Summer 6-7-2012

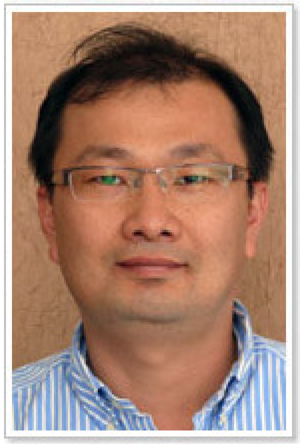
13C Metabolic Flux Analysis of CHO Cells with Parallel Labeling Experiments

Maciek Antoniewicz
University of Delaware

^{13}C Metabolic Flux Analysis of CHO Cells with Parallel Labeling Experiments

Maciek Antoniewicz, Woo Suk Ahn

University of Delaware

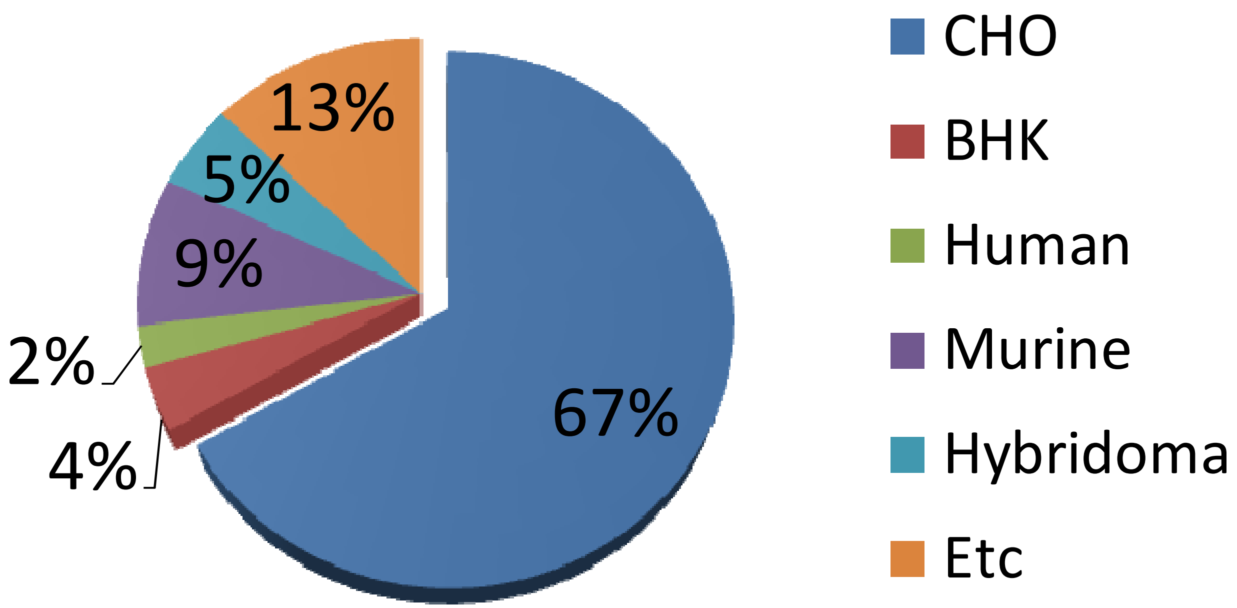


Metabolic Engineering IX

June 7, 2012

UNIVERSITY OF DELAWARE

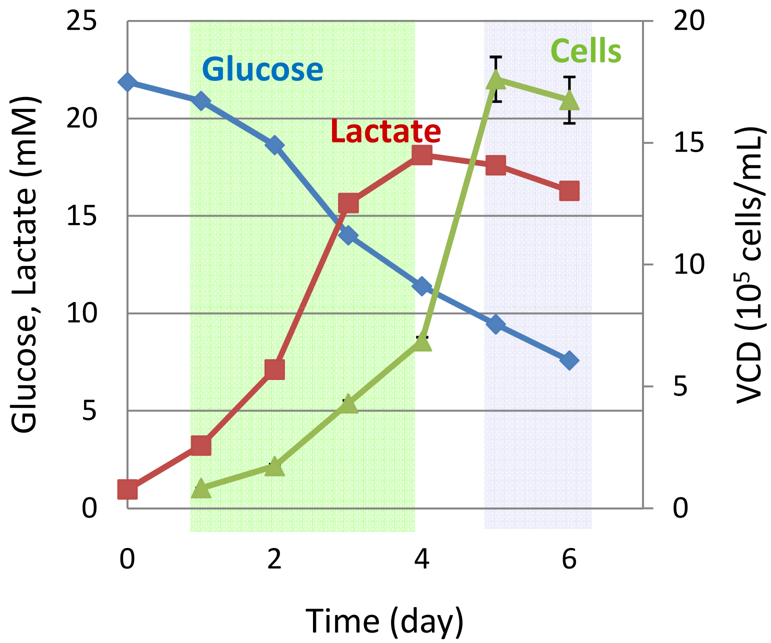
CHO cells



- **Most popular cell line to produce biotherapeutics**
- **CHO metabolism is still poorly understood**
- **New insights needed for medium optimization and cell line screening**

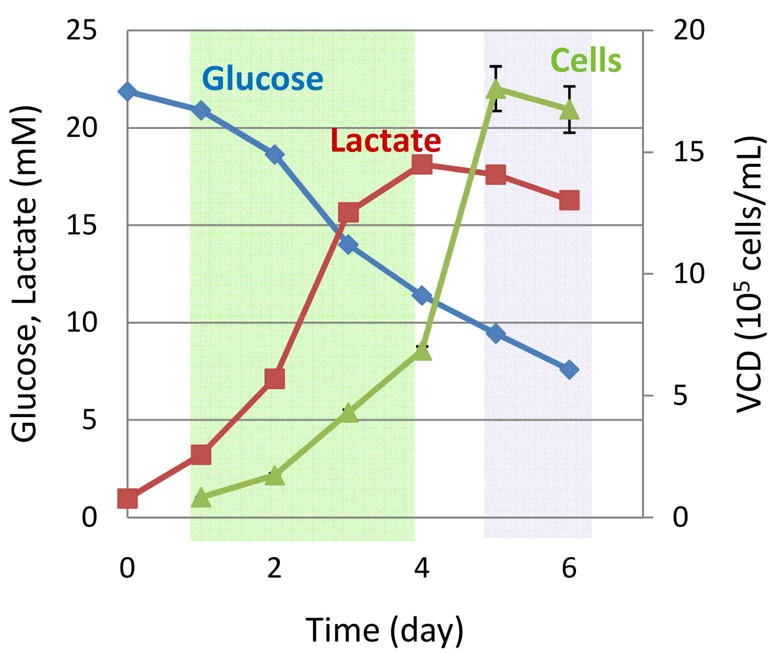
Flux analysis in CHO cells

Metabolism changes in time

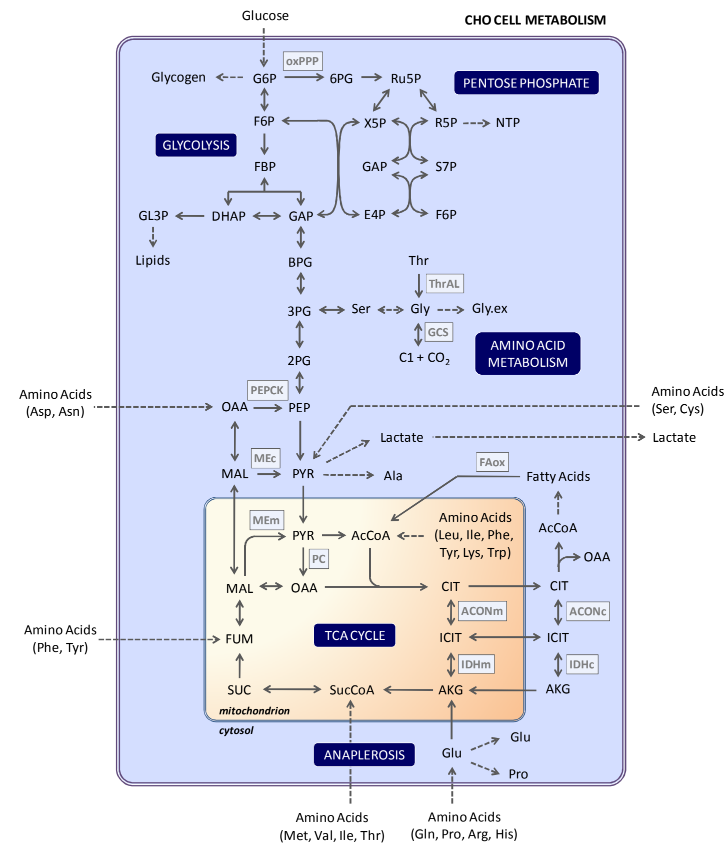


Flux analysis in CHO cells

Metabolism changes in time



Metabolism is complex



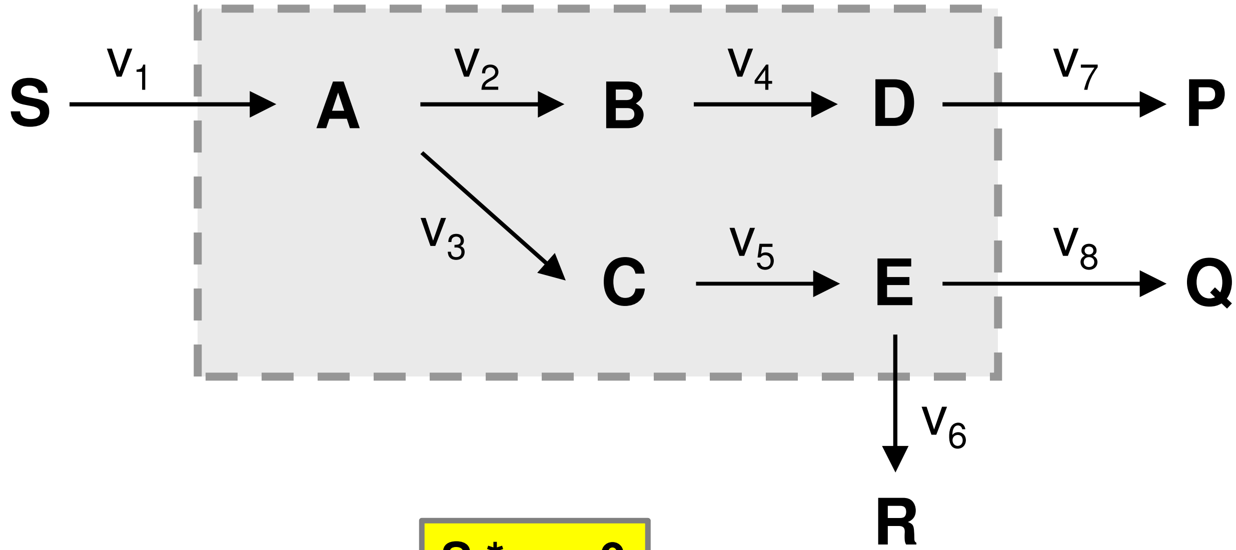
Metabolic Flux Analysis in CHO cells

Table 1. Overview of metabolic flux analysis studies in CHO cells

Cell line	Culture method	Flux analysis method	Major achievements	Year [Ref.]
γ-CHO	Continuous	MFA	MFA validated the metabolism of amino acids derived from peptide in serum-free media.	1999 [101]
γ-CHO	Continuous	MFA	Carbon utilization efficiency was estimated by MFA. IFN-γ glycosylation was related to TCA cycle flux, not glycolysis.	1999 [102]
CHO TF 70R	Continuous	MFA	The efficiency of carbon utilization was estimated by MFA and linked to reduced production rate of t-PA.	2001 [103]
CHO-320	Batch	MFA + kinetic model	Macroscopic dynamical modeling approach was linked to a simplified network model for MFA.	2004 [110]
CHO-320	Batch	MFA + kinetic model	Dynamic modeling was linked to MFA to estimate fluxes during cell growth, transition and stationary phase.	2006 [112]
CHO TF 70R	Batch	MFA	Co-feeding of glucose and galactose resulted in metabolic shift from lactate production to consumption.	2006 [104]
Unknown	Perfusion	MFA	Intracellular fluxes were estimated by quasi real-time MFA in perfusion culture.	2006 [107]
Unknown	Perfusion	MFA	Error propagation from measurements to metabolic fluxes was determined for MFA.	2009 [106]
Unknown	Perfusion	^{13}C-MFA	MFA and ^{13}C-MFA were compared. Flux agreement required oxPPP and PC fluxes to be set by ^{13}C-MFA data.	2010 [114]
Super-CHO	Batch	MFA	Differences in CHO cell metabolism and hybridoma cell metabolism were identified using MFA.	2010 [109]
CHO-320	Batch	MFA	To improve flux observability, the number and type of available measurements were optimized for MFA.	2010 [108]
GS-CHO SF18	Fed-batch	MFA and ^{13}C-MFA	oxPPP flux was estimated by ^{13}C-MFA. MFA was used to estimate fluxes in the rest of network model.	2011 [115]
Unknown	Fed-batch and continuous	MFA	Amino acid composition of culture medium was optimized using MFA. By-product levels were reduced and cell density and antibody production were enhanced.	2011 [100]
CHO-K1	Fed-batch	MFA + kinetic model	MFA was integrated with a kinetic model to simulate metabolic dynamics in fed-batch cultures.	2011 [111]
dhfr-CHO	Fed-batch	MFA + kinetic model	Kinetic models for growing and non-growing subpopulations of cells were integrated with a simplified MFA model.	2011 [113]
CHO-K1	Fed-batch	^{13}C-MFA and ^{13}C-NMFA	Metabolic fluxes were determined for growth phase and stationary phase in a fed-batch culture using ^{13}C-based MFA.	2011 [105]

Ahn and Antoniewicz, *Biotechnol J*, 7: 61-74, 2012

Metabolic Flux Analysis (MFA)

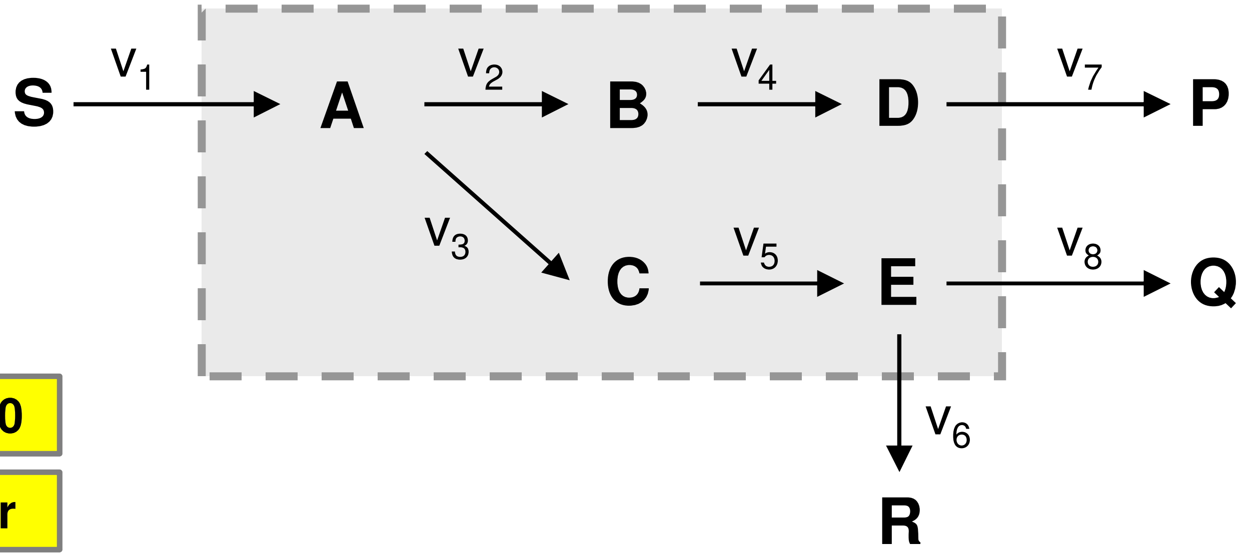


S * v = 0

	v1	v2	v3	v4	v5	v6	v7	v8
A	1	−1	−1	0	0	0	0	0
B	0	1	0	−1	0	0	0	0
C	0	0	1	0	−1	0	0	0
D	0	0	0	1	0	0	−1	0
E	0	0	0	0	1	−1	0	−1

$$* \begin{pmatrix} v1 \\ v2 \\ v3 \\ v4 \\ v5 \\ v6 \\ v7 \\ v8 \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix}$$

Metabolic Flux Analysis (MFA)



$S * v = 0$

$R * v = r$

	v1	v2	v3	v4	v5	v6	v7	v8
A	1	−1	−1	0	0	0	0	0
B	0	1	0	−1	0	0	0	0
C	0	0	1	0	−1	0	0	0
D	0	0	0	1	0	0	−1	0
E	0	0	0	0	1	−1	0	−1
S	−1	0	0	0	0	0	0	0
P	0	0	0	0	0	0	1	0
Q	0	0	0	0	0	0	0	1
R	0	0	0	0	0	1	0	0

