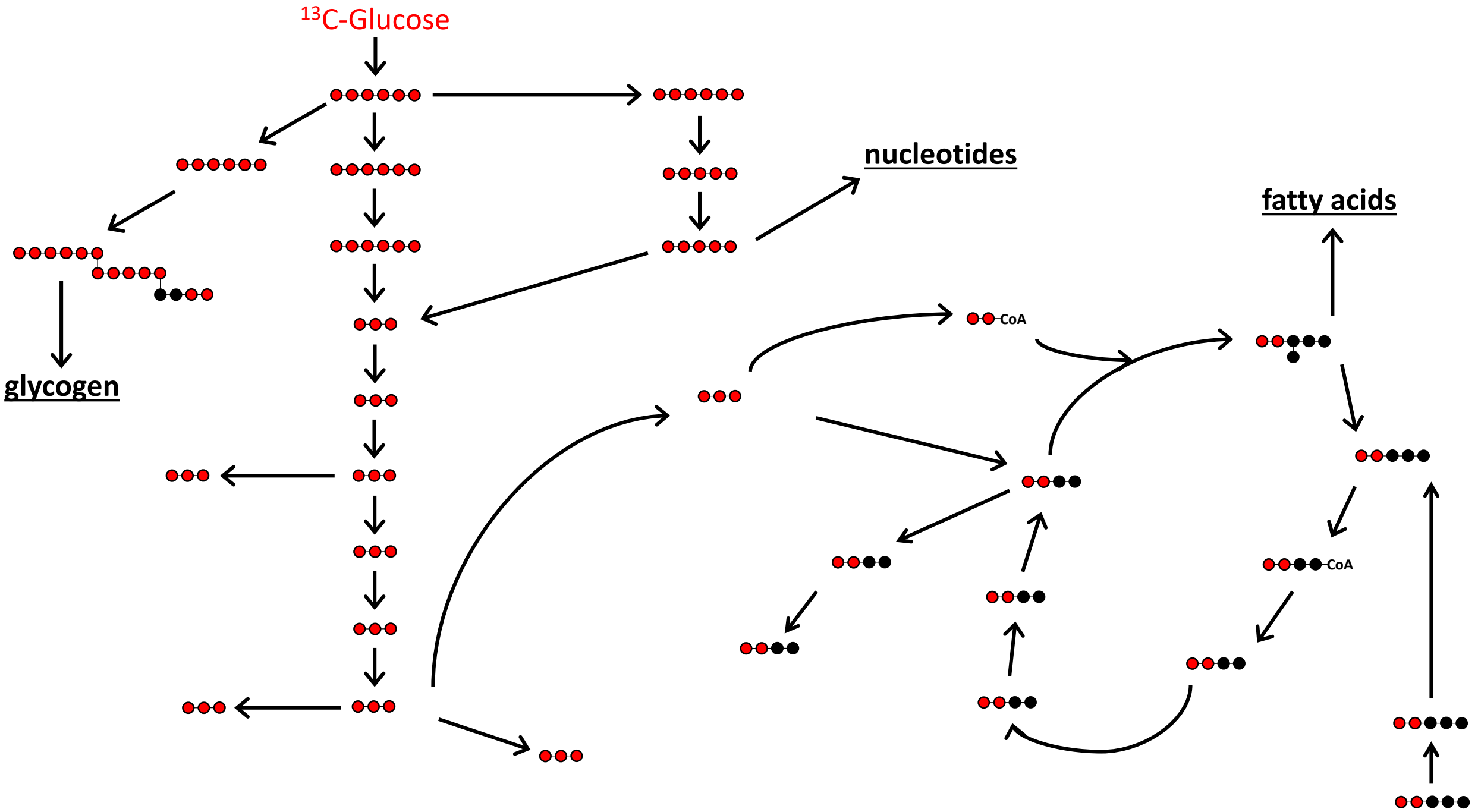
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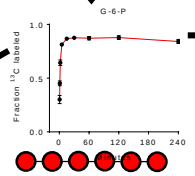
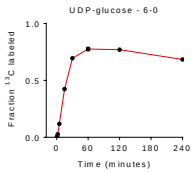
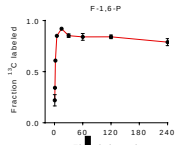
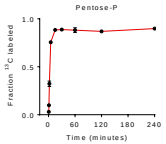




