Metabolic Flux Mapping

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Flux

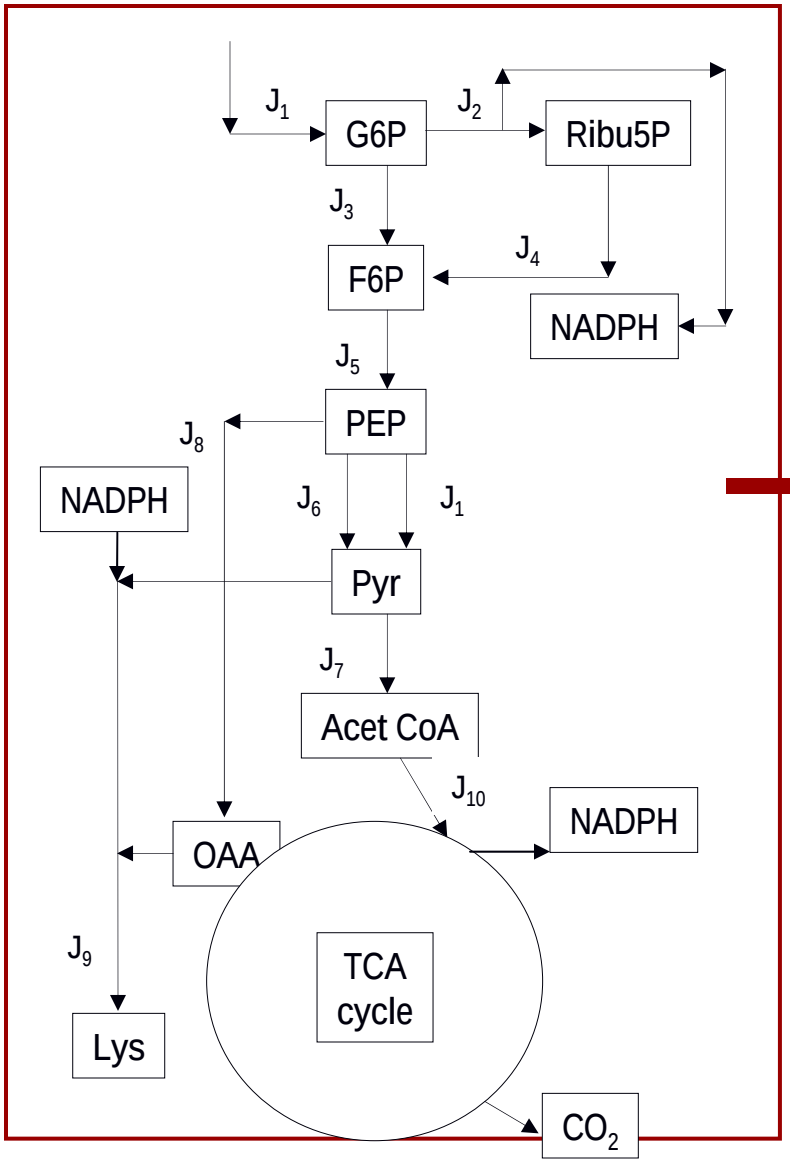
$$A \xrightarrow[J_1]{E_1} B \xrightarrow[J_2]{E_2} C$$

- Enzyme - E -- the biocatalyst
- Metabolic flux - *J*
  - Net rate of conversion of one metabolic precursor to a product
  - Based upon a material balance:

    $d[B]/dt = J_1 - J_2$
- Fluxes reflect integration of genetic and metabolic regulation

Metabolic Flux Analysis

Fluxes are obtained using material balances



$$\frac{d[\mathrm{G6P}]}{dt} = J_1 - J_2 - J_3$$

$$\frac{d[\mathrm{Ribu5P}]}{dt} = J_2 - J_4$$

$$\ldots$$

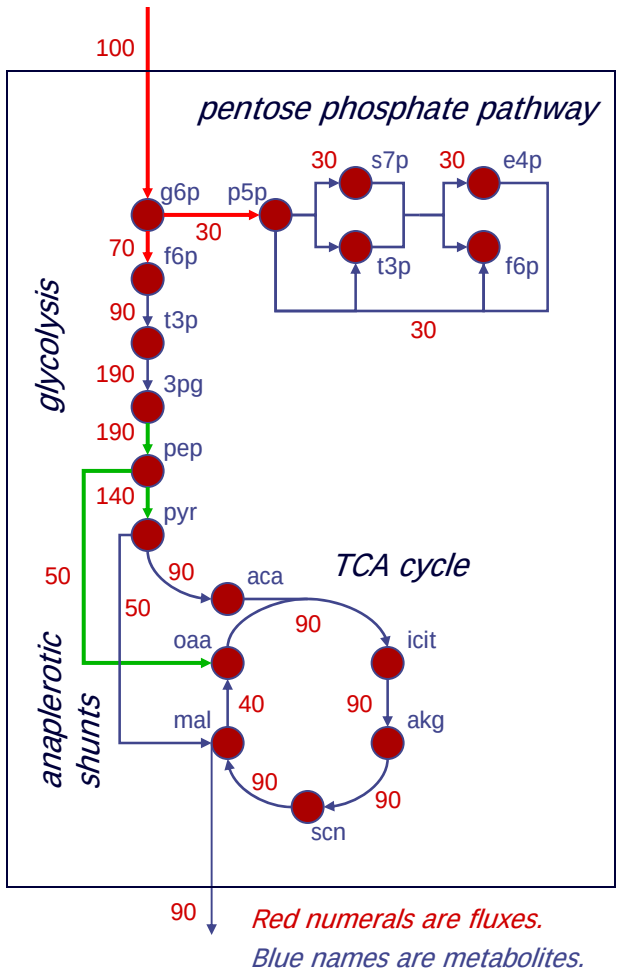
$$\frac{d[\mathrm{NADPH}]}{dt} = 2J_2 + J_{10} - 4J_9$$

$$\frac{d\overline{\mathbf{X}}}{dt} = \overline{\overline{\mathbf{A}}} \cdot \overline{\mathbf{J}}$$

assume
quasi -steady state

$$\boxed{\overline{\overline{\mathbf{A}}} \cdot \overline{\mathbf{J}} = \mathbf{0}}$$

Models and measurements



Hypothetical flux map, for purpose of illustration.

Stoichiometric model has *m* unknown fluxes and *n* internal metabolite balances

$m>n$ — more complex networks

$m=n$ — simpler networks

$m<n$

Insufficient number of measurements. Multiple solutions.

Sufficient (or greater) number of measurements. Unique flux distribution.

How do we choose the "true" solution?

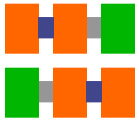
Choose a solution that **optimizes** a logical cellular objective.

Add internal measurements (GC-MS, NMR). Expensive, but more accurate.

GC-MS vs 2D NMR

^{13}C, ^{12}C

GC-MS



1 metabolite

more sensitive

2D NMR

2 metabolites

less sensitive

^{13}C Labeling Data

(from proteinogenic amino acids or metabolites)

Comparative

Bioinformatics

Analytical

Algebraic equations

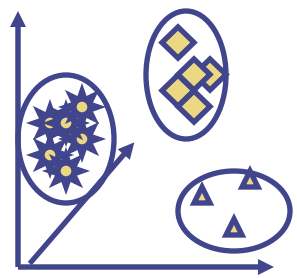
Quantitative physiological data

External fluxes, Biomass composition

Integrated

Iterative isotopomer balancing

Mutant/condition discrimination



Flux ratios (%)

PEP 40%

Krebs cycle 60%

Oxaloacetate

Glycolysis 85%

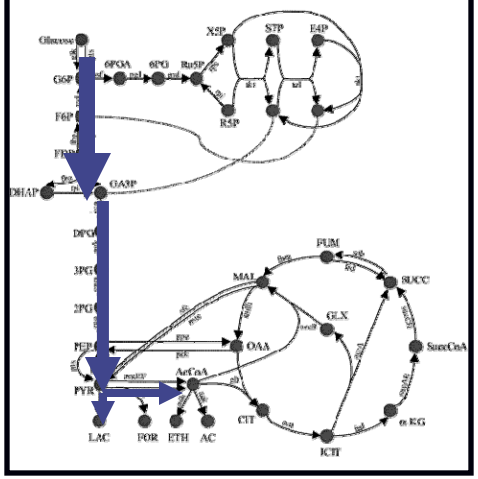
Pentose-P pathway 15%

PEP

etc.