*

v1
v2
v3
v4
v5
v6
v7
v8

=

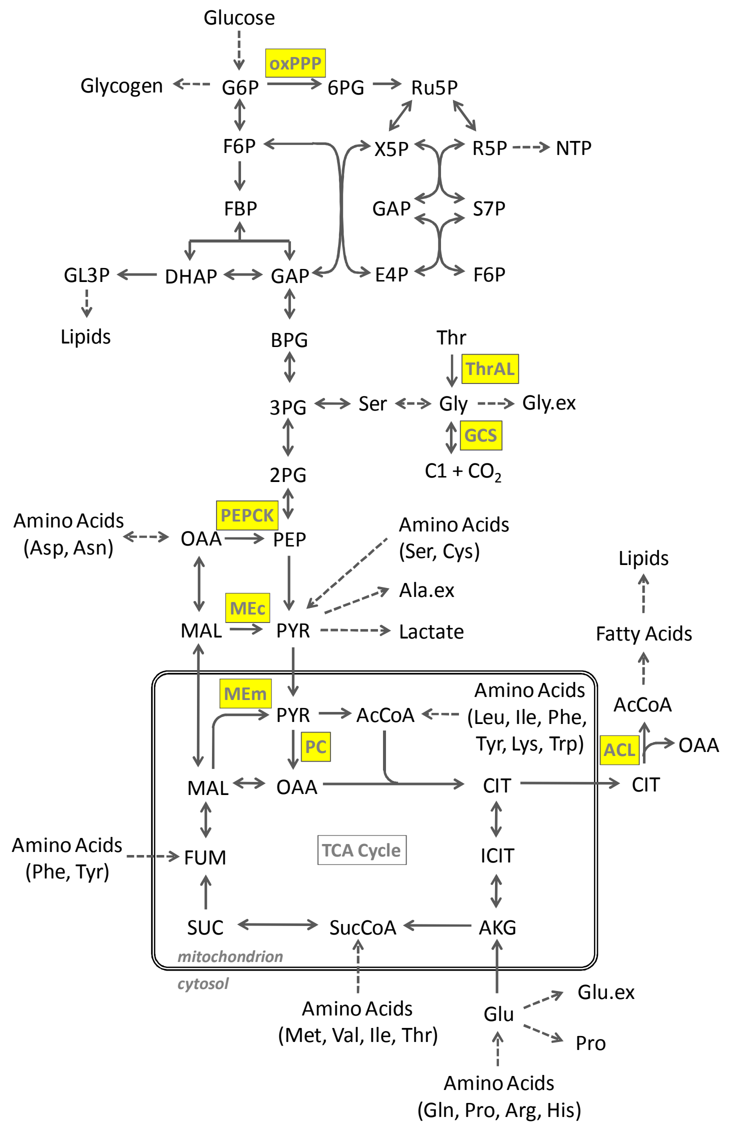
0
0
0
0
0
r_S
r_P
r_Q
r_R

Metabolic Flux Analysis (MFA)

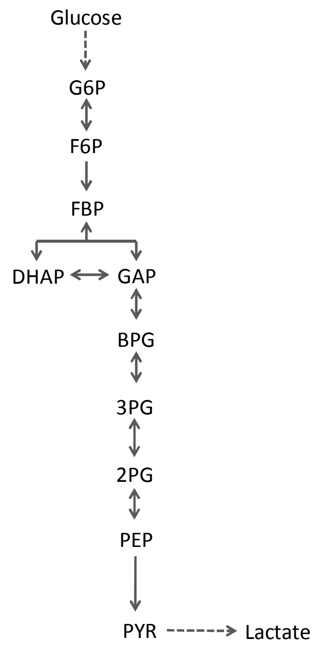
Metabolic flux analysis is <u>only as reliable</u> as your data and <u>modeling assumptions</u>

- **Observability Problem**
  - If rank(data) = number of fluxes → all fluxes can be determined
  - If rank(data) < number of fluxes → not all fluxes can be uniquely determined

Model of CHO metabolism

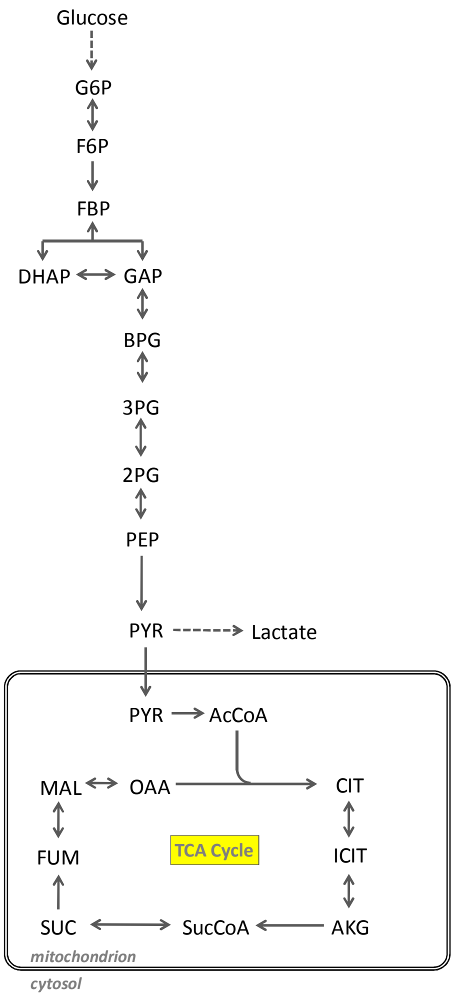


Model of CHO metabolism



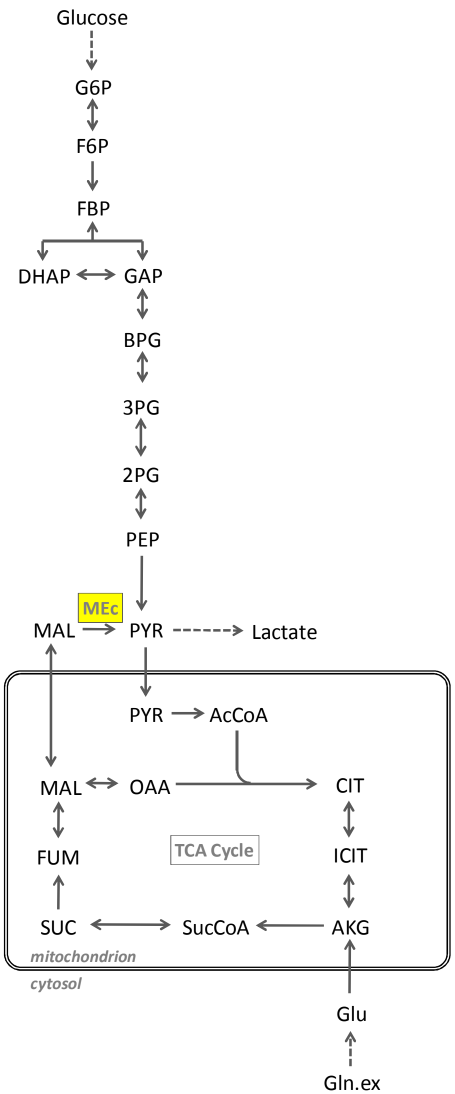
- **External Measurements**
  - **Glucose**

Model of CHO metabolism



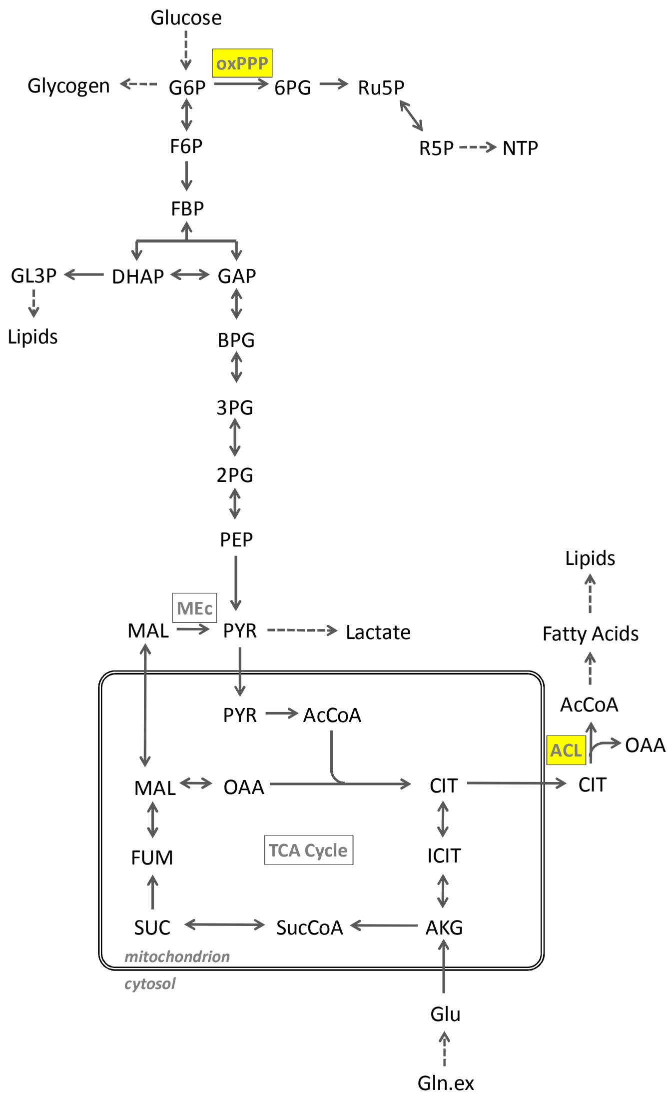
- **External Measurements**
  - **Glucose**
  - **Lactate**

Model of CHO metabolism



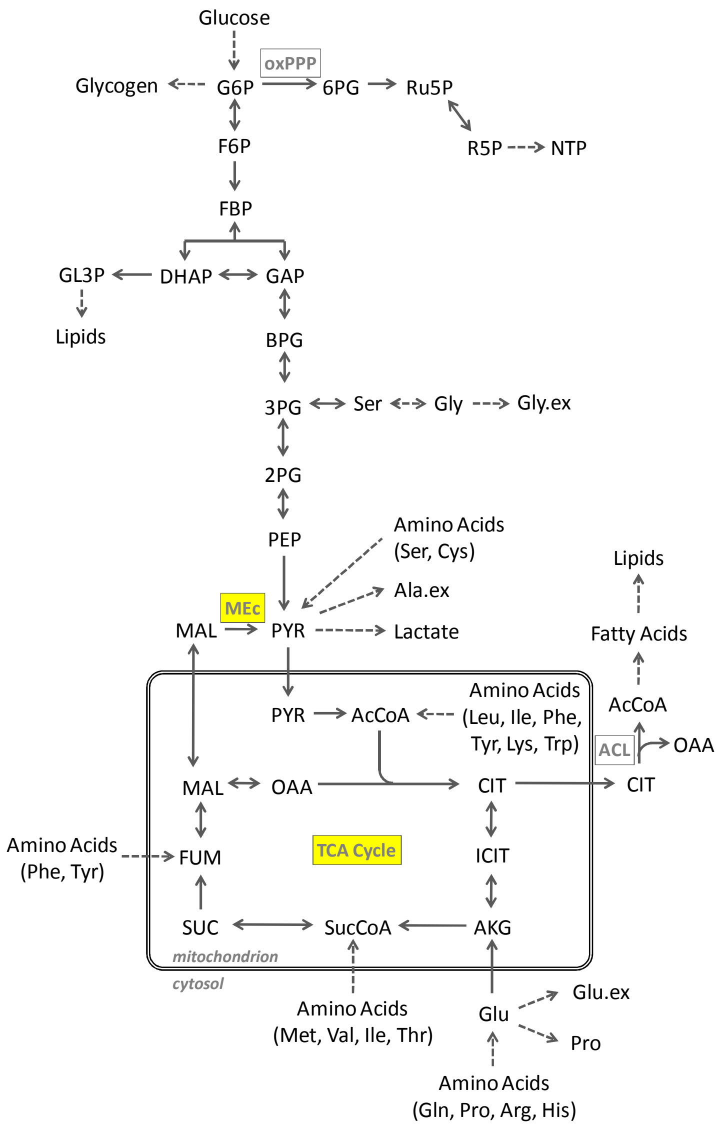
- **External Measurements**
  - **Glucose**
  - **Lactate**
  - **Glutamine**

Model of CHO metabolism



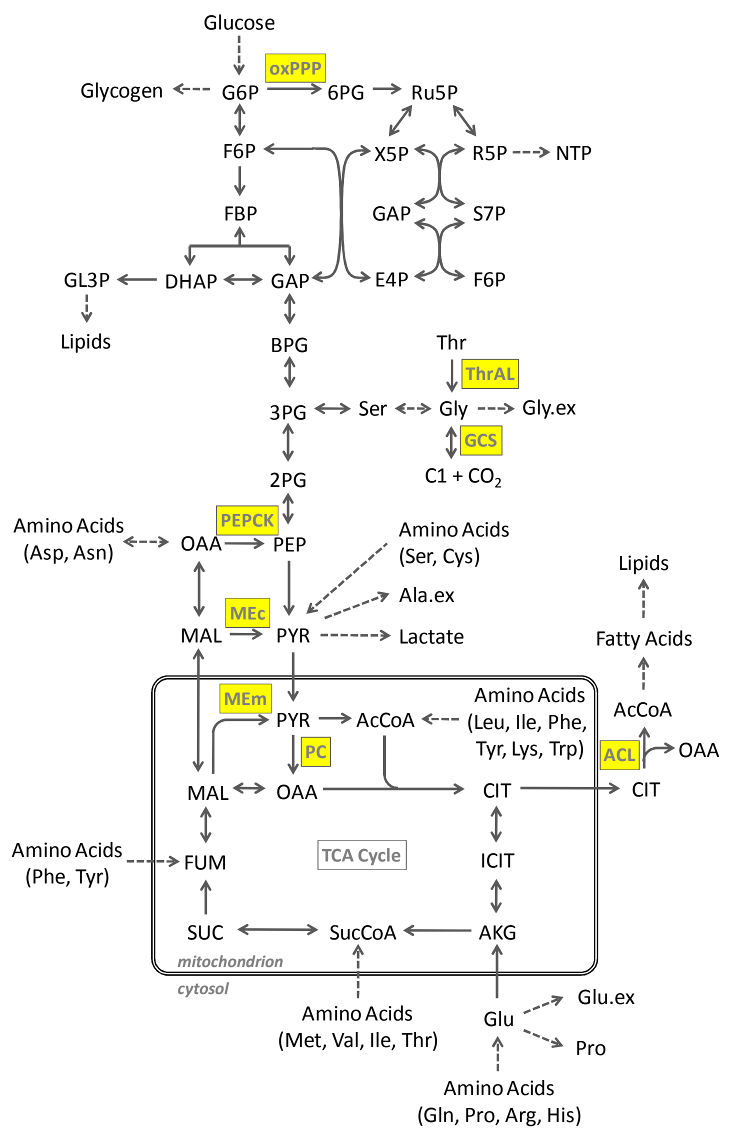
- **External Measurements**
  - Glucose
  - Lactate
  - Glutamine
  - Growth rate

Model of CHO metabolism



- **External Measurements**
  - Glucose
  - Lactate
  - Glutamine
  - Growth rate
  - Amino acids

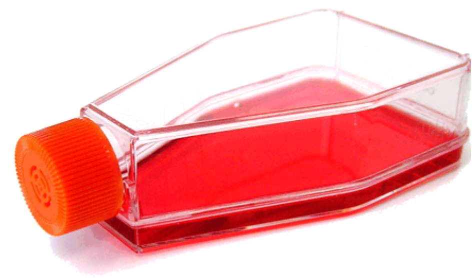
Model of CHO metabolism



- **External Measurements**
  - **Glucose**
  - **Lactate**
  - **Glutamine**
  - **Growth rate**
  - **Amino acids**
- **^{13}C-Labeling GC-MS**
  - **Typical 200+ mass isotopomers measured**

CHO Cell Culture

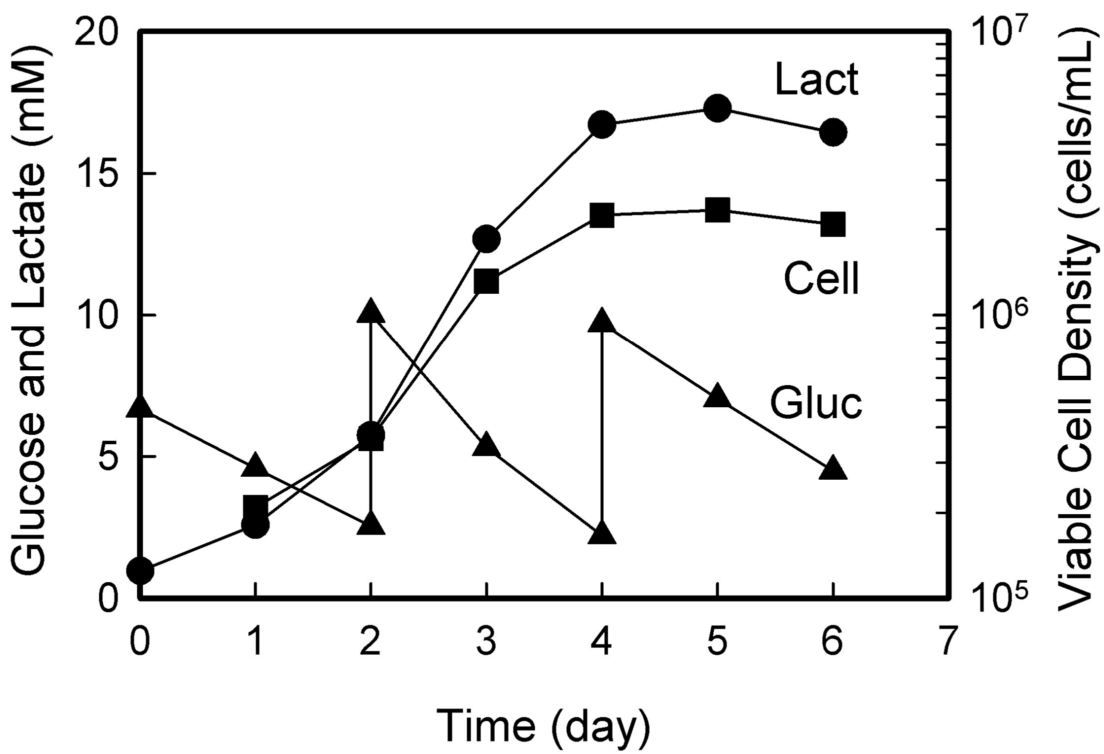
- **CHO cell fed-batch culture**
  - **CHO-K1 cells (ATCC CCL-61) in T-25 flasks**
  - **Humidified incubator, 5% CO_2, 37 deg.C**



- **Medium composition**
  - **DMEM + 10% FBS + 1% PS**
  - **6.7 mM [1,2-^{13}C]glucose**