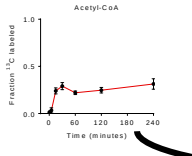


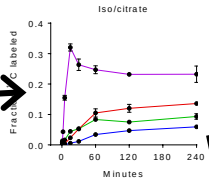
glycogen

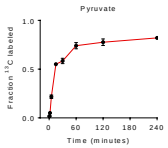
nucleotides

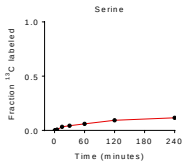


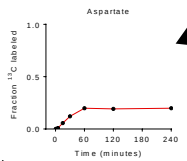
fatty acids



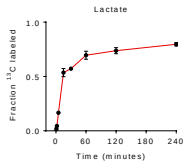


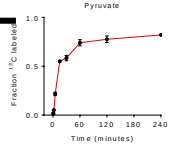


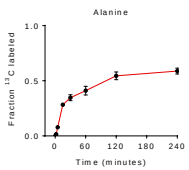


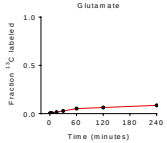


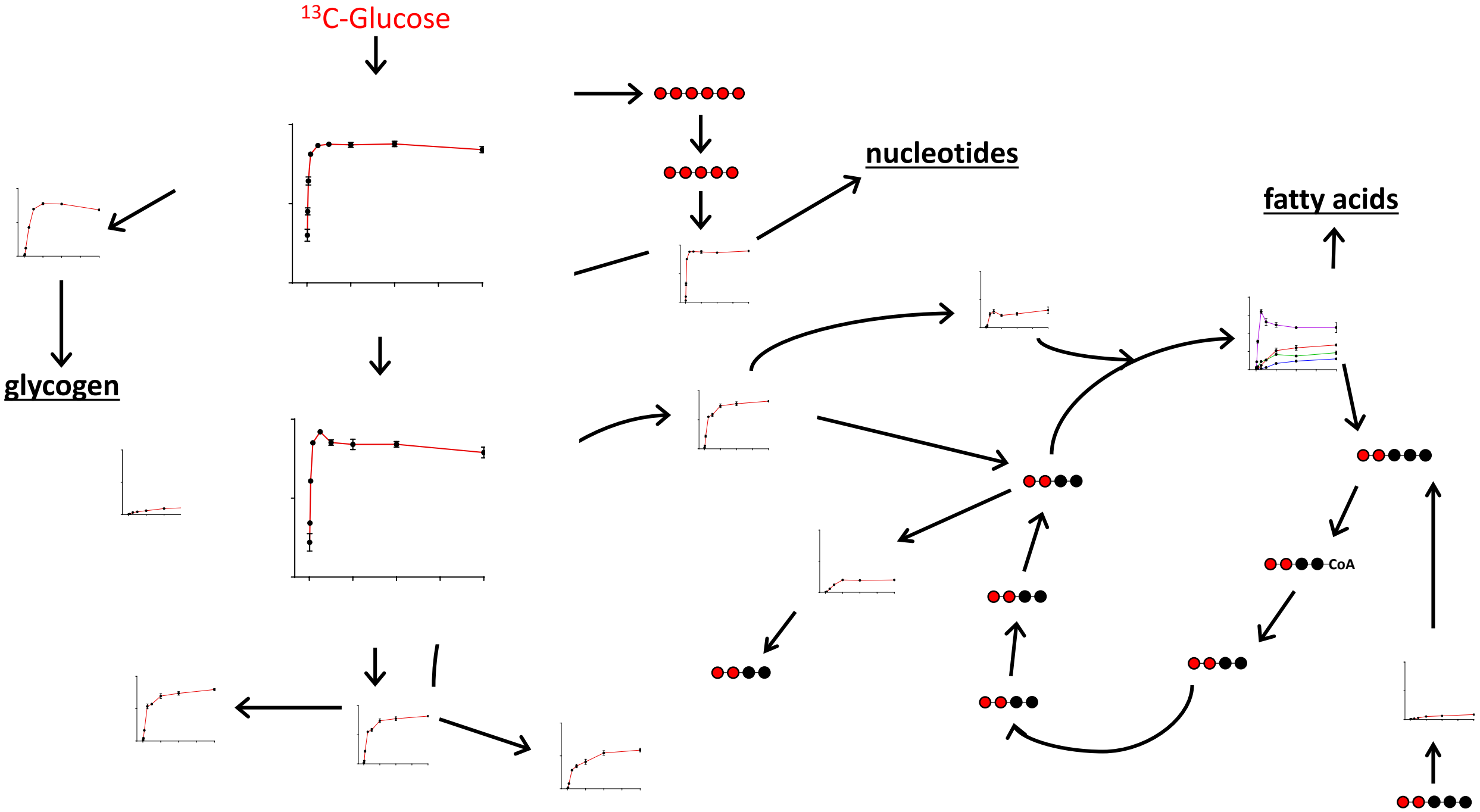
CoA

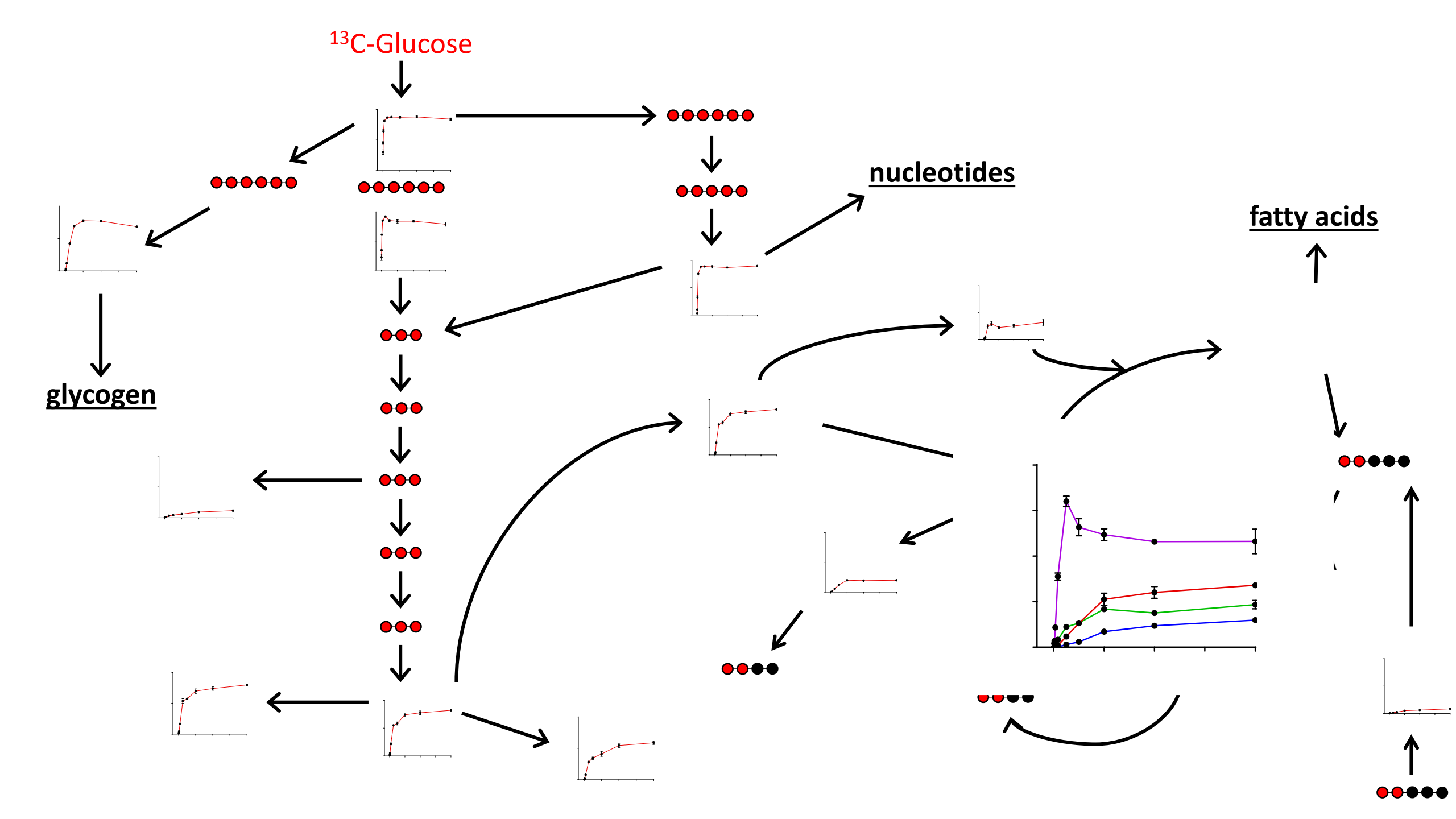









^{13}C-Glucose
glycogen
nucleotides
fatty acids
CoA


^{13}C-Glucose
glycogen
nucleotides
fatty acids

How to analyze all this data?