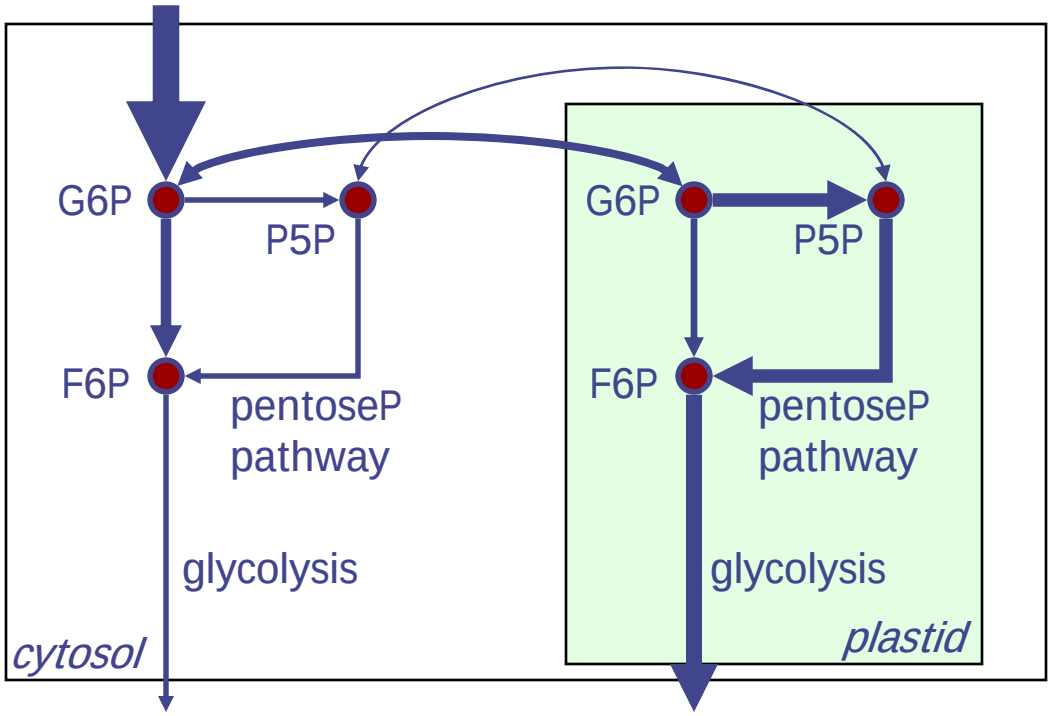
^{13}C-constrained flux analysis

Absolute fluxes (mmol/g h)



Flux estimation is difficult in plants

- Plasticity
  - Compartmentation:
    - Parallel pathways, different fluxes
- Different tissues
- Cell types within tissues



In vitro culture for developing soybean embryos

carbon-labeling experiment

growth chamber

12 DAF

In vitro culture

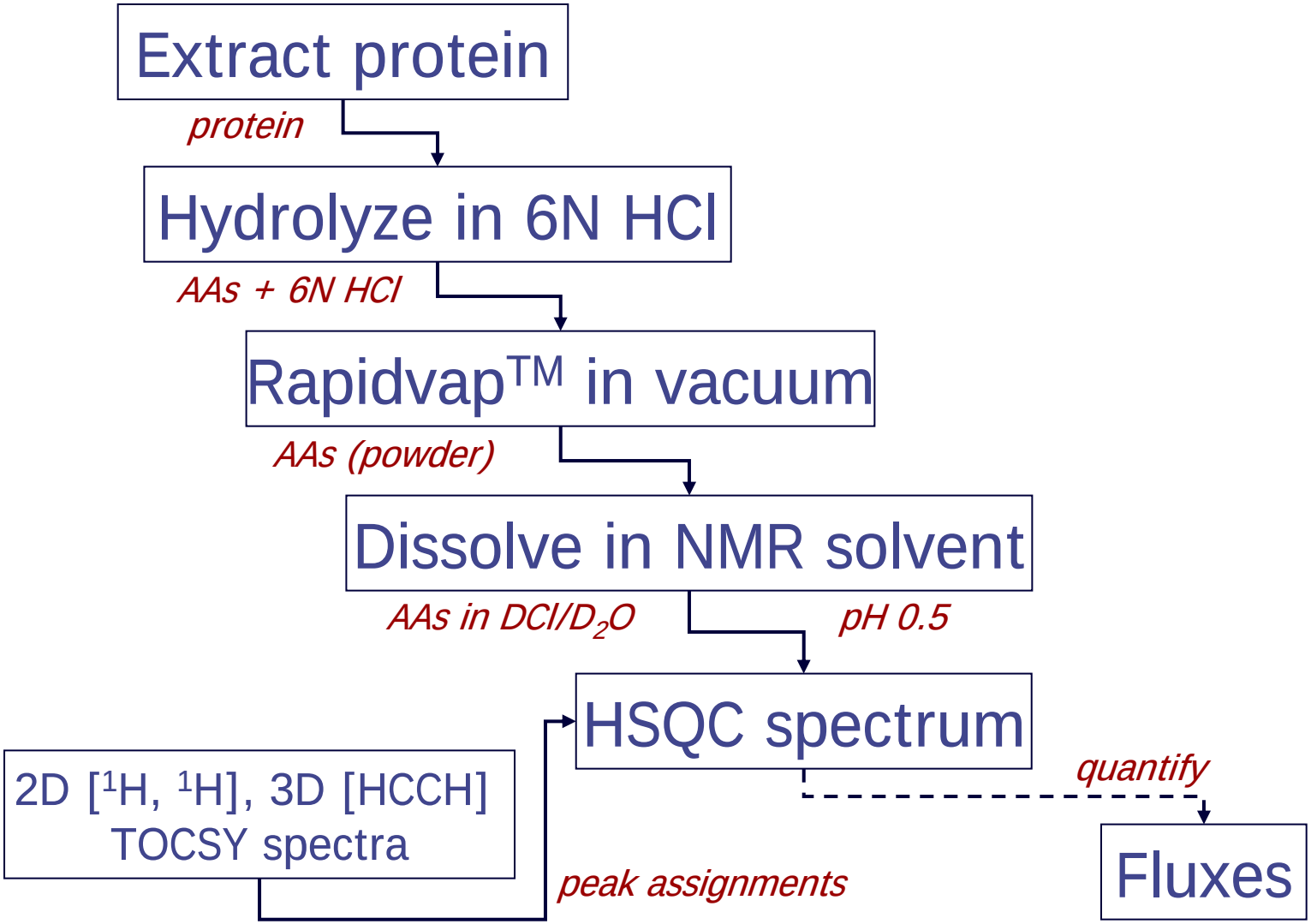
146 mM sucrose (**10% U-^{13}C sucrose**)
37 mM glutamine
vitamins, micronutrients, pH=5.5)