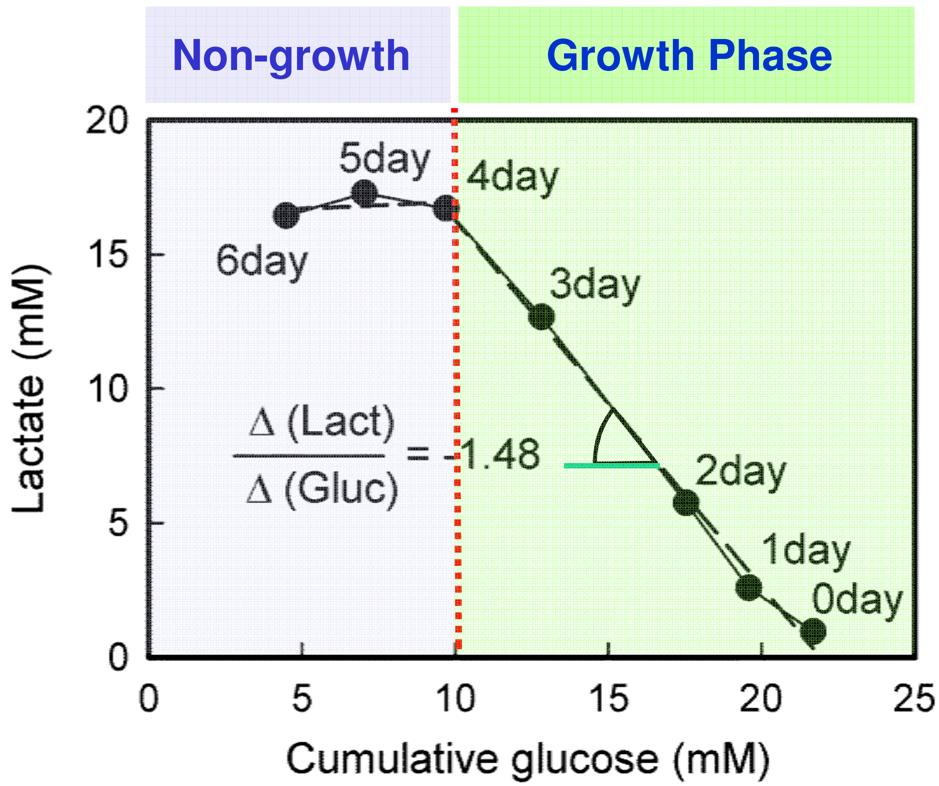
- **Measurements**
  - **Glucose, lactate (YSI)**
  - **Amino acid concentrations (GC-MS)**
  - **^{13}C-Labeling of intracellular metabolites (GC-MS)**

CHO Fed-batch culture



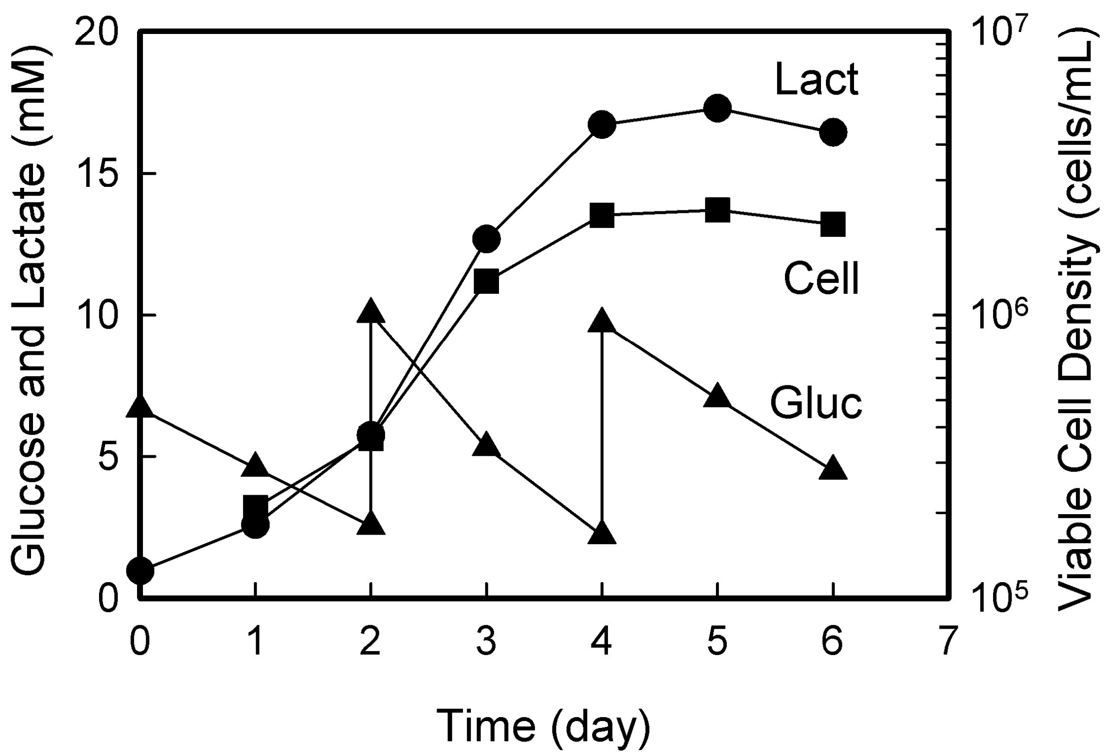
Ahn WS & Antoniewicz MR, Metab Eng, 2011

Shift in Lactate Metabolism



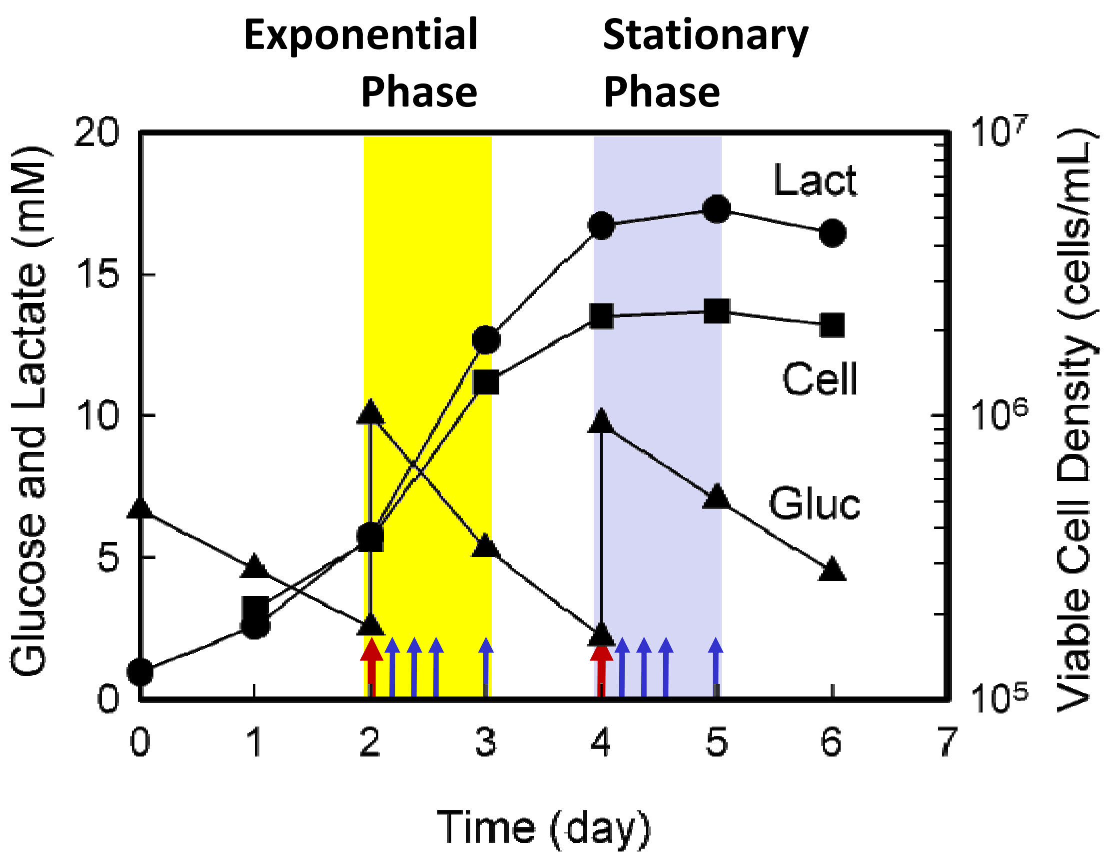
Ahn WS & Antoniewicz MR, Metab Eng, 2011

CHO Fed-batch culture



Ahn WS & Antoniewicz MR, Metab Eng, 2011

CHO Fed-batch culture

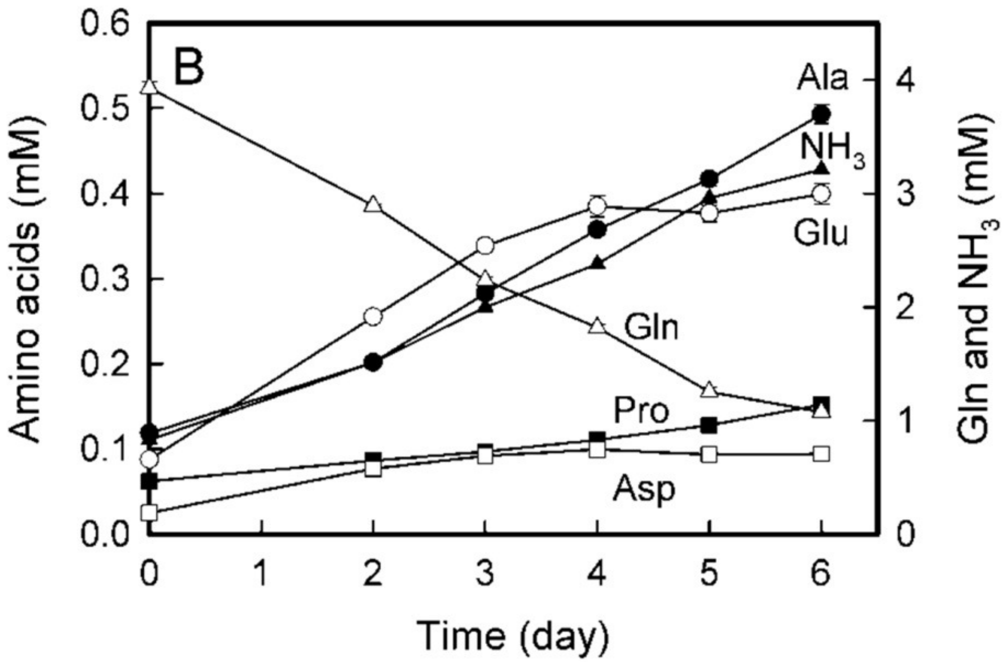


[1,2-^{13}C]glucose addition

Metabolism quenching and GC-MS analysis

Ahn WS & Antoniewicz MR, Metab Eng, 2011

Extracellular metabolite profiling



Ahn WS & Antoniewicz MR, Metab Eng, 2011

Biomass specific fluxes

Biomass specific uptake and excretion rates of extracellular metabolites (nmol/10^6cells/hr)

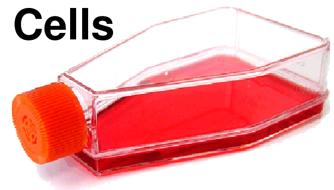
	Exponential Phase	Stationary Phase
Glucose	-201	-48.8
Lactate	299	-2.5
NH_3	24.5	5.2
Alanine	2.2	0.5
Glycine	2.8	0.6
Valine	-5.2	-0.1
Leucine	-7.0	-0.3
Isoleucine	-5.6	-0.3
Proline	-0.3	0.0
Methionine	-1.9	-0.1
Serine	-8.1	-0.9
Threonine	-4.4	-0.1
Phenylalanine	-2.7	-0.2
Aspartate	-0.0	-0.3
Glutamate	5.4	-0.4
Tyrosine	-0.9	-0.1
Glutamine	-35.0	-5.7

Ahn WS & Antoniewicz MR, Metab Eng, 2011

Intracellular Metabolite Analysis

Mass isotopomer distribution quantification

Cells



Tracer experiments with CHO cell culture in T25 flasks

Cold methanol

Removal of media
1.5 mL cold methanol
5 min in ice

Chloroform/water

1 v $CHCl_3$
1 v H_2O

pH 7~8, overnight in cold chamber, centrif.

Non-polar metabolites

Lipids in $CHCl_3$
Drying

SPE separation

fractionation

Triglyceride/FFA

Derivatization
Lipid esters, TMS der. of FFA

GC-MS Analysis

Polar metabolites

Amino acids/organic acids
Drying; 37ºC, 9hr

Organic/Amino acids

MOX/TBDMS der. of metabolites

Derivatization
MOX: 37ºC, 90 min
TBDMS; 60ºC, 30 min

GC-MS Analysis

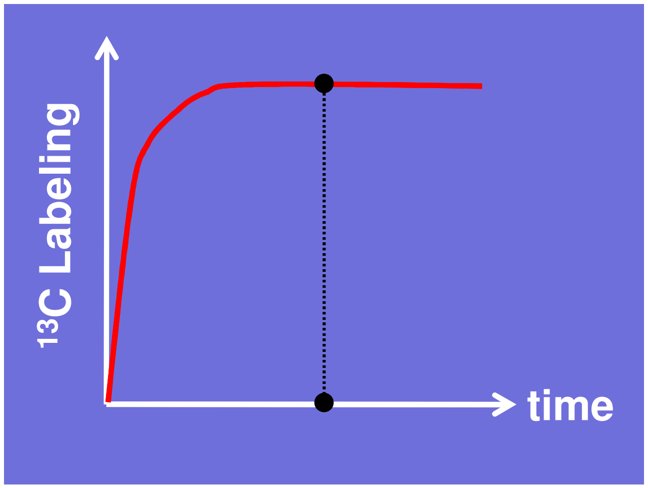
GC-MS Measurements

Metabolite	Base mass	Carbon atoms	Fragment formula
Organic acids			
Pyruvate	174	1-2-3	$C_6H_{12}O_3NSi$
Lactate	233	2-3	$C_{10}H_{25}O_2Si_2$
Lactate	261	1-2-3	$C_{11}H_{25}O_3Si_2$
Succinate	289	1-2-3-4	$C_{12}H_{25}O_4Si_2$
Fumarate	287	1-2-3-4	$C_{12}H_{23}O_4Si_2$
AKG	346	1-2-3-4-5	$C_{14}H_{28}O_5NSi_2$
Malate	419	1-2-3-4	$C_{18}H_{39}O_5Si_3$
PEP	453	1-2-3	$C_{17}H_{38}O_6Si_3P$
GAP	484	1-2-3	$C_{18}H_{43}O_6NSi_3P$
Glyc3P	571	1-2-3	$C_{23}H_{56}O_6Si_4P$
Citrate	459	1-2-3-4-5-6	$C_{20}H_{39}O_6Si_3$
3PG	585	1-2-3	$C_{23}H_{54}O_7Si_4P$
Amino acids			
Alanine	232	2-3	$C_{10}H_{26}ONSi_2$
Alanine	260	1-2-3	$C_{11}H_{26}O_2NSi_2$
Glycine	246	1-2	$C_{10}H_{24}O_2NSi_2$
Valine	260	2-3-4-5	$C_{12}H_{30}ONSi_2$
Leucine	274	2-3-4-5-6	$C_{13}H_{32}ONSi_2$
Isoleucine	274	2-3-4-5-6	$C_{13}H_{32}ONSi_2$
Proline	286	1-2-3-4-5	$C_{13}H_{28}O_2NSi_2$
Methionine	320	1-2-3-4-5	$C_{13}H_{30}O_2NSi_2S$
Serine	390	1-2-3	$C_{17}H_{40}O_3NSi_3$
Threonine	404	1-2-3-4	$C_{18}H_{42}O_3NSi_3$
Phenylalanine	302	1-2	$C_{14}H_{32}O_2NSi_2$
Aspartate	302	1-2	$C_{14}H_{32}O_2NSi_2$
Aspartate	390	2-3-4	$C_{17}H_{40}O_3NSi_3$
Aspartate	418	1-2-3-4	$C_{18}H_{40}O_4NSi_3$
Glutamate	330	2-3-4-5	$C_{16}H_{36}O_2NSi_2$
Glutamate	432	1-2-3-4-5	$C_{19}H_{42}O_4NSi_3$
Glutamine	431	1-2-3-4-5	$C_{19}H_{43}O_3N_2Si_3$
Tyrosine	302	1-2	$C_{14}H_{32}O_2NSi_2$