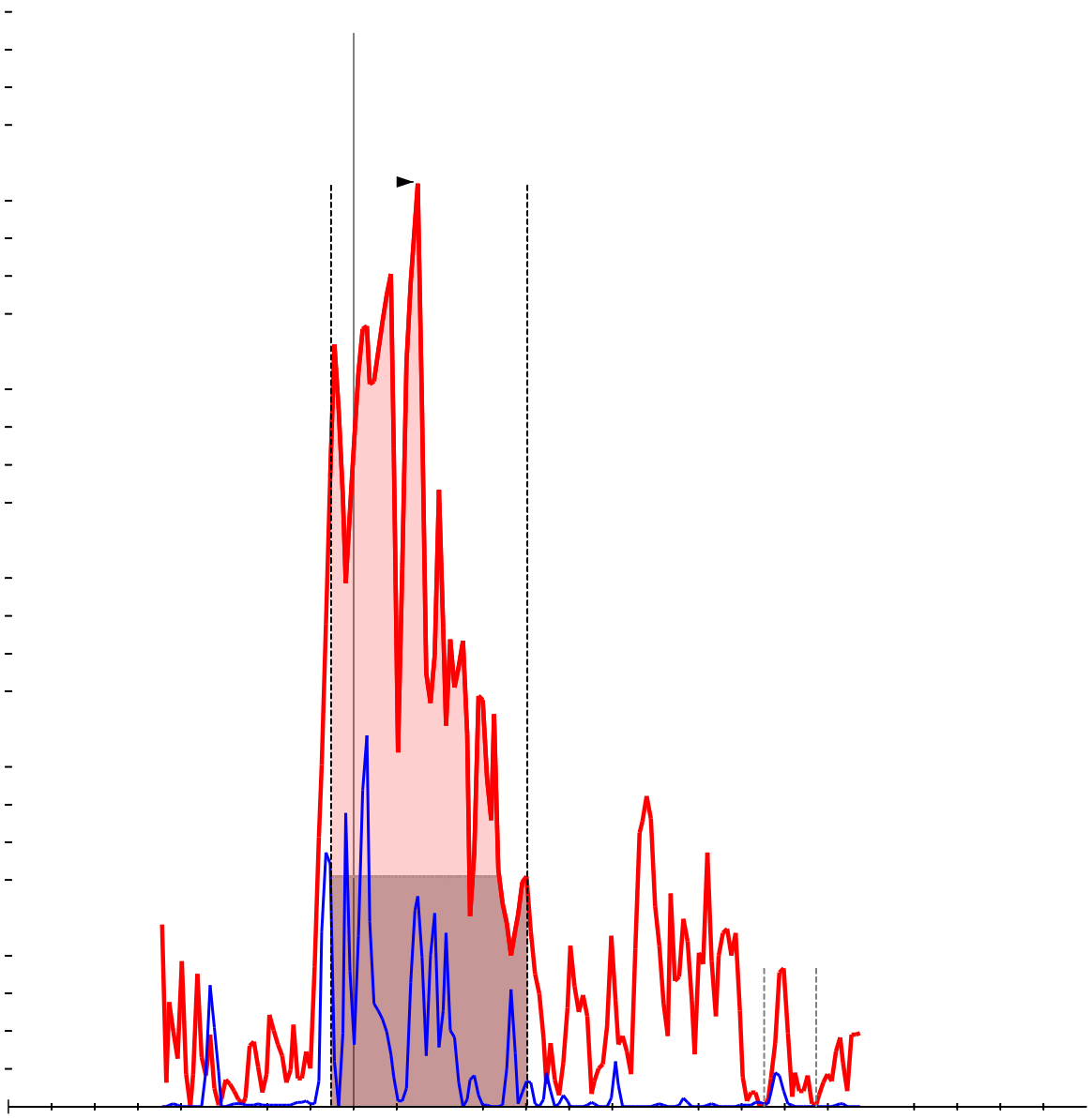
We have a lot of peaks to integrate

100 samples x 295 SRMs ~ 30,000 independent peaks

Benefits of Skyline for processing quantitative, small molecule data

Several aspects of Skyline help to streamline analysis of small molecule data:

- **Easy and quick to alter peak integration**
- Integration of multiple products from single parent is linked
- Data output close to ideal format
- Good peak picking
- User friendly visualization



Fructose-1,6-bisphosphate in liver cell extract

Poor peak shapes do not integrate well by default

But when integrated completely, these peaks often result in reproducible flux data