6 days

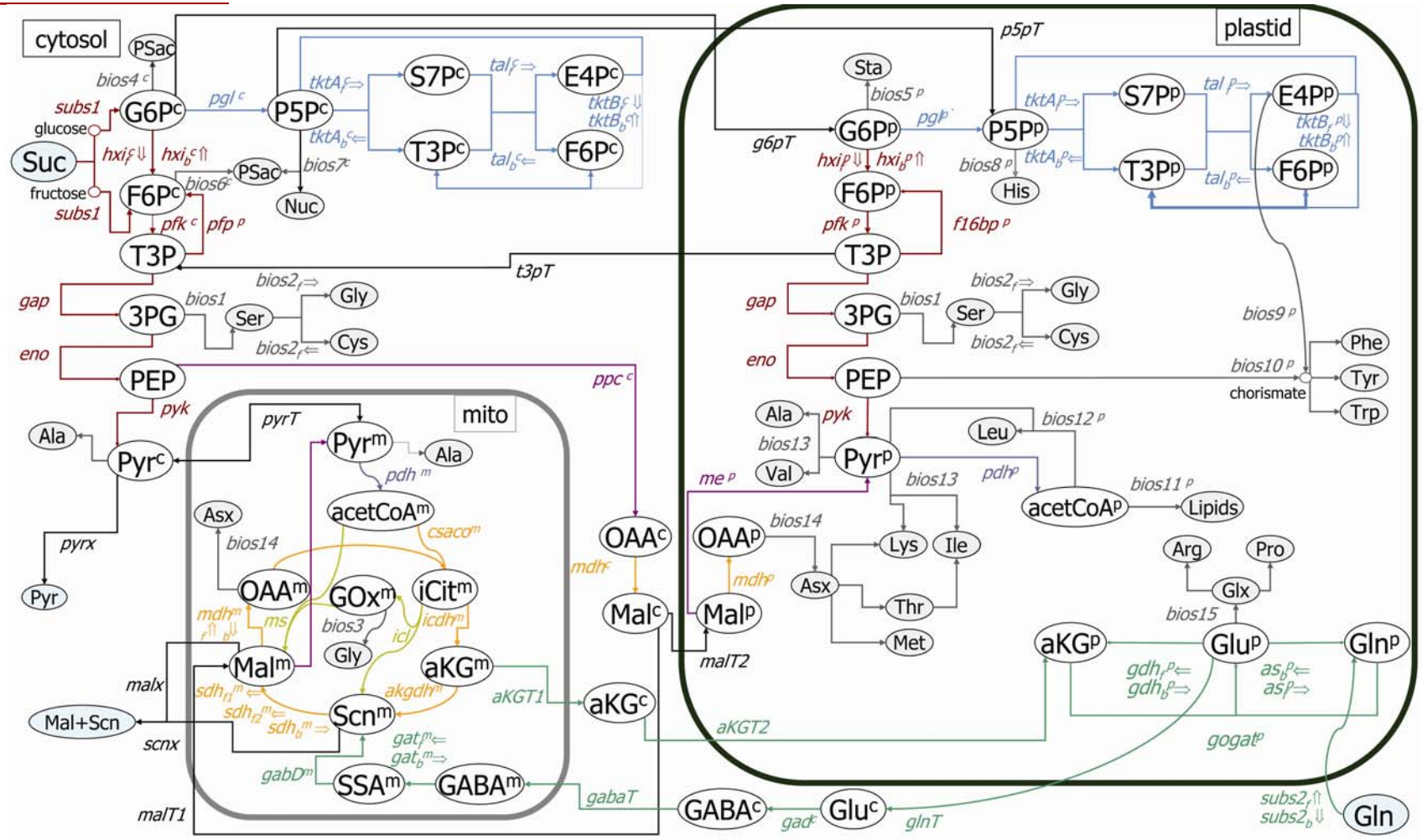
- Extract protein, starch, oil.
- Obtain NMR spectrum of hydrolyzed protein and starch
- Analyze spectrum, use mathematical model to obtain metabolic fluxes.

- Biomass accumulation
- Substrate and product rates
- Biomass composition
- HPLC analysis of protein

Sample Preparation

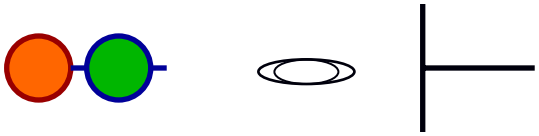


Metabolic network

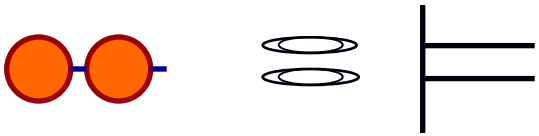


(Recent literature; Singh, 1999; Soybase; KEGG encyclopedia)

^{13}C fine structures

^{13}C attached to ^{12}C  singlet

Most likely formed by a biosynthetic bond between a ^{13}C and a ^{12}C molecule

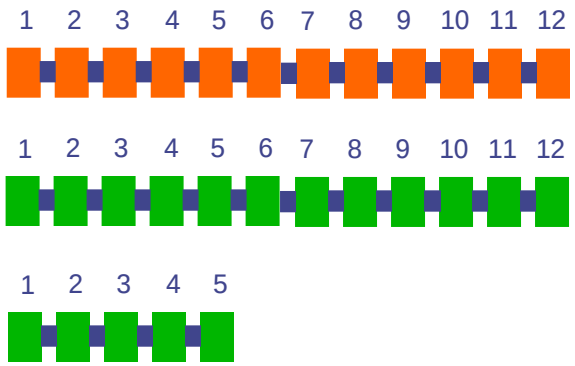
^{13}C attached to ^{13}C  doublet

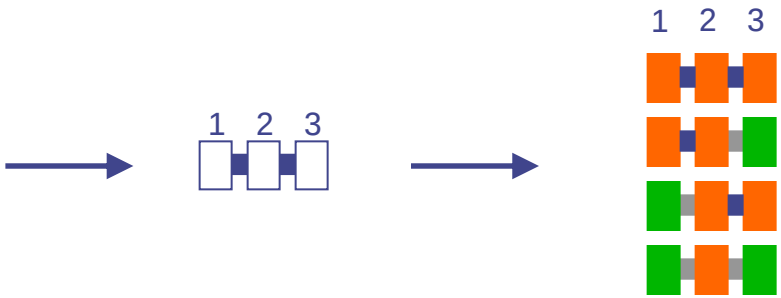
Most likely formed from an intact ^{13}C molecule

Different metabolic histories

Relative abundance of doublets and singlets represents the relative concentrations of intact and biosynthetic bonds in the same metabolite molecule (e.g. different pathways)

Isotopomer

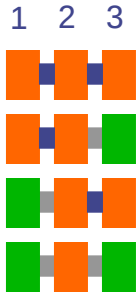




substrate mixture
sucrose (^{13}C, ^{12}C) + glutamine (^{12}C)