Ahn WS & Antoniewicz MR, Metab Eng, 2011

^{13}C-Labeling in Metabolites

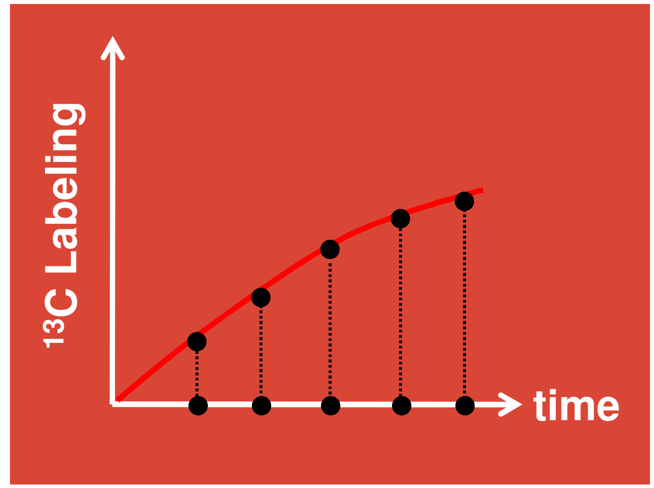
Isotopic steady state



Stationary ^{13}C-MFA

Wiechert et al., Met. Eng. 1999

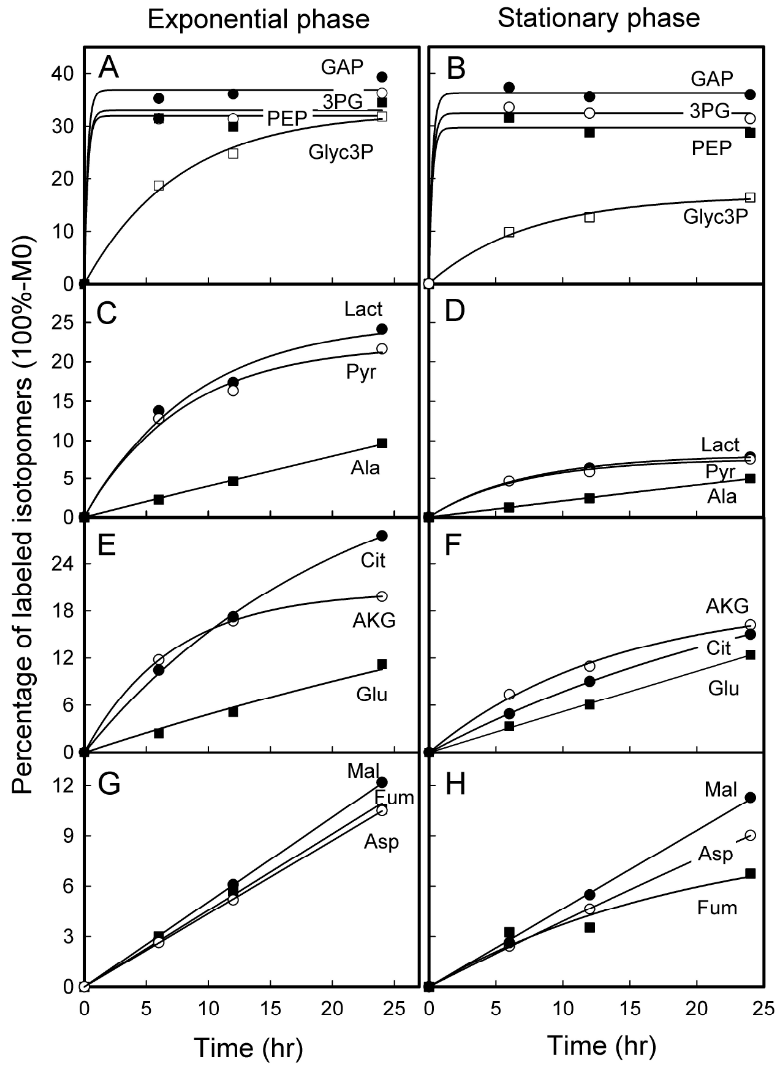
Isotopic non-steady state



Non-stationary ^{13}C-MFA

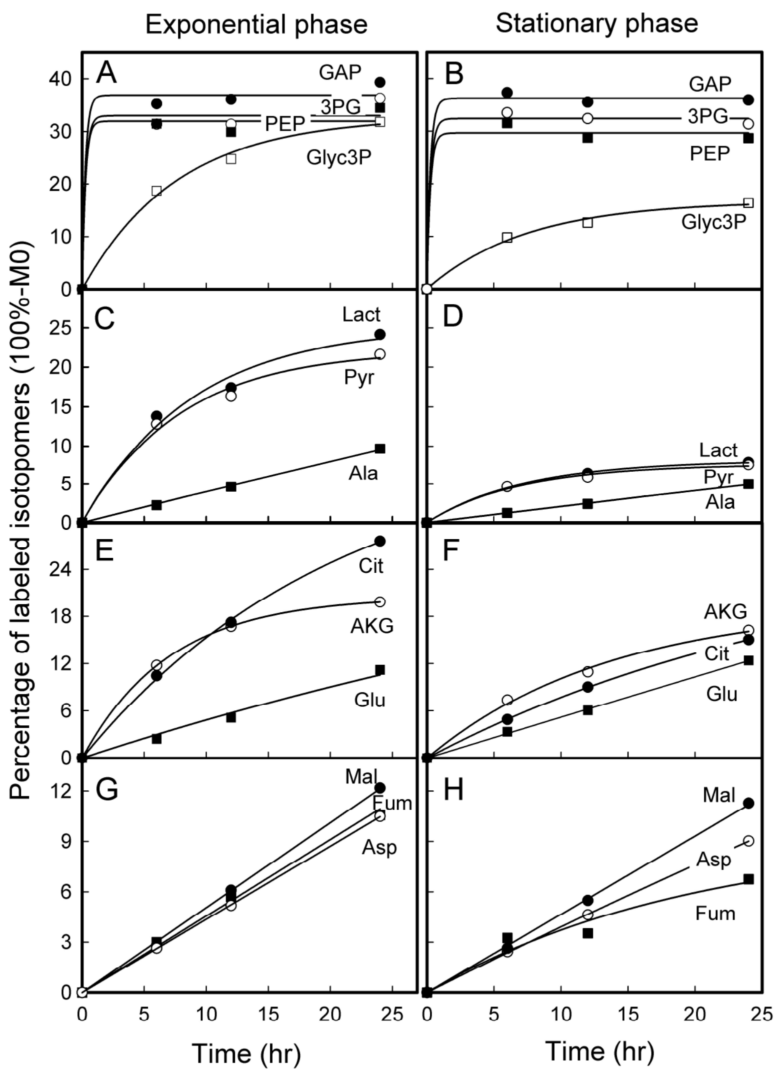
Young et al., Biotech. Bioeng. 2008

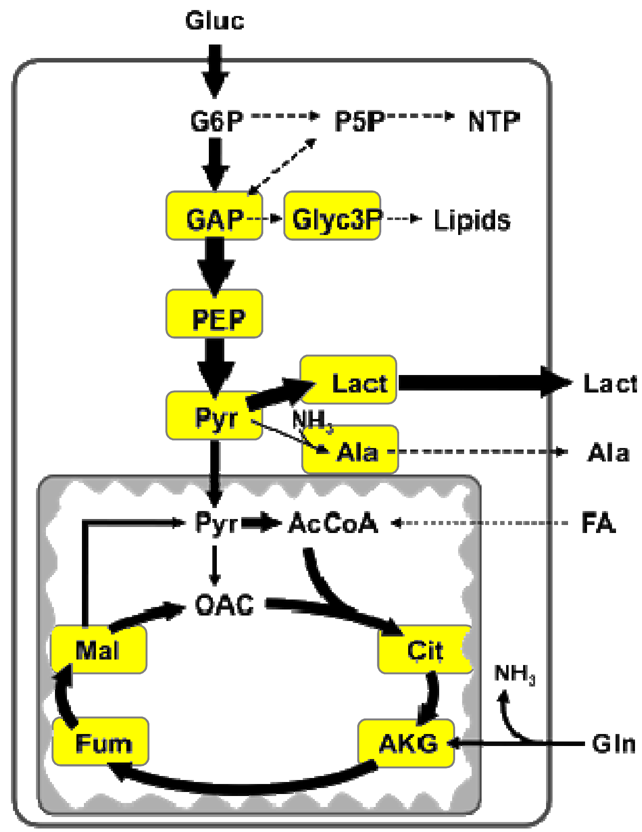
Labeling dynamics of intracellular metabolites



Ahn WS & Antoniewicz MR, Metab Eng, 2011

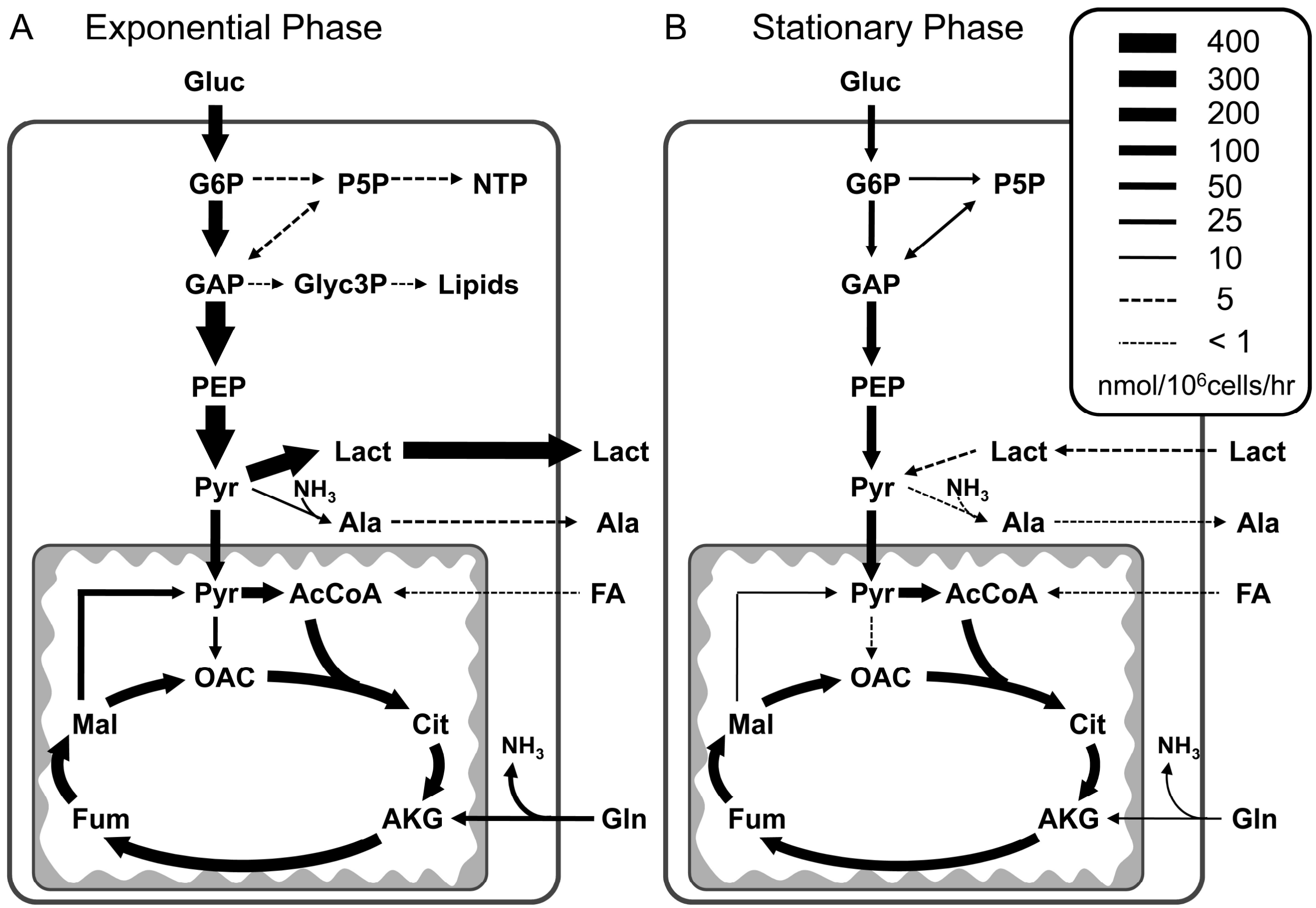
Labeling dynamics of intracellular metabolites





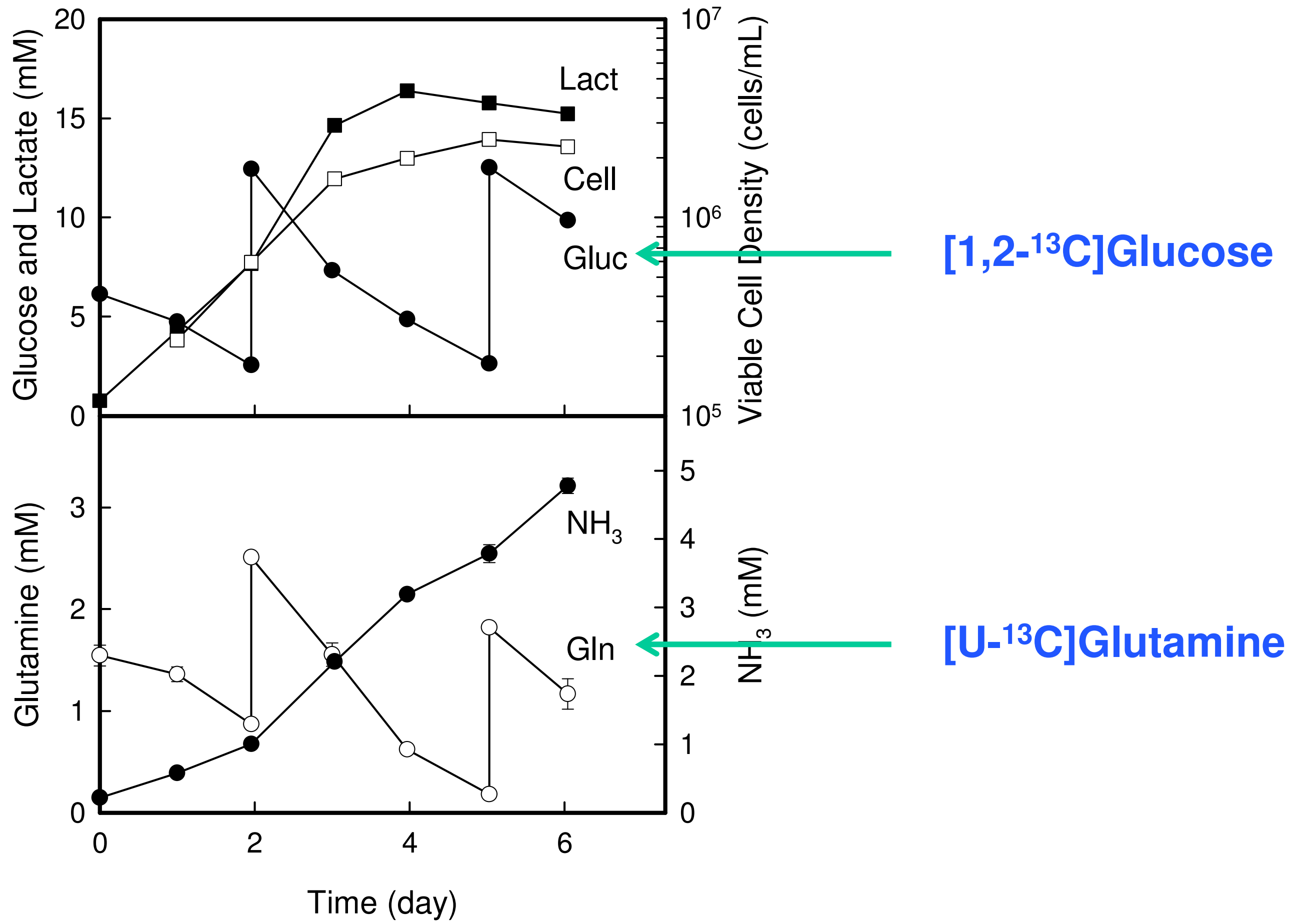
Ahn WS & Antoniewicz MR, Metab Eng, 2011

Isotopic non-stationary ^{13}C-MFA



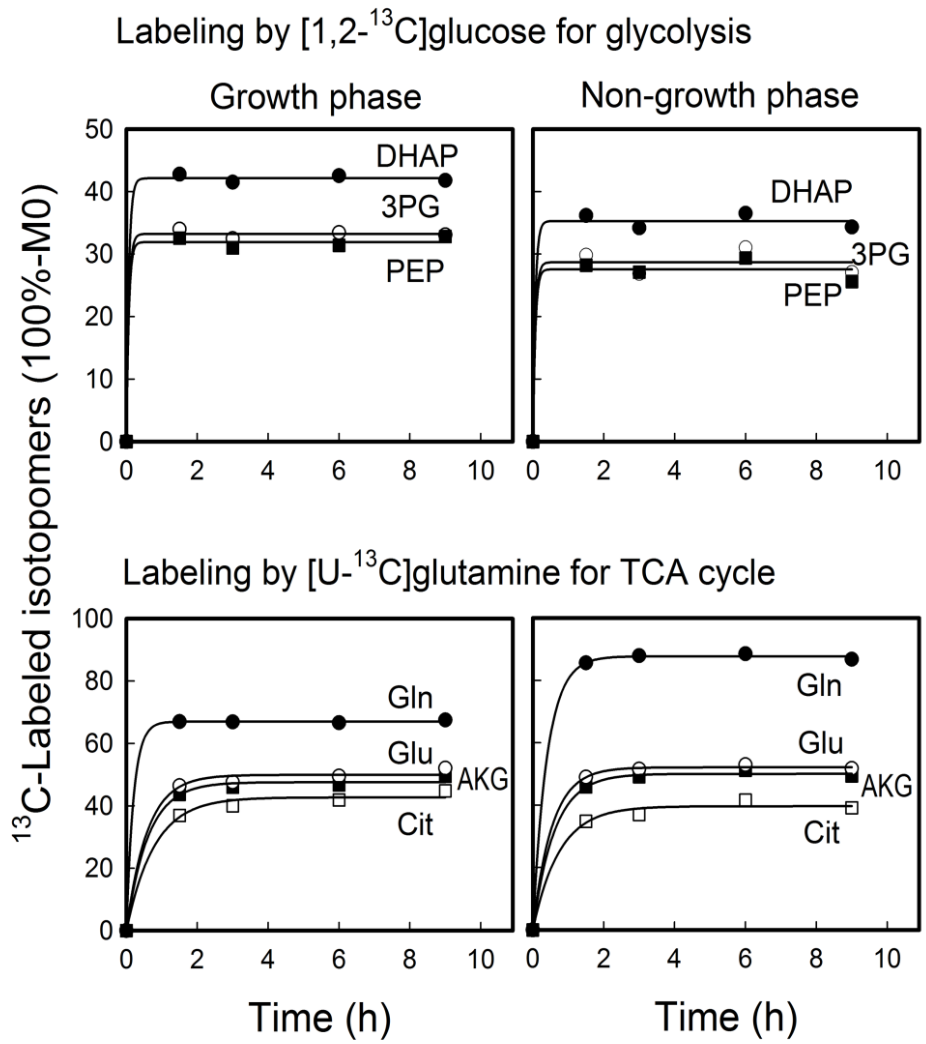
Ahn WS & Antoniewicz MR, Metab Eng, 2011