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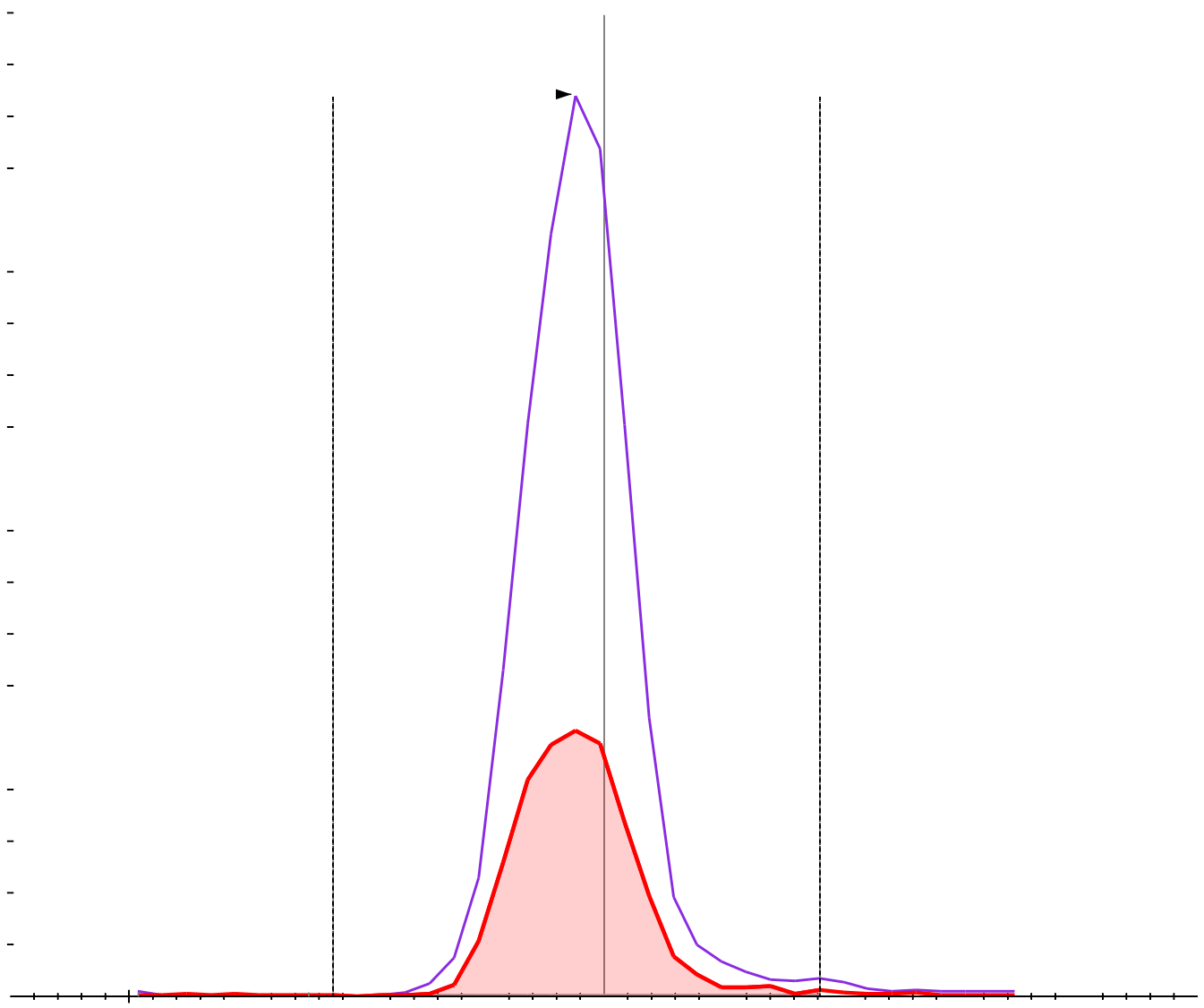

SRM #1 175 → 115
SRM #2 175 → 88

Integration of SRM #1 is linked to SRM #2

Time required to integrate these two transitions is cut in half

UDP-glucose
9 ^{13}Cs in parent requires 7 SRM transitions to monitor, all with same parent ion