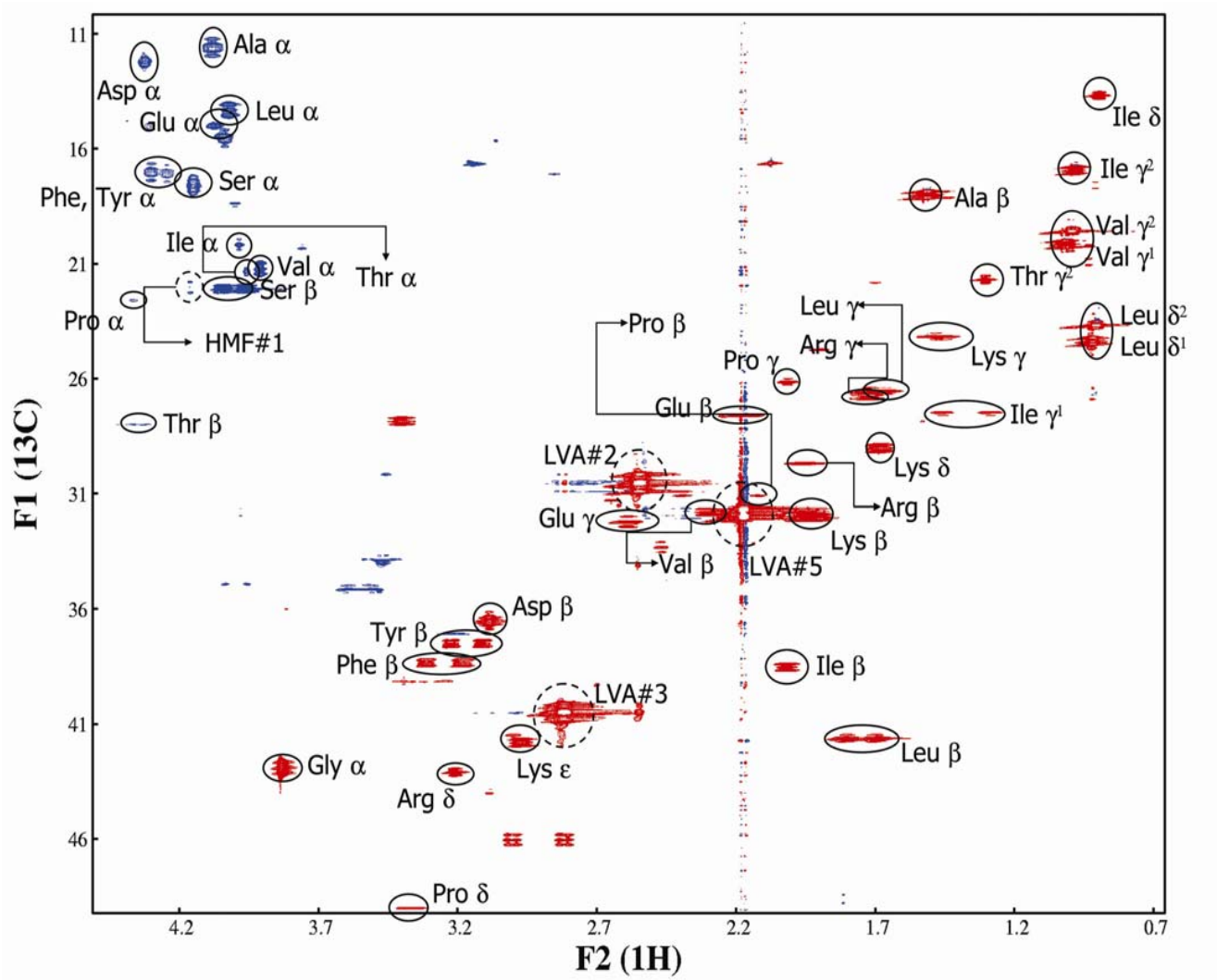
metabolite
e.g. T3P

Isotopomers of T3P



1-1-1, $T3P_7$
1-1-0, $T3P_6$
0-1-1, $T3P_3$
0-1-0, $T3P_2$

2-D [^{13}C,^{1}H] HSQC spectrum

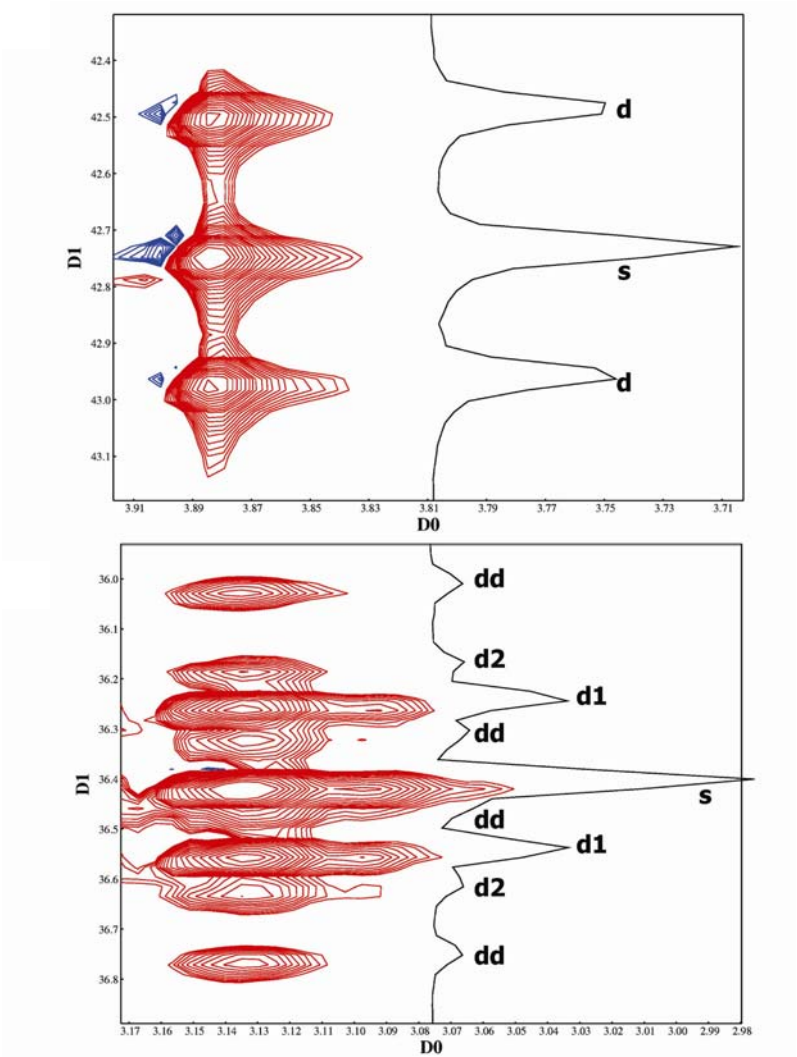


Multiplets and isotopomers

Peak fine structure shows multiplets

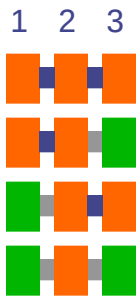
Multiplets are proportional to isotopomers

- singlet
  - ^{12}C-^{13}C-^{12}C
- doublet
  - ^{12}C-^{13}C-^{13}C
  - ^{13}C-^{13}C-^{12}C
- double doublet
  - ^{13}C-^{13}C-^{13}C



Flux evaluation methodology

- 156 isotopomers were measured
- Isotopomer abundances are related to fluxes
- Relationship is nonlinear and nontrivial to solve
- A computational framework was developed to automatically convert raw data to fluxes



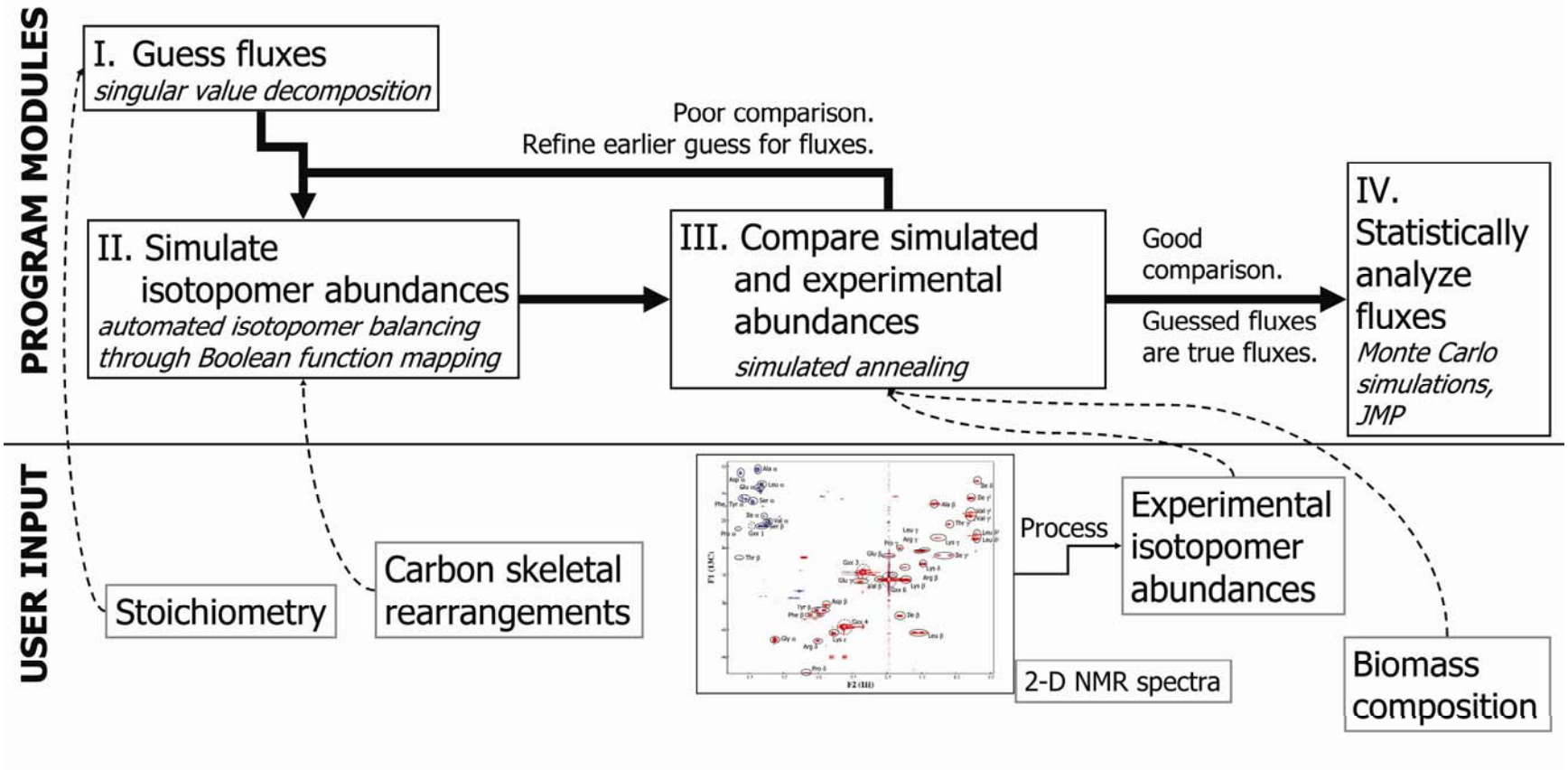
^{13}C-^{13}C coupling indicates intact bonds