Parallel Labeling Experiments



Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

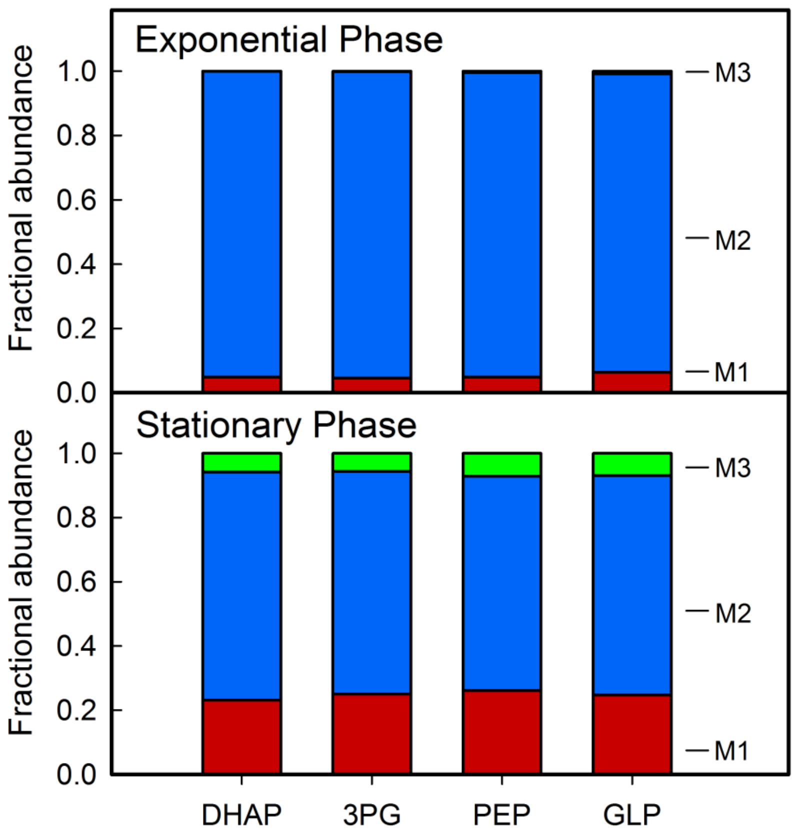
Isotopic steady state < 3 hr



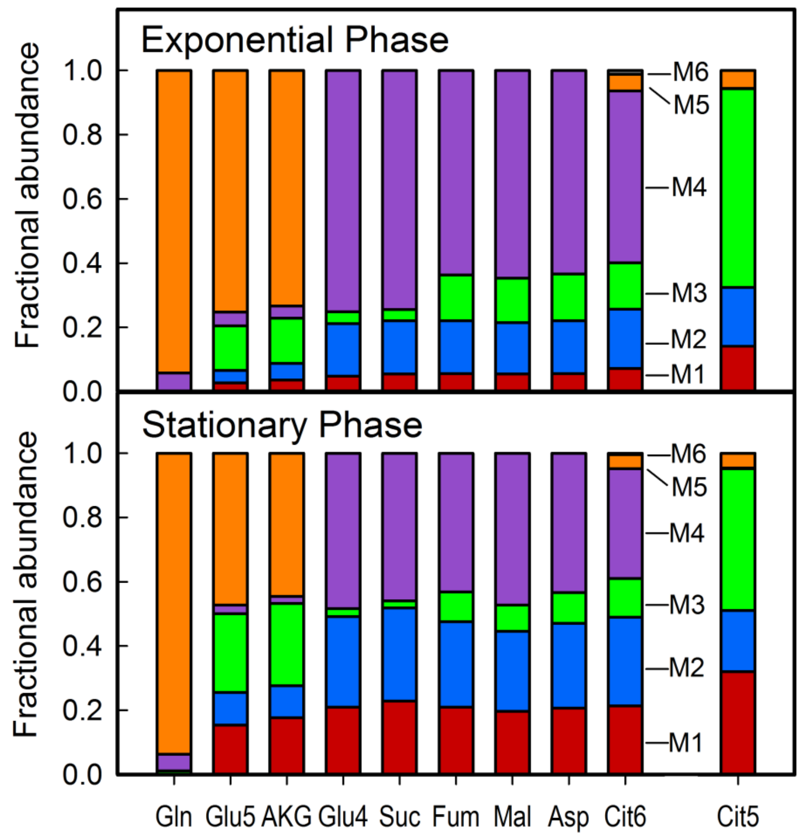
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Intracellular Mass Isotopomers

[1,2-^{13}C]Glucose

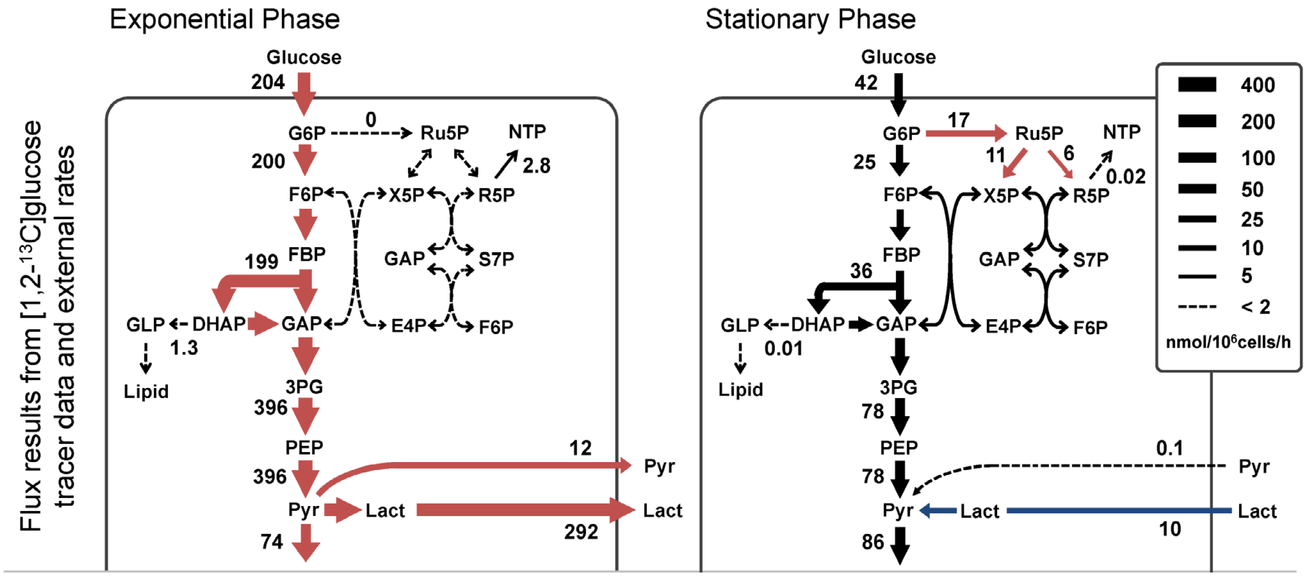


[U-^{13}C]Glutamine



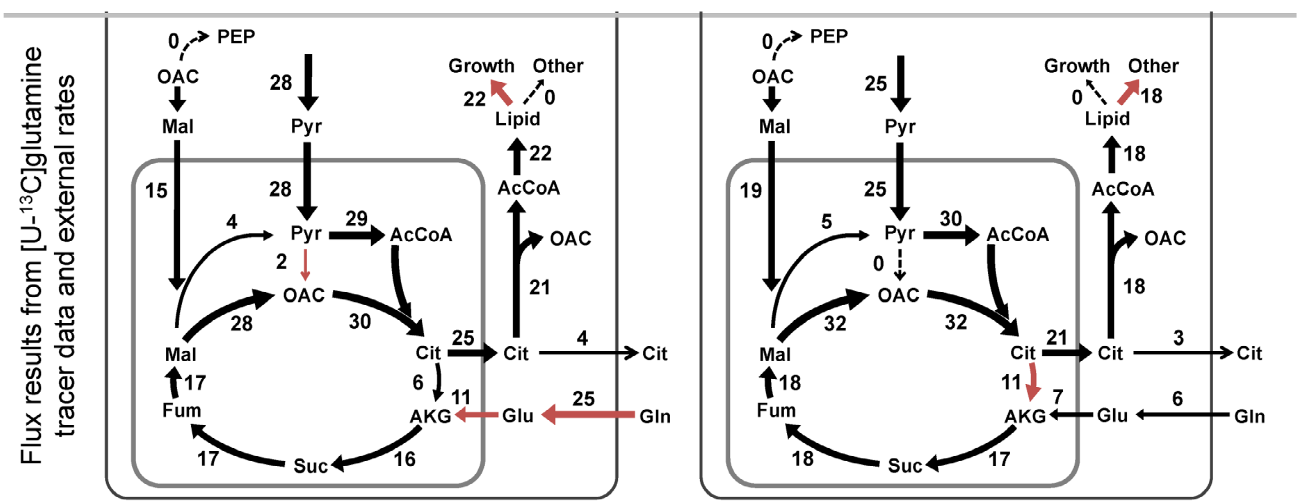
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Metabolic Fluxes Growth vs. Stationary



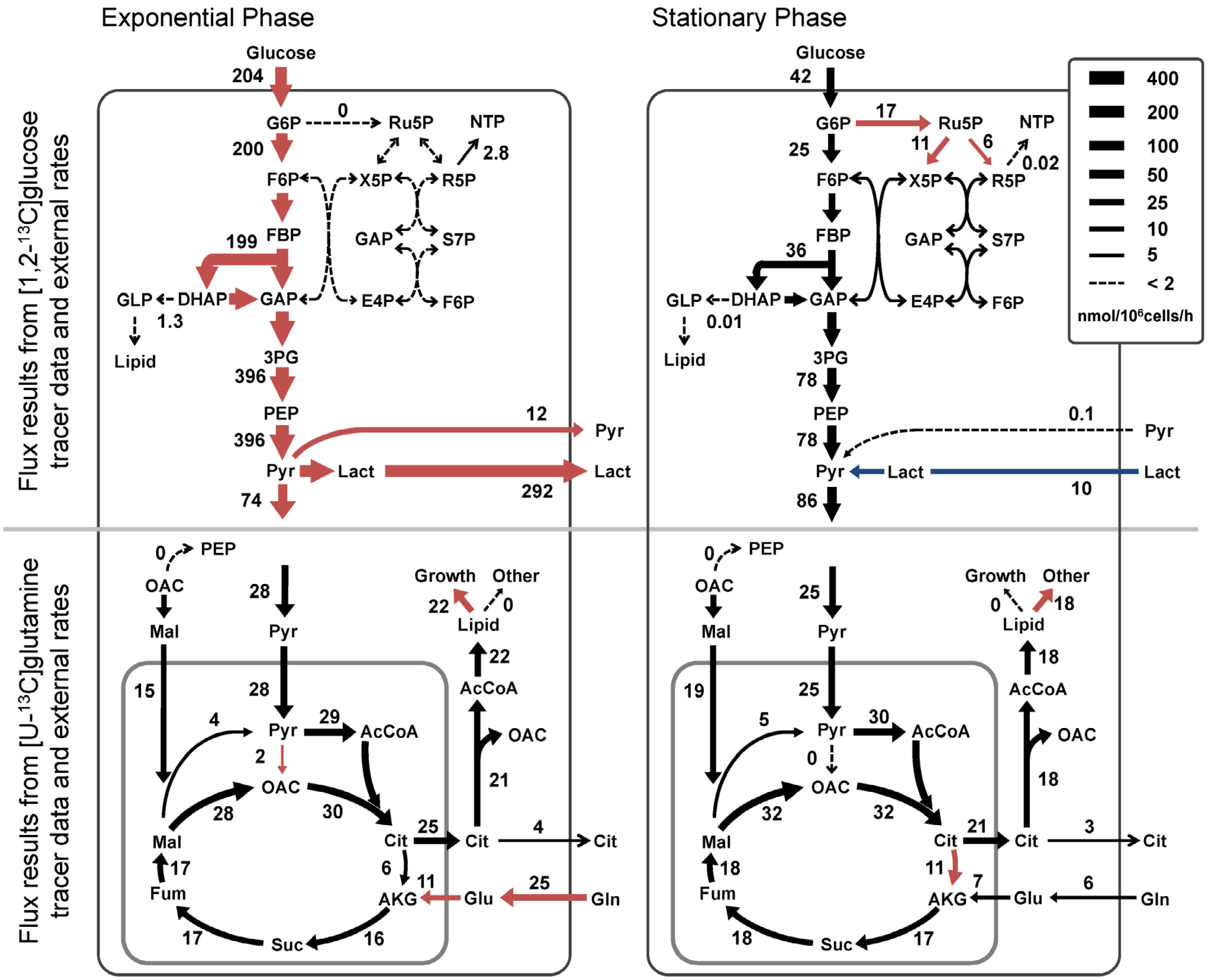
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Metabolic Fluxes Growth vs. Stationary



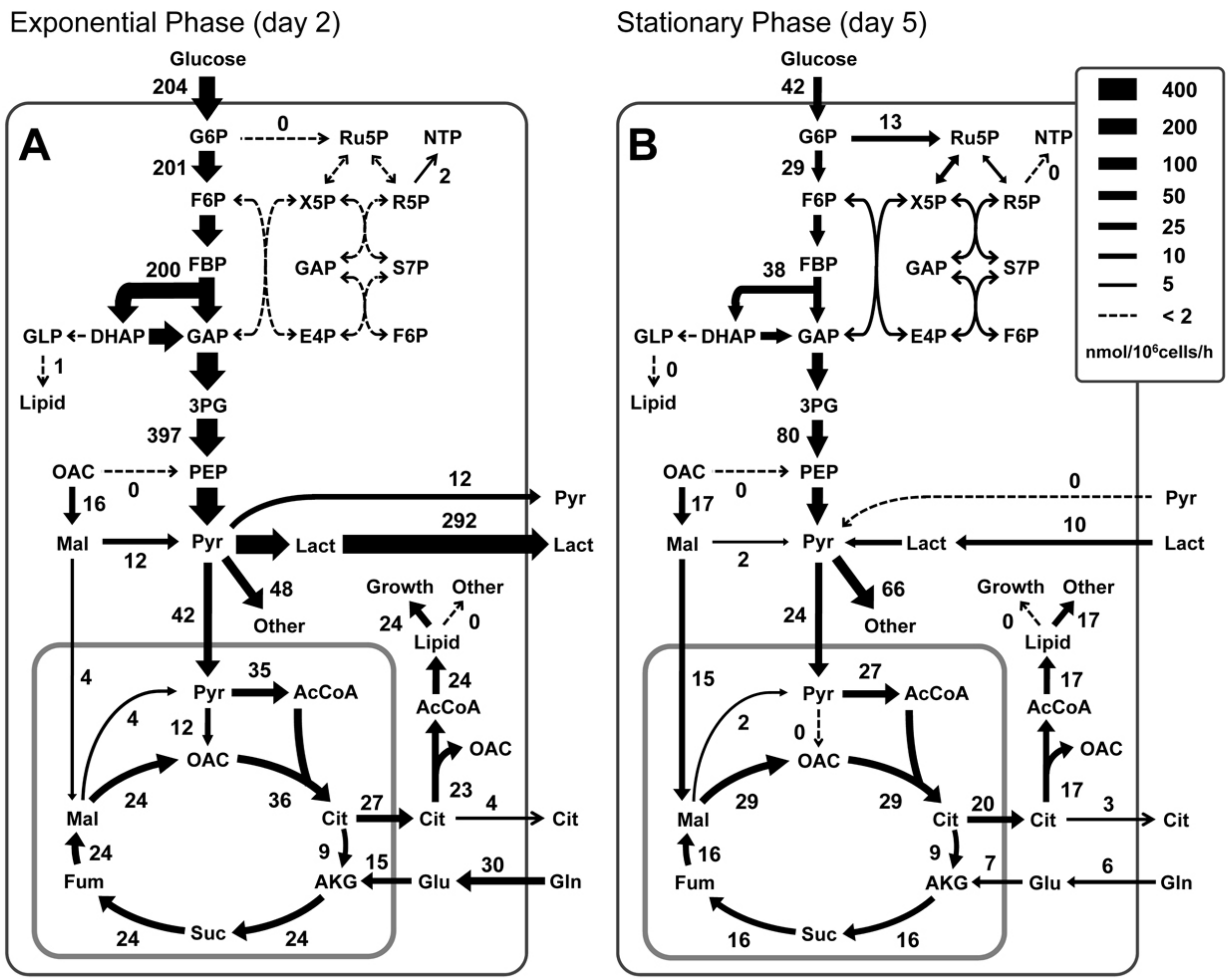
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Metabolic Fluxes Growth vs. Stationary



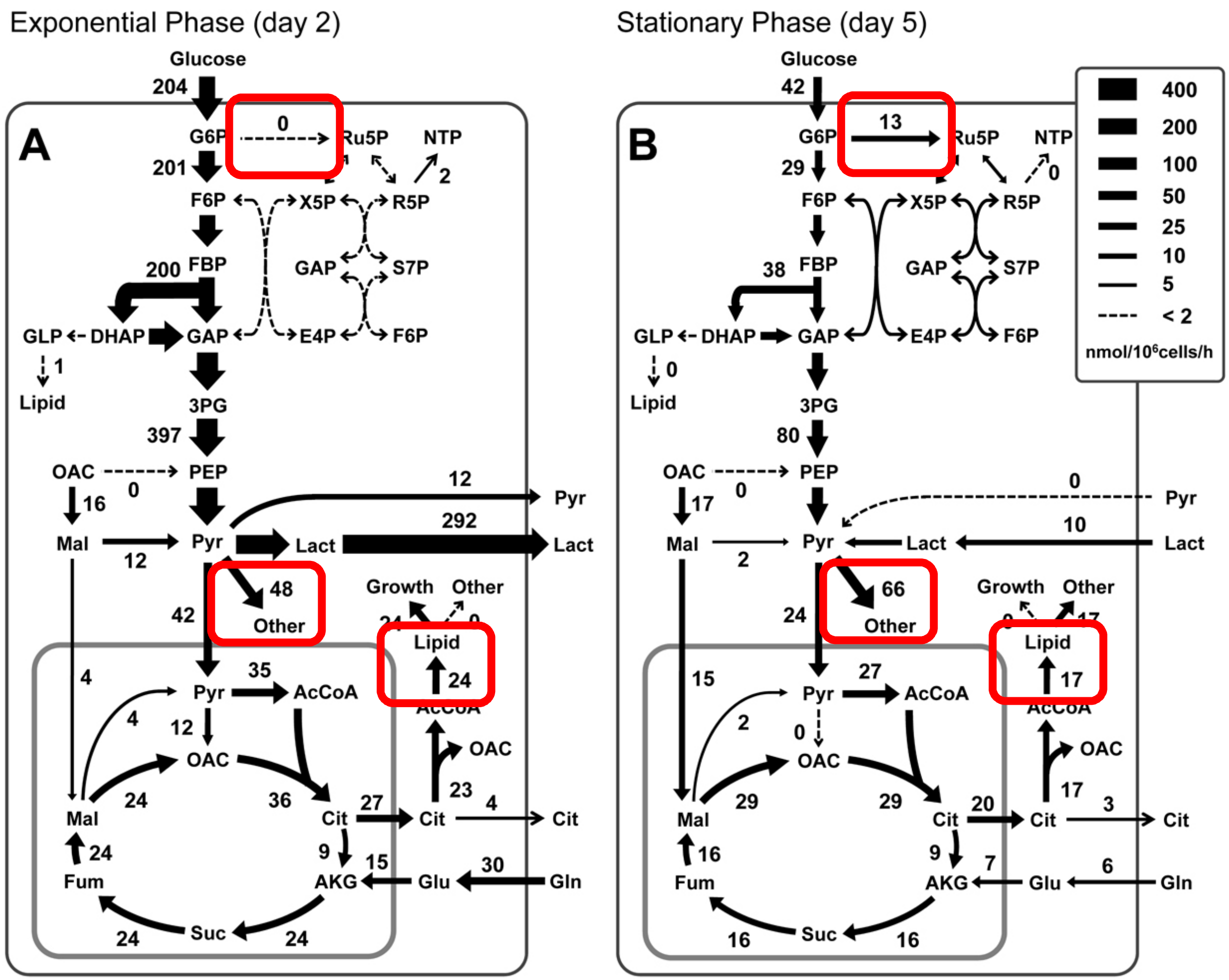
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Combined ^{13}C-MFA of parallel experiments