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	metabolite x	metabolite y
sample a	data	data
sample b	data	data
sample c	data	data

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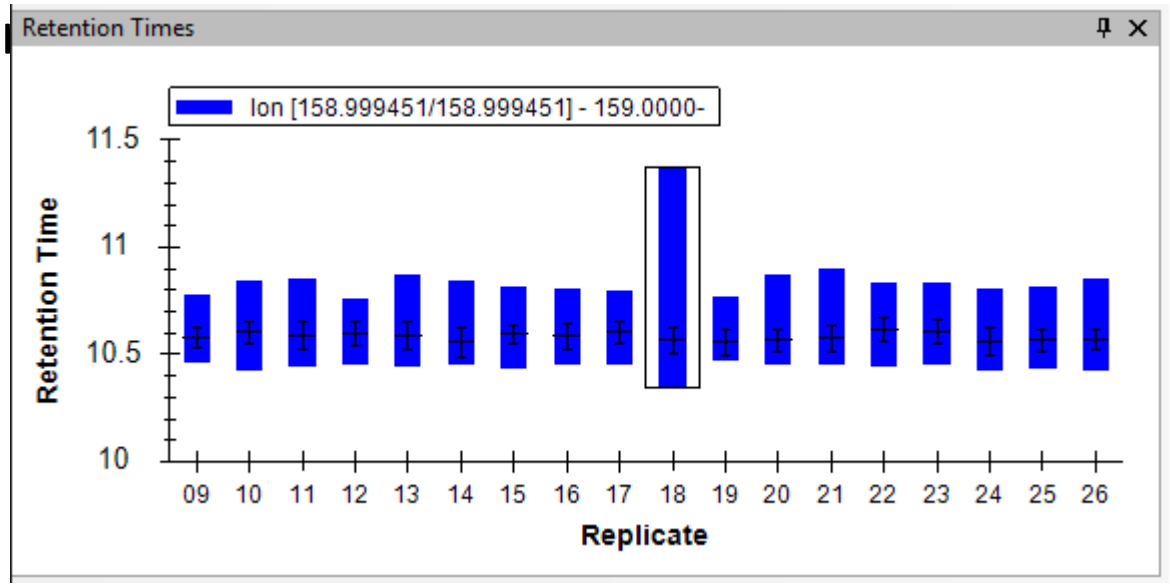
RT min and max values