^{13}C-^{12}C coupling indicates broken bonds

Natural abundance needs to be considered

Exact models can be constructed

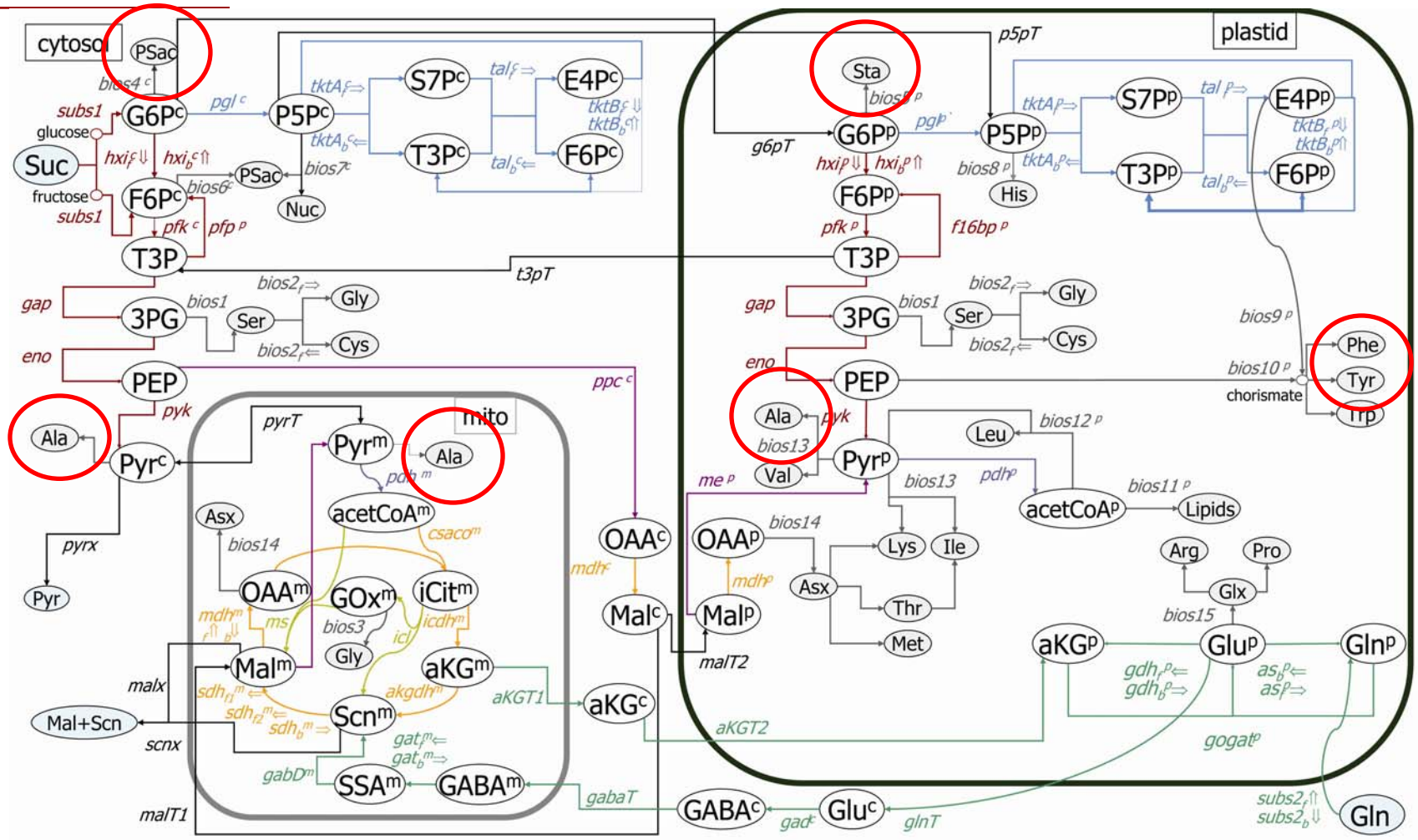
Flux evaluation methodology



Computer program, NMR2flux (C, Red Hat Linux)

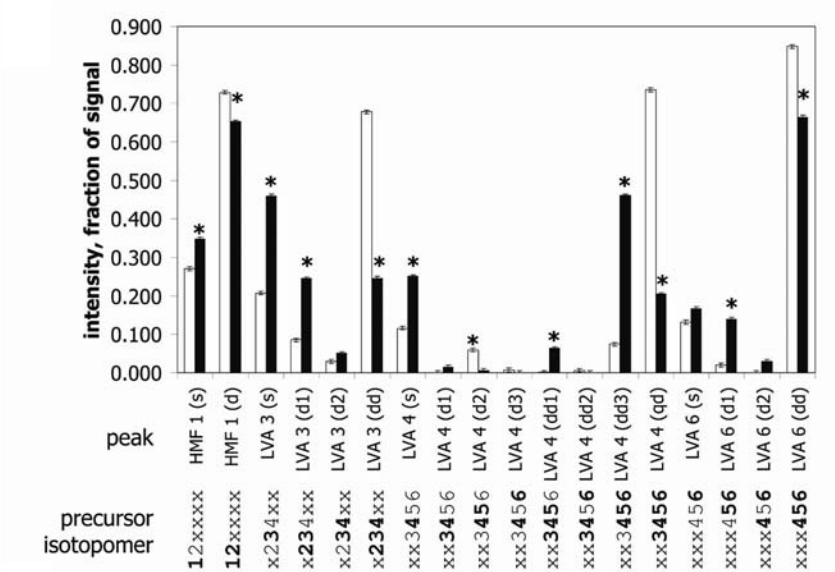
A posteriori model modification is possible

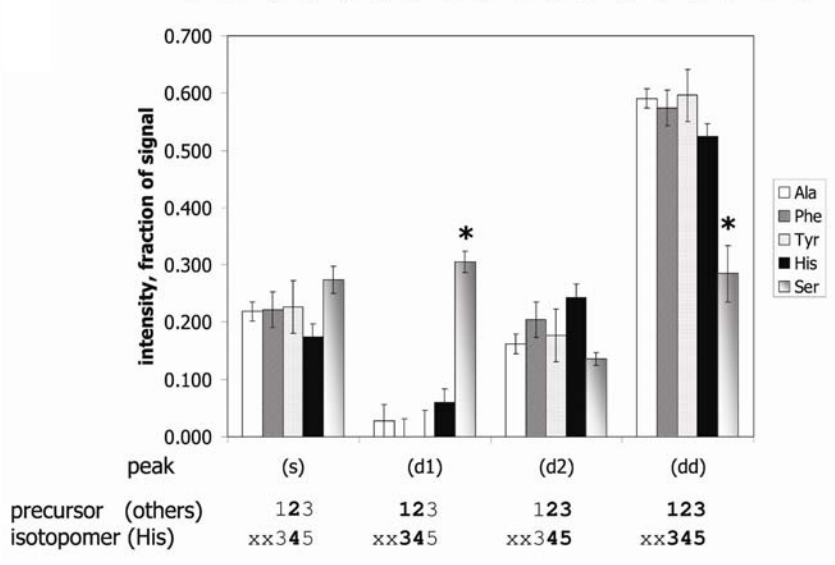
Strategy to identify segregation of pathways