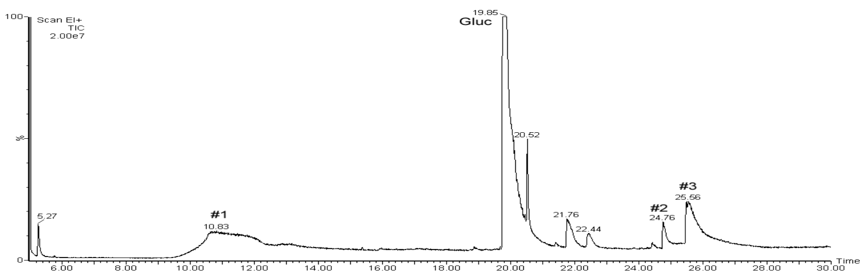
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Combined ^{13}C-MFA of parallel experiments



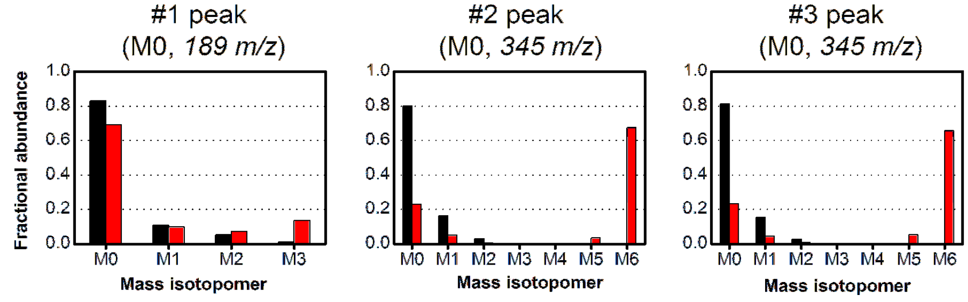
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

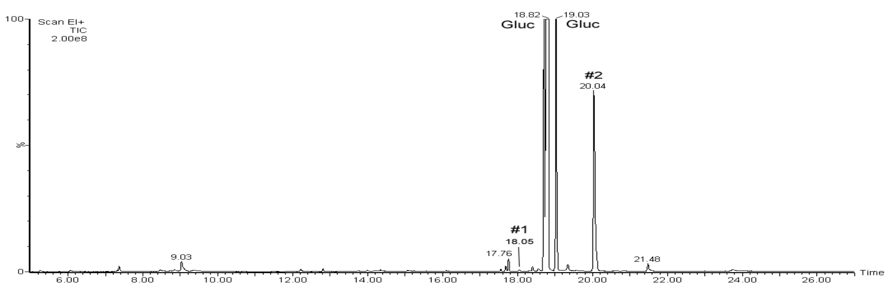
Validating loss of metabolites in glycolysis



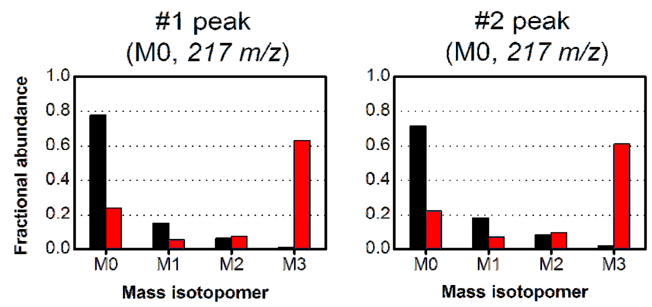
Aldonitrile pentaacetate derivatization of media metabolites

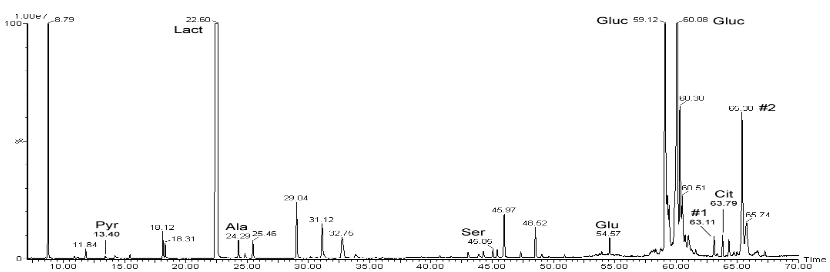
Natural Gluc
[U]Gluc



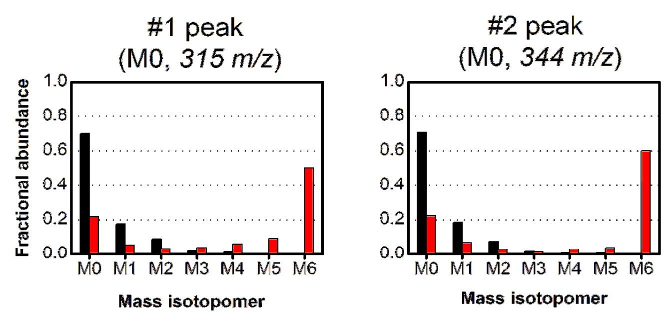


Methyloxime trimethylsilylation derivatization of media metabolites

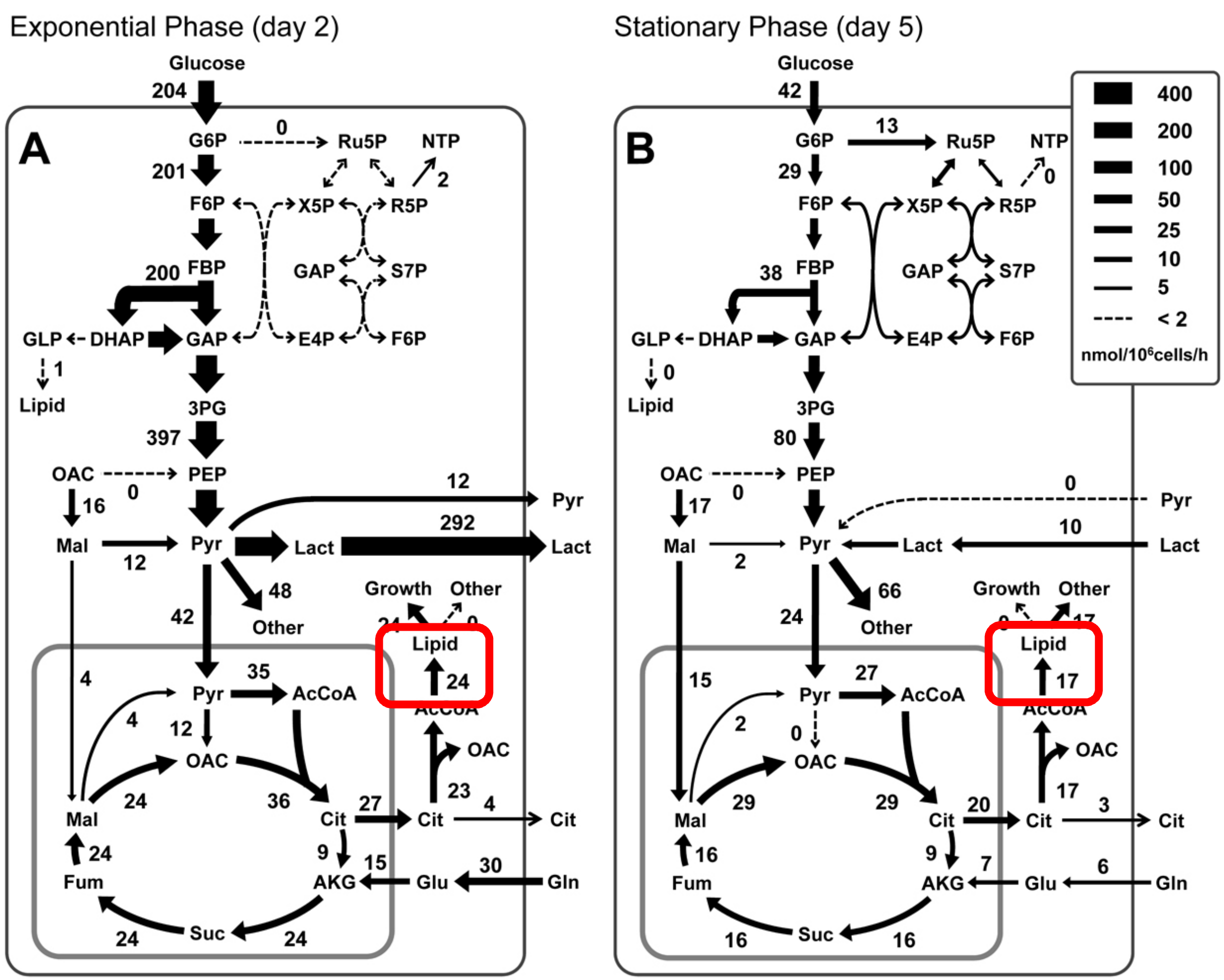




Methyloxime tert-butyldimethylsilylation derivatization of media metabolites

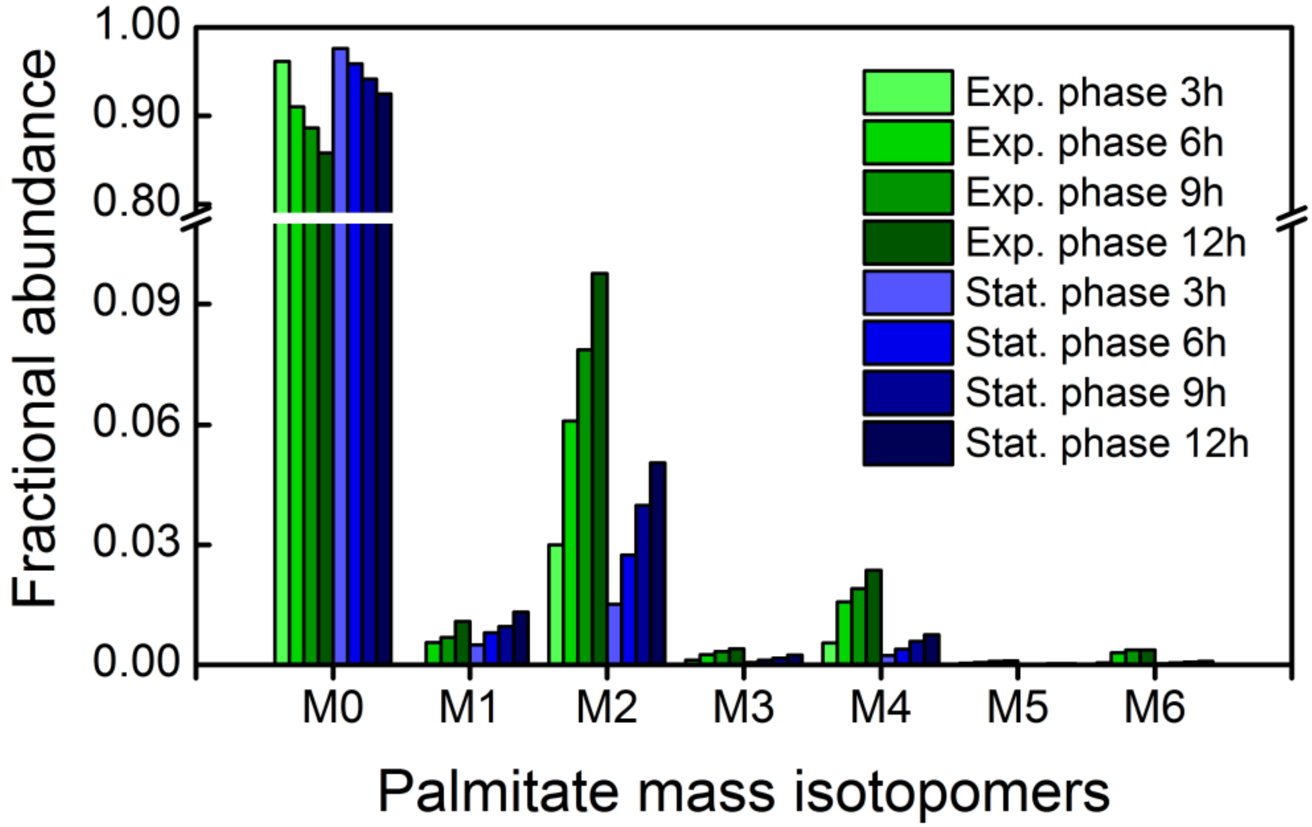


Validating Lipid Metabolism



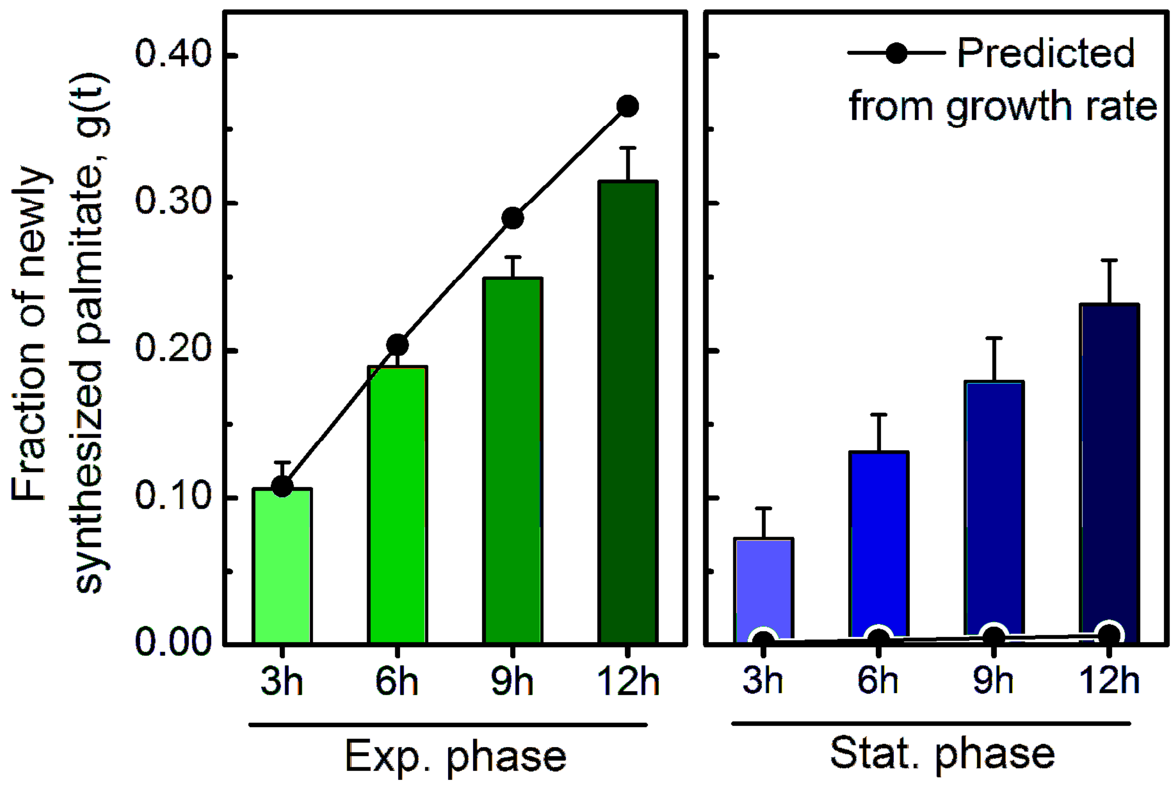
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