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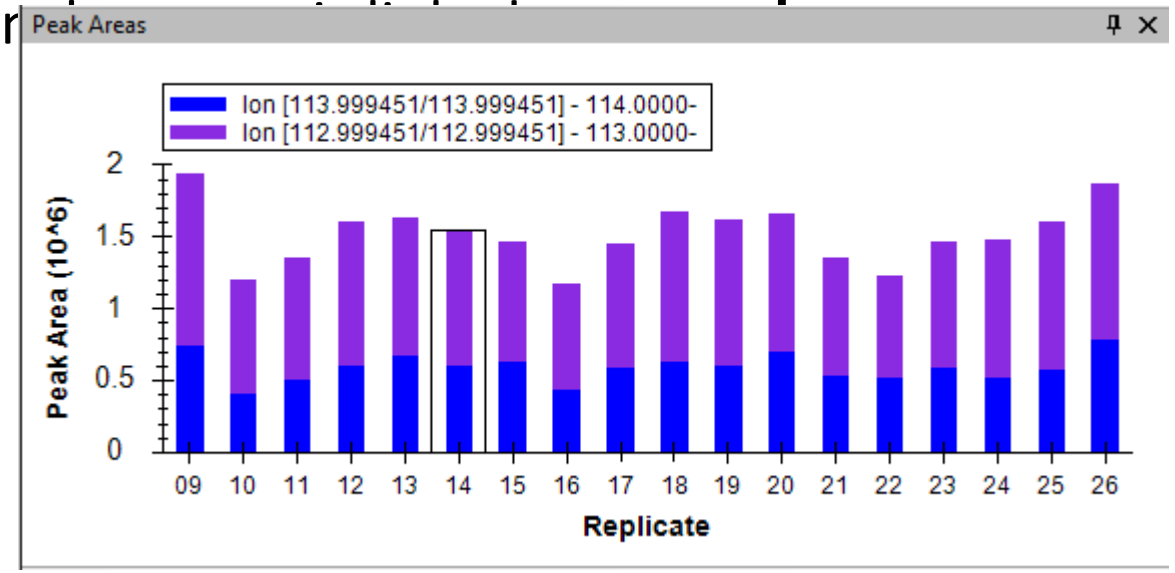
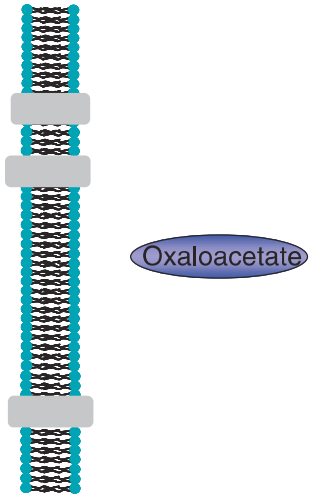
- **User friendly visualization**