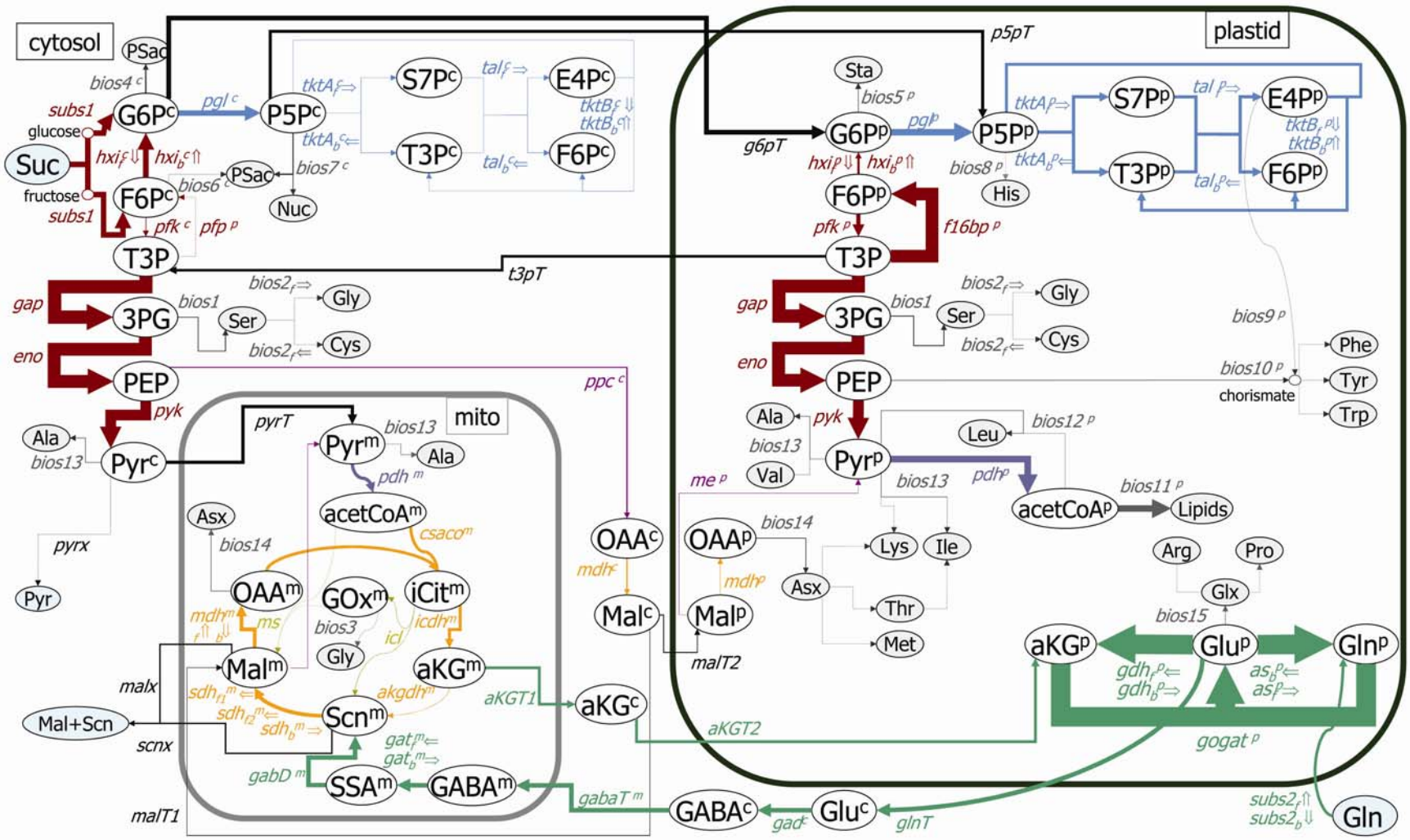
Hexose phosphates are segregated

- Between cytosol and plastid:
  - Hexose pools are isotopically segregated
  - Distinct glycolytic and pentose phosphate pathways exist
  - Triose pools are exchanging rapidly (except Ser)



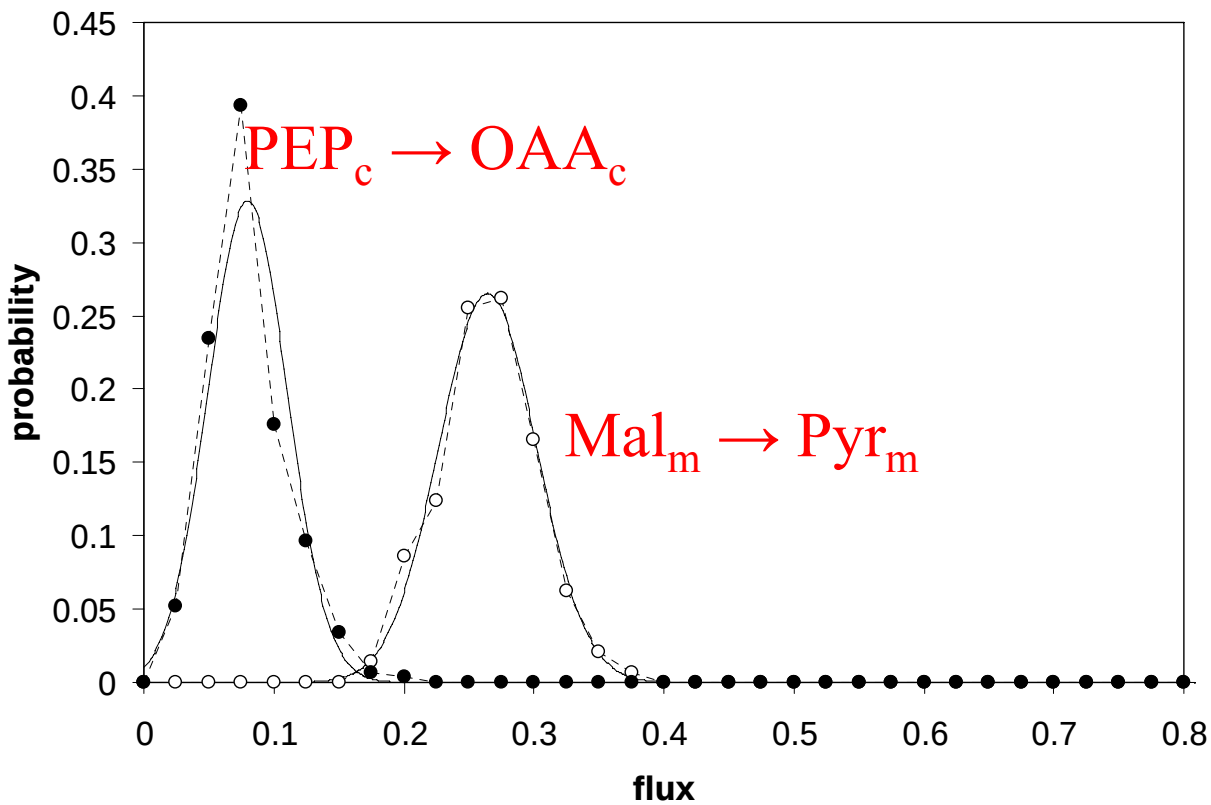


Flux map



Sriram et al.2004. Plant Physiology, 136, 3043-3057.