^{13}C-Labeling in Lipids



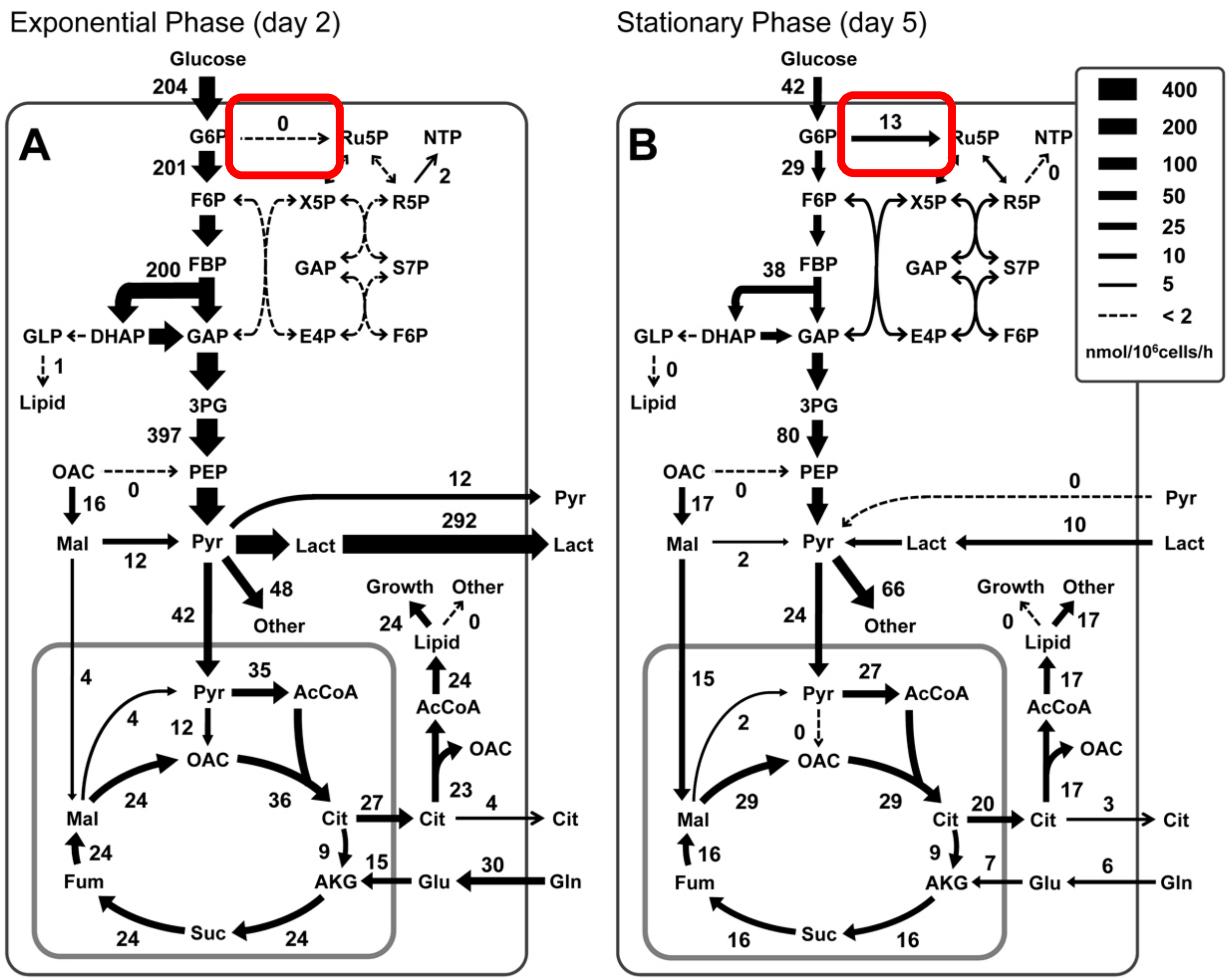
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

De novo lipid biosynthesis



Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Validating Pentose Phosphate Pathway



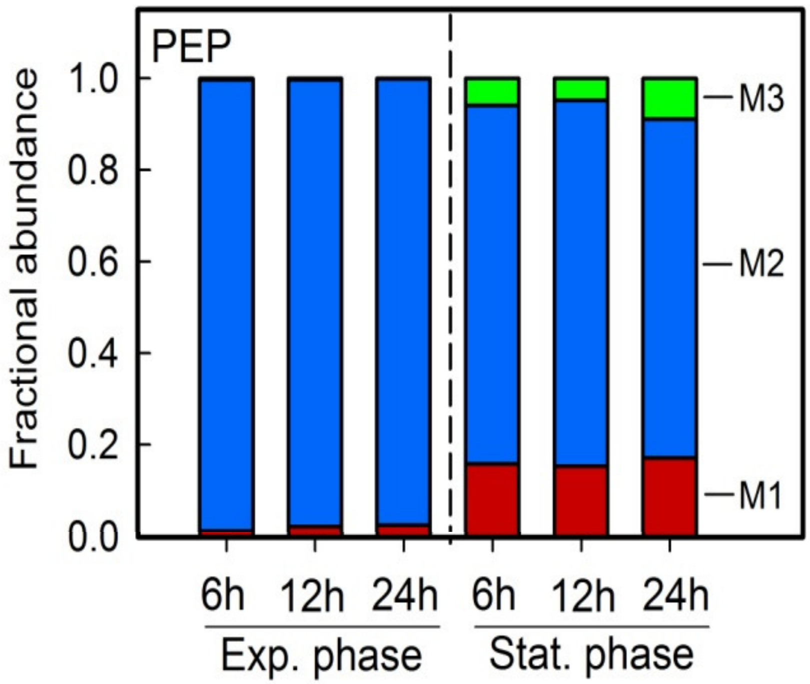
Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

Estimating oxPPP flux using ^{13}C-MFA

- [1,2-^{13}C]glucose tracer
  - Glycolysis produces M2
  - oxPPP produces M1
  - non-oxPPP produces M3

- Exponential growth phase
  - Low oxPPP (1% of glucose)

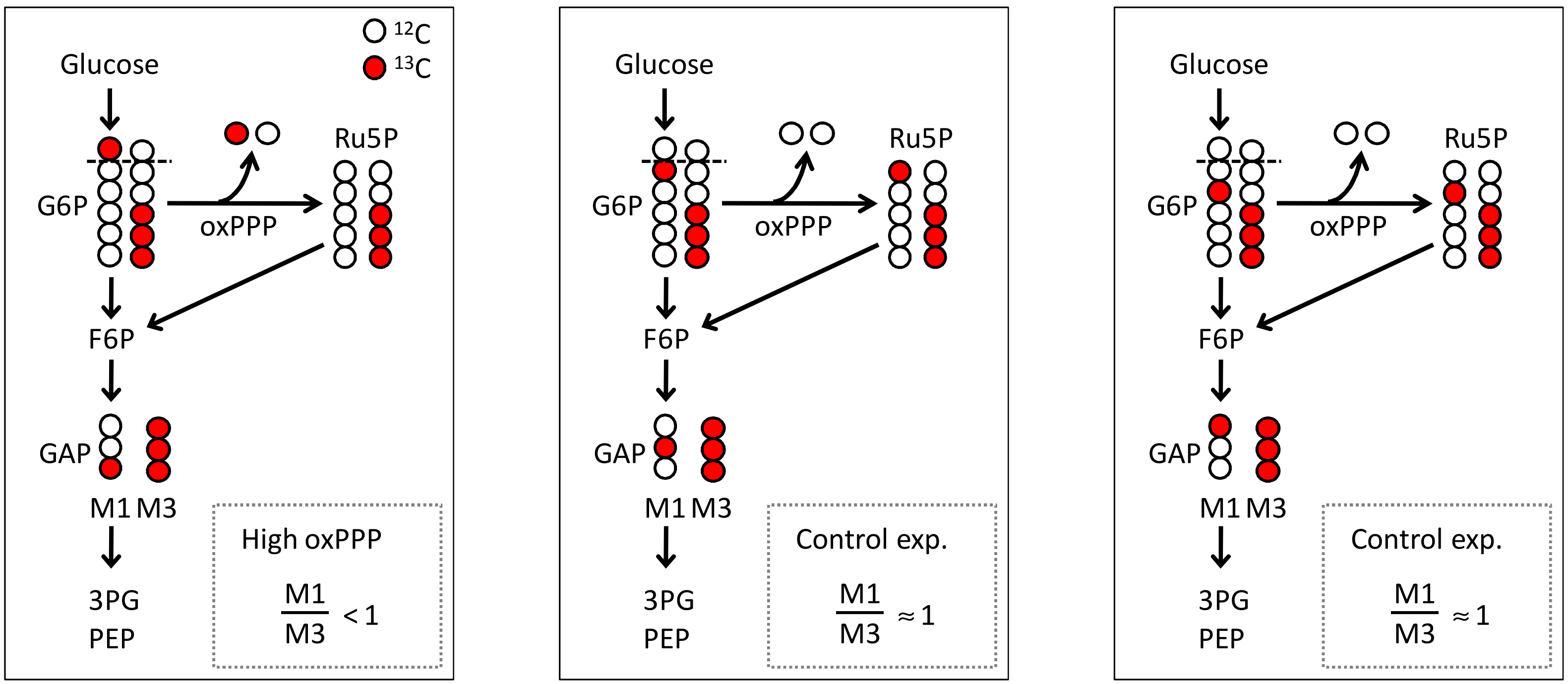
- Stationary phase
  - Increased oxPPP (20% of glucose)



Ahn WS & Antoniewicz MR, Metab Eng (Submitted)

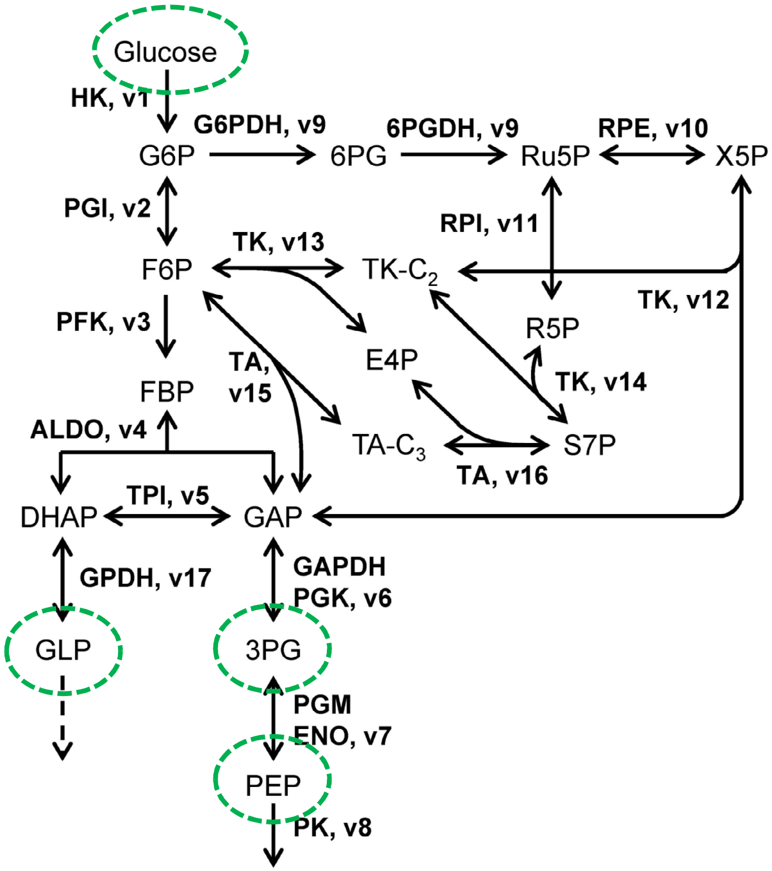
Parallel Labeling Experiments for PPP

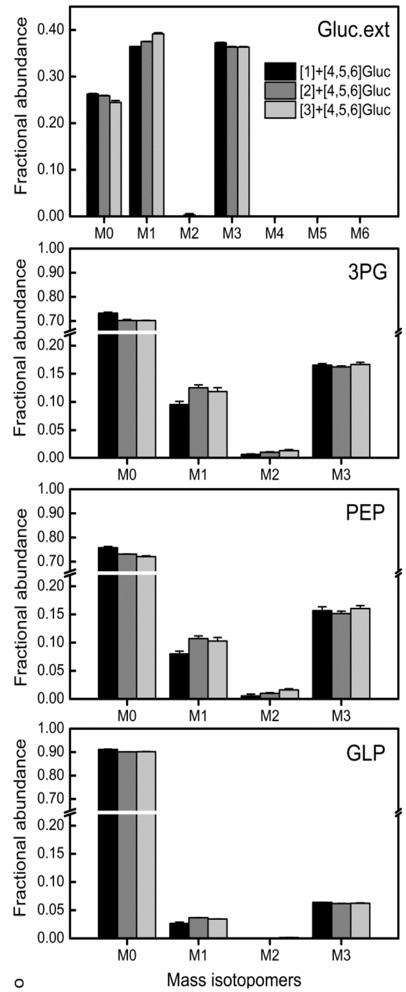
[1]+[4,5,6]Gluc | [2]+[4,5,6]Gluc | [3]+[4,5,6]Gluc



Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

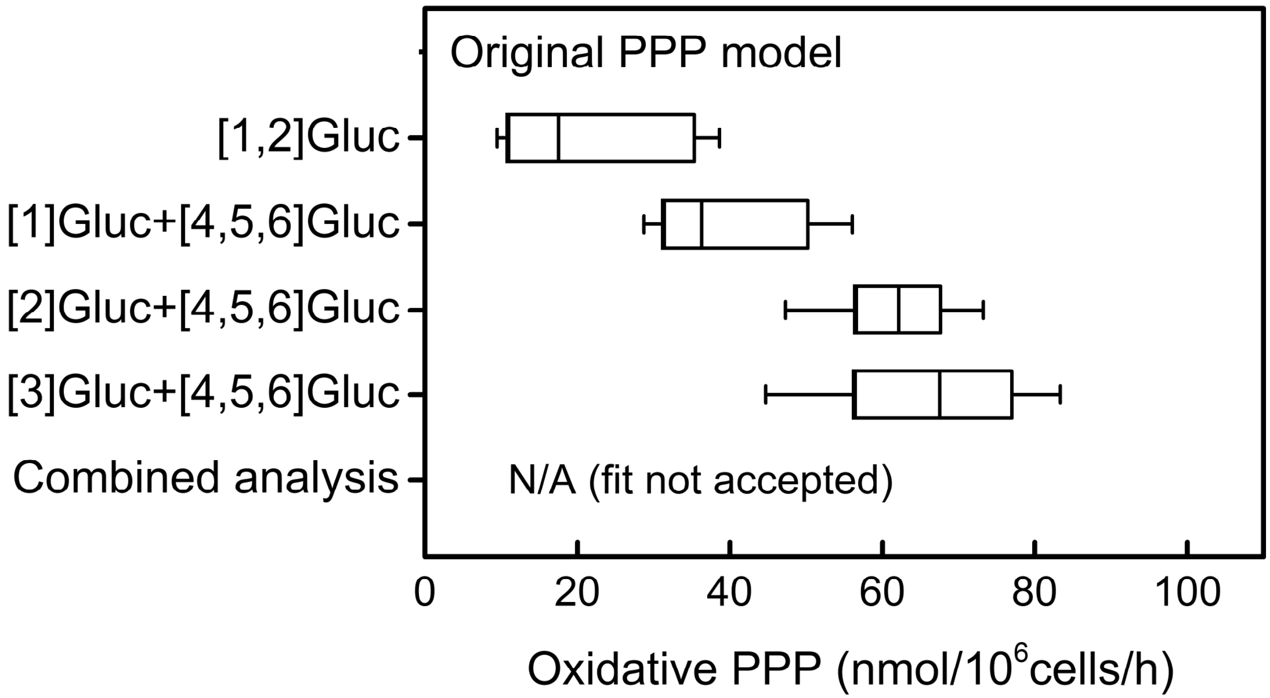
Mass Isotopomer Distributions





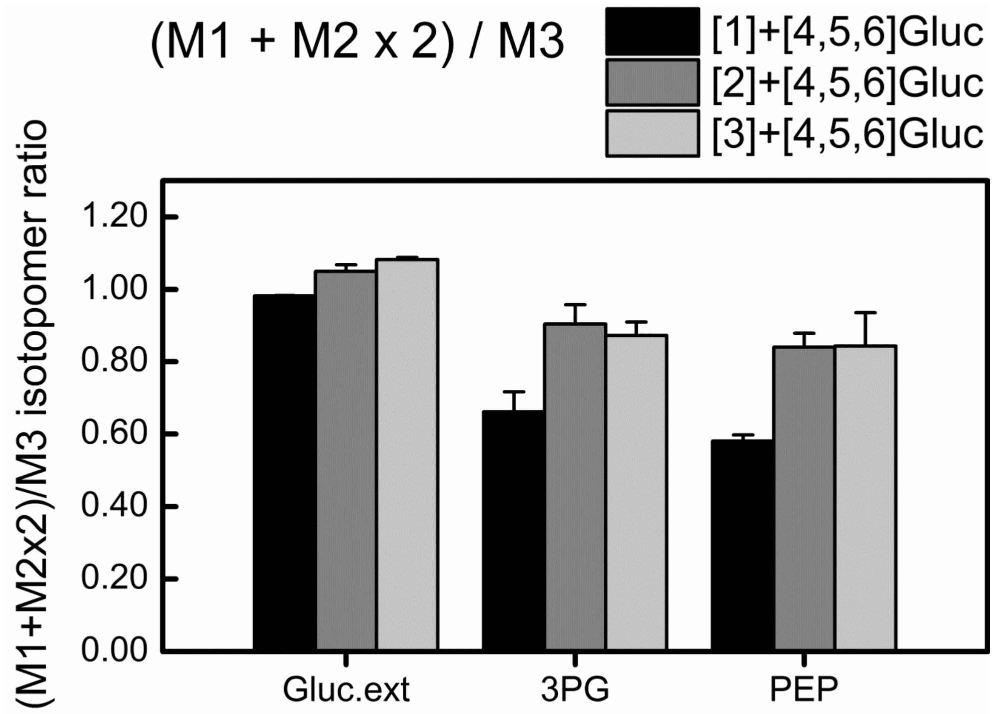
Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

oxPPP estimated with different tracers



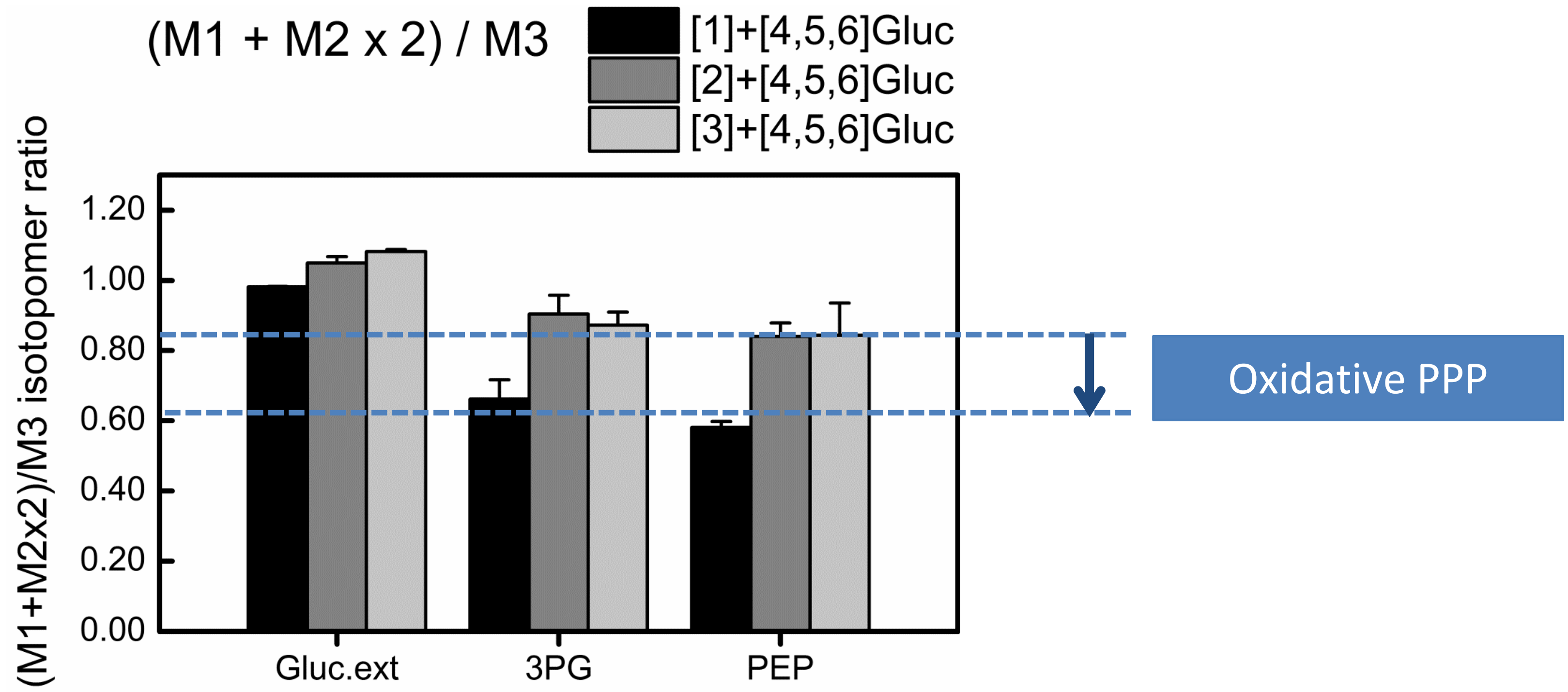
Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