Quick view of peak areas across samples

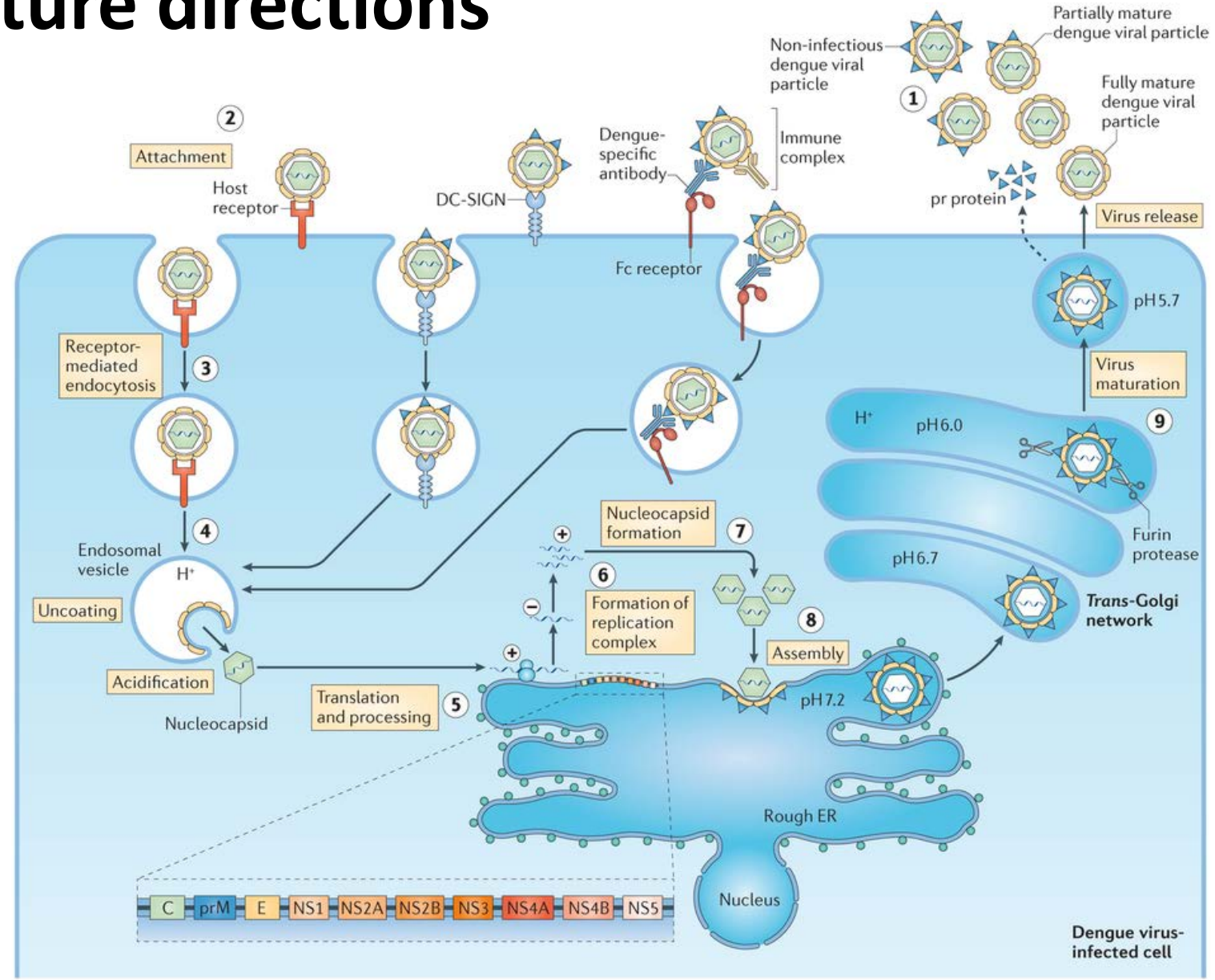


Future directions

Does successful dengue viral infection rely on transformations of host cell metabolism?



Munger et al, *Nat Biotech* 2008



Screaton et al, *Nat Rev Immunology* 2015

Nature Reviews | Immunology

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The Skyline team

Others
Doc Brown

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