Statistical analysis of evaluated fluxes

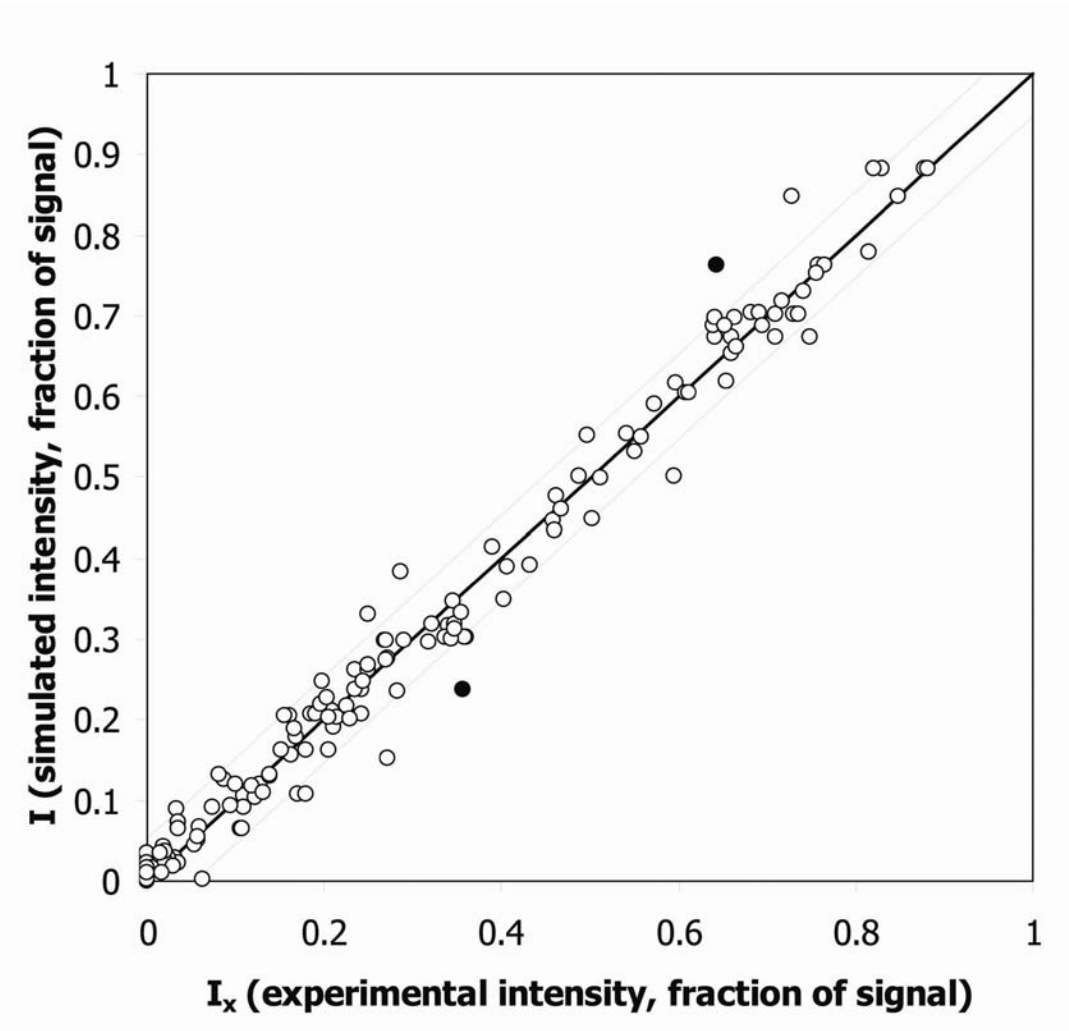


Takes into account experimental error of NMR measurements

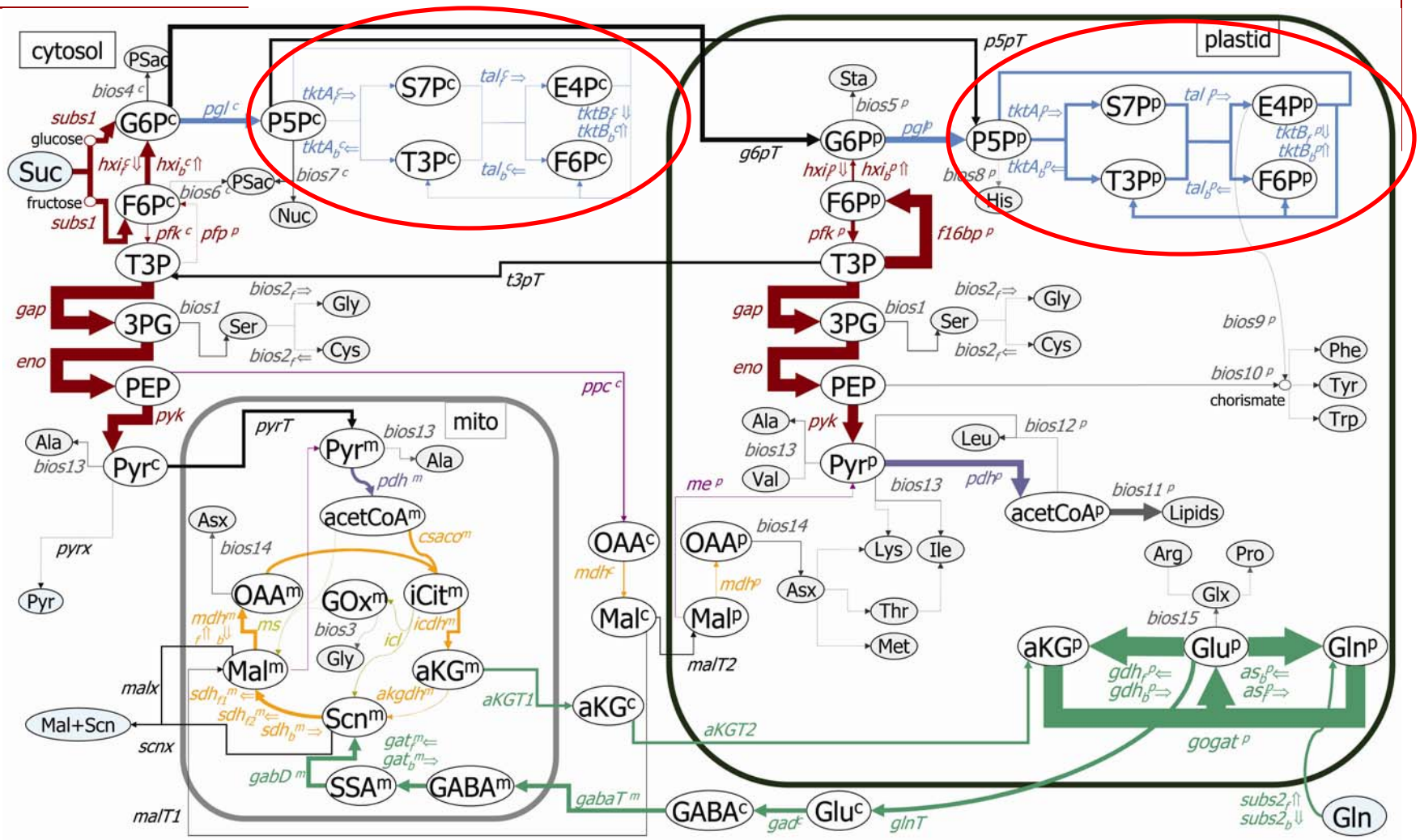
Fluxes, reversibilities, std. deviations

Reaction name	Stoichiometry	Net flux Mean	Net flux SD	Reversibility Mean	Reversibility SD	Comment
Glycolysis and oxPPP						
hxi_f^c	$G6P^c \rightarrow F6P^c$	-113.4	18.0	51.8	26.7	Flux is in the direction $F6P^c \rightarrow G6P^c$
hxi_b^c	$F6P^c \rightarrow G6P^c$					
hxi_f^p	$G6P^p \rightarrow F6P^p$	-43.4	50.2	97.0	3.2	Flux is in the direction $F6P^p \rightarrow G6P^p$
hxi_b^p	$F6P^p \rightarrow G6P^p$					
hxi_f	$G6P \rightarrow F6P$	-156.8	57.3	—	—	Combined *hxi* flux (cytosol+plastid)
pgl^c	$G6P^c \rightarrow P5P^c + CO_2$	107.9	45.7	irrev.	—	
pgl^p	$G6P^p \rightarrow P5P^p + CO_2$	129.3	58.9	irrev.	—	
pgl	$G6P^c \rightarrow P5P + CO_2$	237.0	52.0	irrev.	—	Combined *pgl* flux (cytosol+plastid)
$tktA_f^c$	$P5P^c + P5P^c \rightarrow S7P^c + T3P^c$	7.2	3.0	90.4	17.9	
$tktA_b^c$	$S7P^c + T3P^c \rightarrow P5P^c + P5P^c$					
$tktA_f^p$	$P5P^p + P5P^p \rightarrow S7P^p + T3P^p$	64.6	17.8	88.5	14.6	
$tktA_b^p$	$S7P^p + T3P^p \rightarrow P5P^p + P5P^p$					
tal_f^c	$S7P^c + T3P^c \rightarrow F6P^c + E4P^c$	7.2	3.0	46.7	25.1	
tal_b^c	$F6P^c + E4P^c \rightarrow S7P + T3P^c$					
tal_f^p	$S7P^p + T3P^p \rightarrow F6P^p + E4P^p$	64.6	17.8	76.2	16.1	
tal_b^p	$F6P^p + E4P^p \rightarrow S7P^p + T3P^p$					
$tktB_f^c$	$P5P^c + E4P^c \rightarrow F6P^c + T3P^c$	7.2	3.0	6.7	10.9	
$tktB_b^c$	$F6P^c + T3P^c \rightarrow P5P^c + E4P^c$					
$tktB_f^p$	$P5P^p + E4P^p \rightarrow F6P^p + T3P^p$	60.2	17.8	34.2	30.7	
$tktB_b^p$	$F6P^p + T3P^p \rightarrow P5P^p + E4P^p$					
pfk^c	$F6P^c \rightarrow T3P^c + T3P^c$	14.3	17.3	irrev.	—	
pfk^p	$F6P^p \rightarrow T3P^p + T3P^p$	81.4	20.6	irrev.	—	
pfk	$F6P \rightarrow T3P + T3P$	95.7	16.9	irrev.	—	Combined *pfk* flux (cytosol+plastid)
pfp^c	$T3P^c + T3P^c \rightarrow F6P^c$	3.7	4.3	irrev.	—	
$f16bp^p$	$T3P^p + T3P^p \rightarrow F6P^p$	257.5	59.3	irrev.	—	
gap	$T3P \rightarrow 3PG$	267.4	16.8	n. d.	—	For *gap*, *eno* and *pyk*, cytosolic and
eno	$3PG \rightarrow PEP$	253.5	16.8	n. d.	—	plastidic fluxes are indistinguishable. There-
pyk	$PEP \rightarrow Pyr$	219.4	13.4	n. d.	—	fore, only combined fluxes are reported.
pdh^p	$Pyr^p \rightarrow ACA^p + CO_2$	136.0	< 1.0	irrev.	—	
pdh^m	$Pyr^m \rightarrow ACA^m + CO_2$	66.3	16.0	irrev.	—	