Mass isotopomer analysis



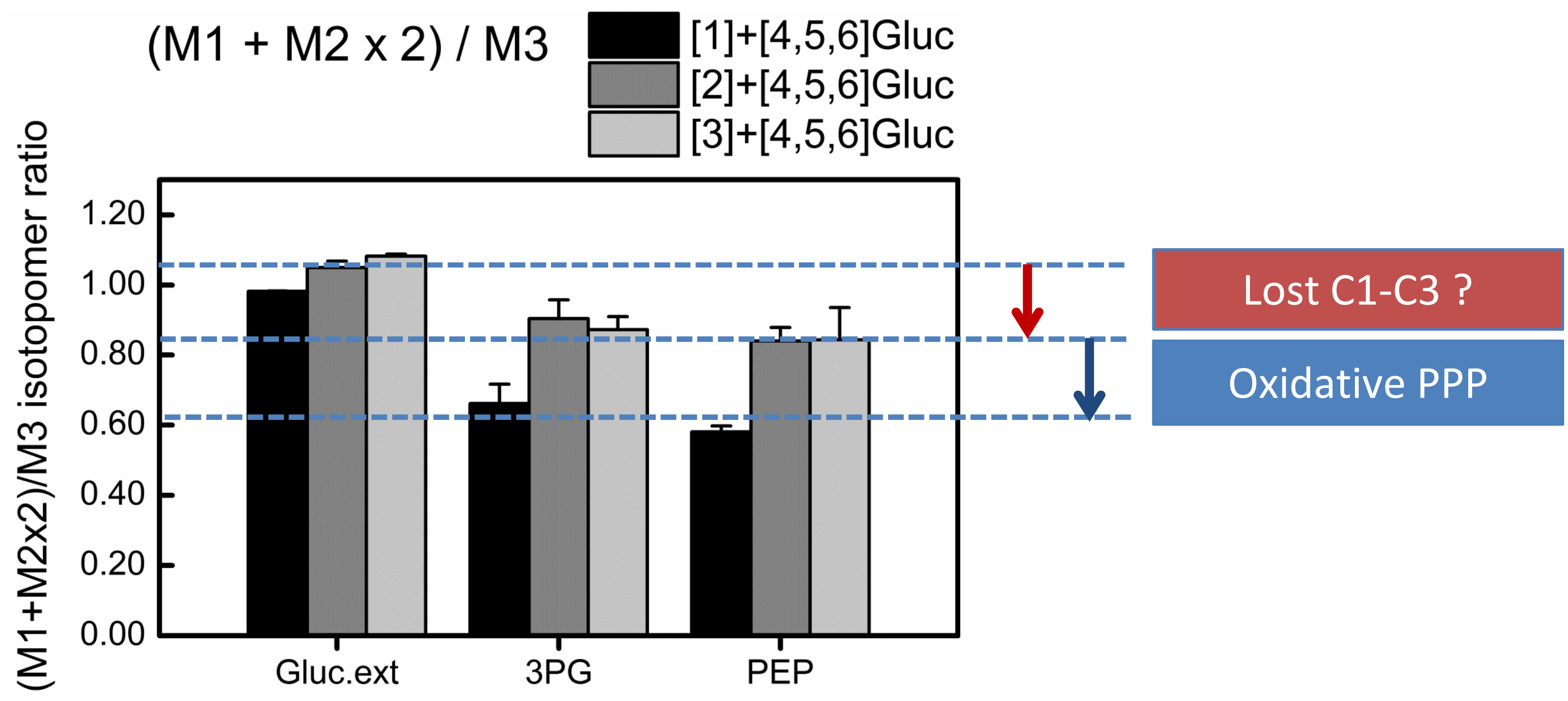
Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

Mass isotopomer analysis



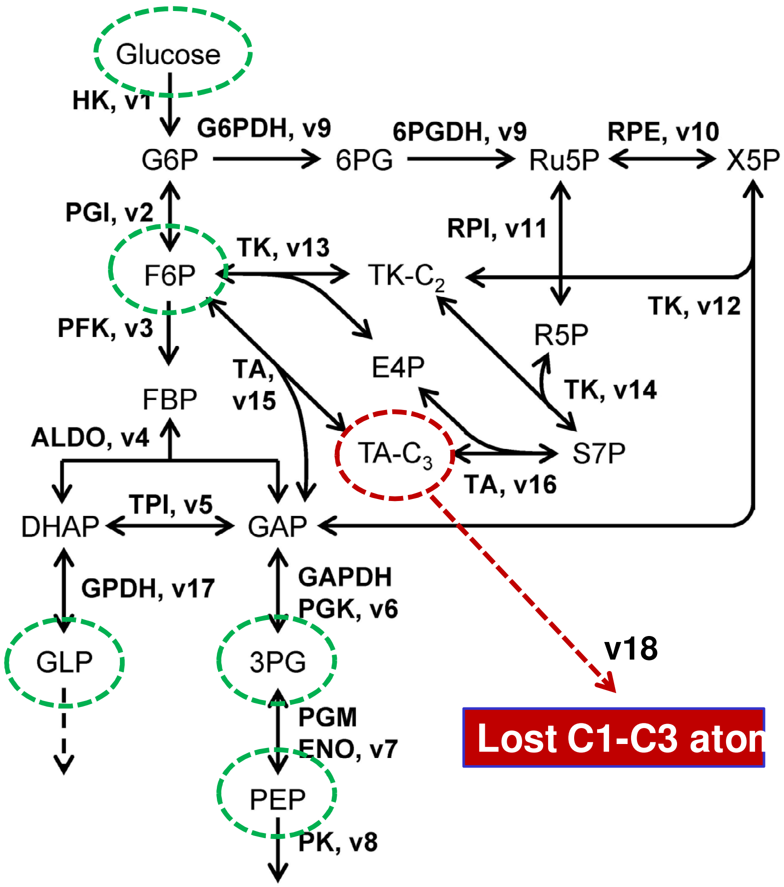
Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

Mass isotopomer analysis



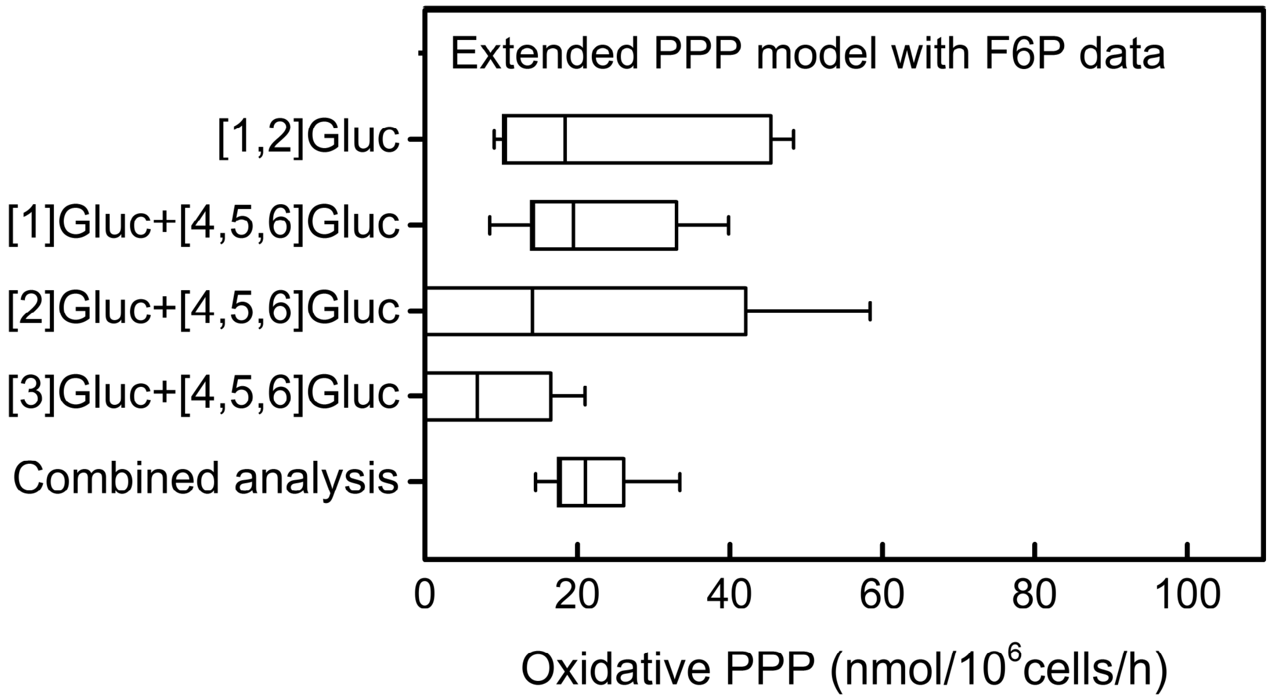
Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

Loss of carbon atoms in non-oxPPP



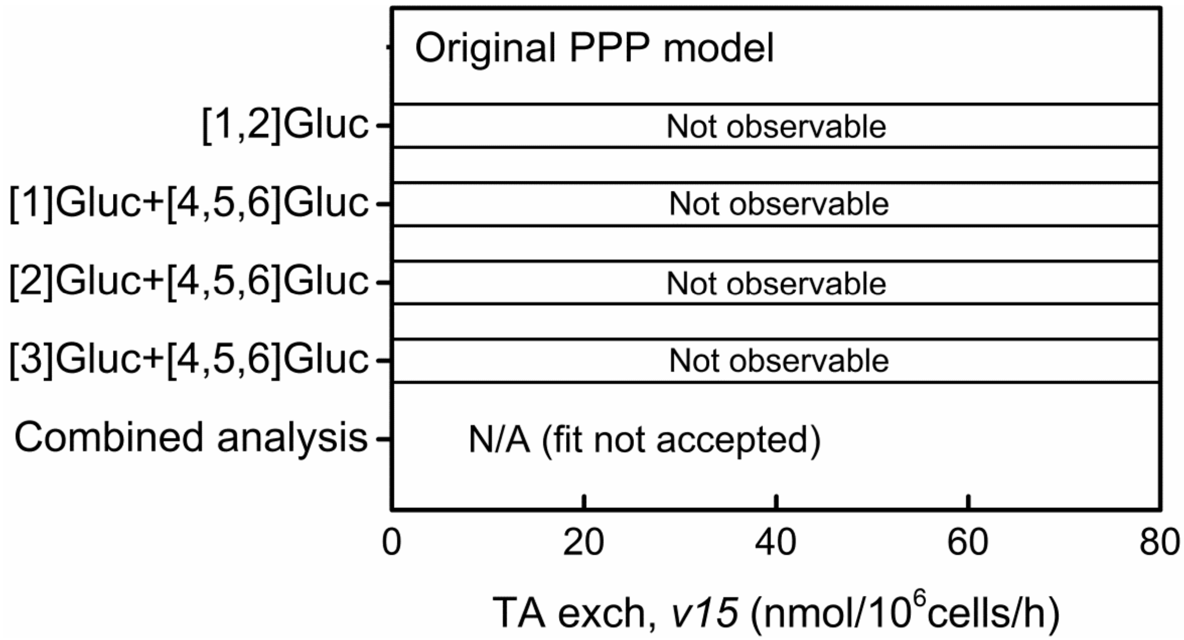
Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

Validated oxPPP flux

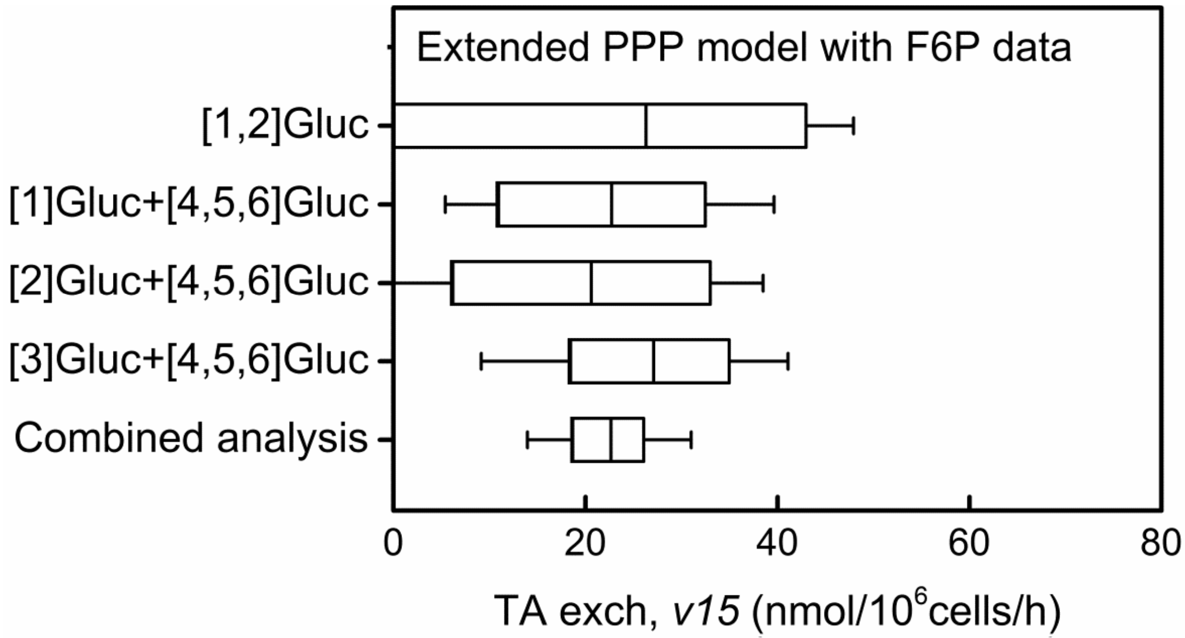


Ahn WS & Antoniewicz MR, J Biol Chem (Submitted)

Reversible non-oxPPP fluxes



Reversible non-oxPPP fluxes



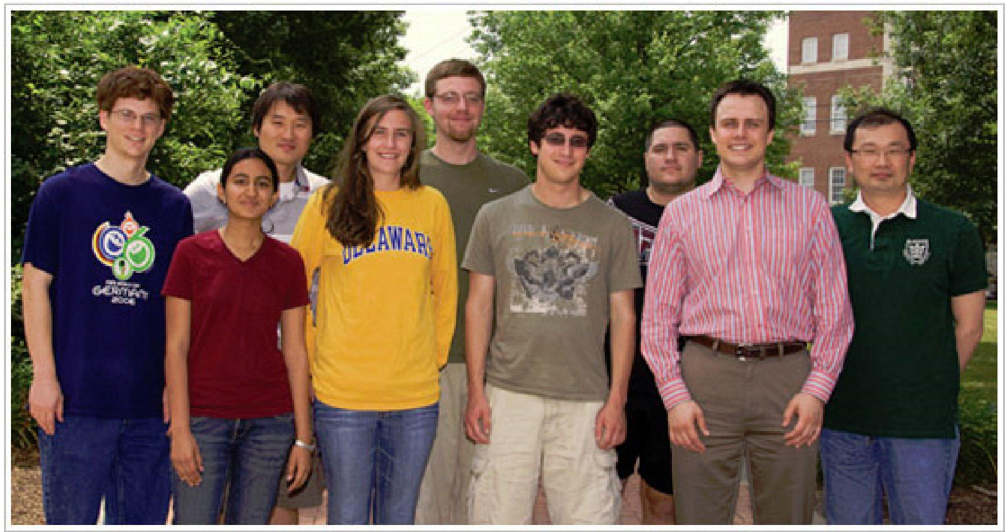
Conclusions CHO cells

- **Flux analysis is only as reliable as the model**
- **^{13}C-MFA for model validation**

- **In the transition from growth phase to stationary phase, CHO cells dramatically changed metabolism**

- **New insights into CHO metabolism:**
  - **Oxidative Pentose Phosphate**
  - **Lipid metabolism**
  - **Loss of carbon atoms in glycolysis**

Acknowledgments



Graduate Students

<u>Woo Suk Ahn</u>

Rob Leighty

Aditi Swarup

Jungik Choi

Scott Crown

Undergrad Students

Nick Marze

Carolyn Slusser

Matt Grossbach

Casey DeWoody

Christine Smith

Jing Lu

Funding

DuPont Young Professor Grant

UD Research Foundation

NIH COBRE

NSF Graduate Fellowship

NSF CAREER Award

NSF MCB