Simulation and experiment



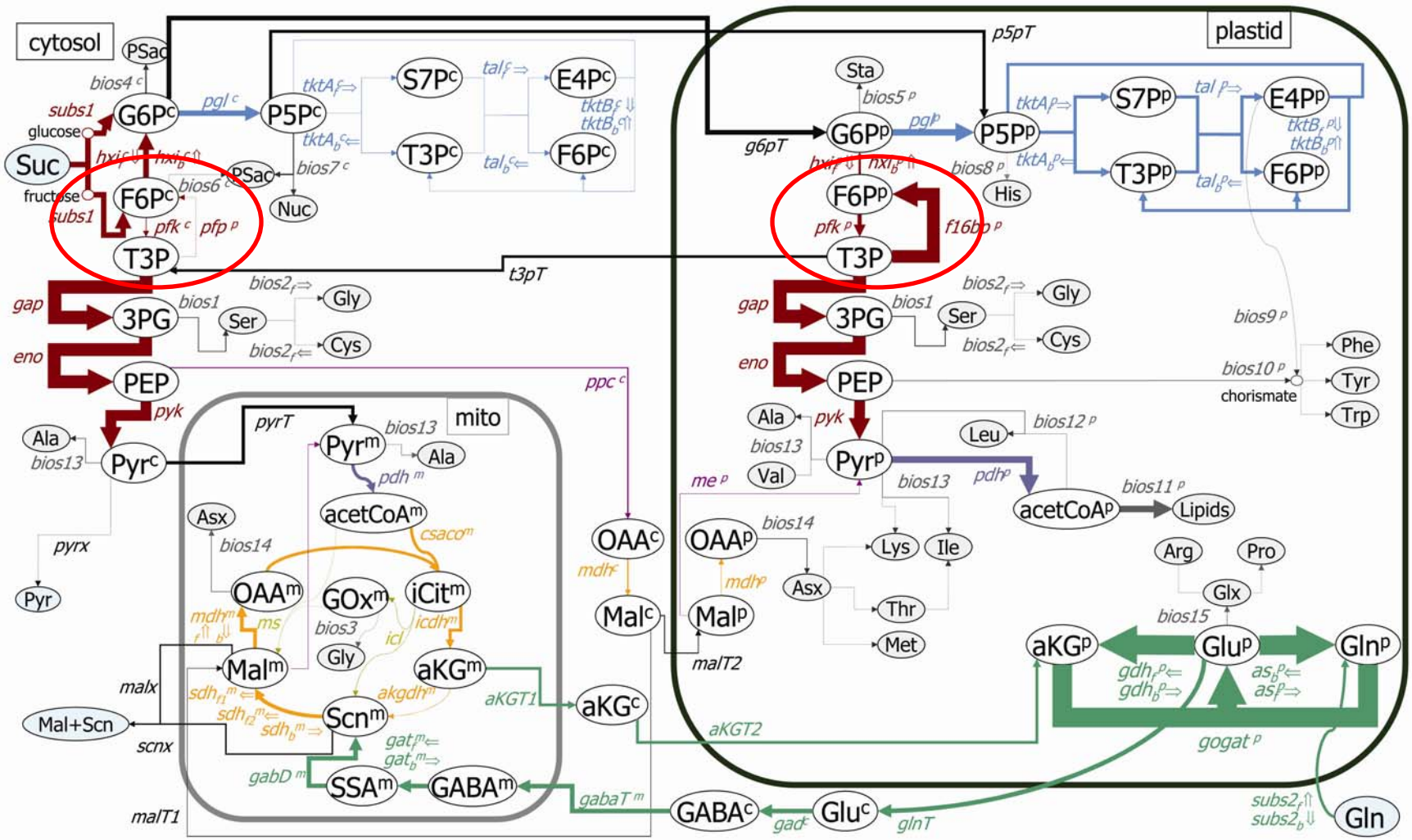
Reductive PPP



Reductive PPP compartmentation

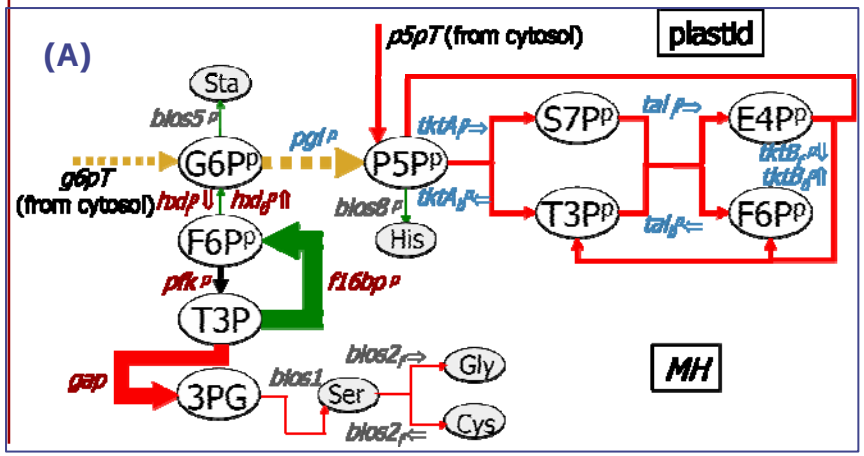
- Reductive PPP fluxes negligible in cytosol (7.4 ± 3.0 units), substantial in plastid (64.6 ± 17.8 units)
- Consistent with genetic and enzymatic data from several higher plants:
  - Arabidopsis genome lacks cytosolic genes for reductive PPP (Eicks *et al.*, 2002)
  - Spinach and pea did not contain cytosolic enzymes for reductive PPP (Debnam and Emes, 1999)
- Reductive PPP may not be present in the cytosol in most plants (Kruger and von Schaewen, 2003)

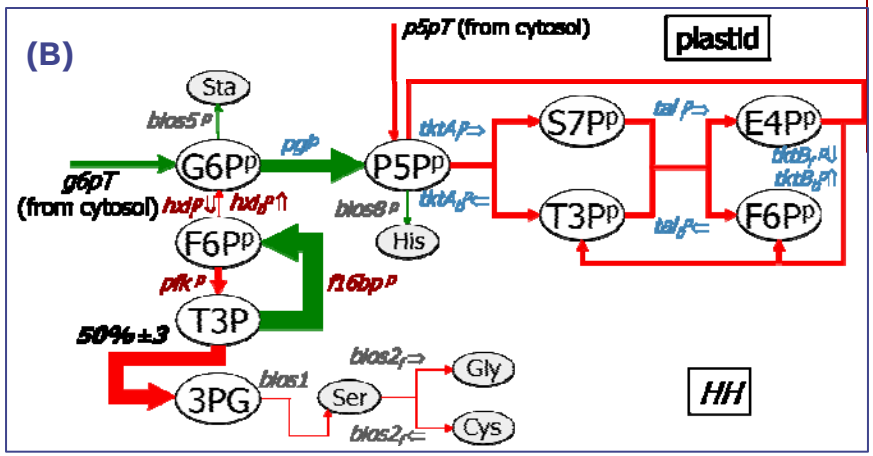
Network Addition of Fructose-1,6-bisphosphatase



A posteriori model modification

Flexibility and Rigidity of Nodes





oxPPP node for MH temp. condition (Case 1- MH, MM, ML)

oxPPP node for HH temp. condition (Case 2- HH, MM, LL)

RIGID

FLEXIBLE

E. coli

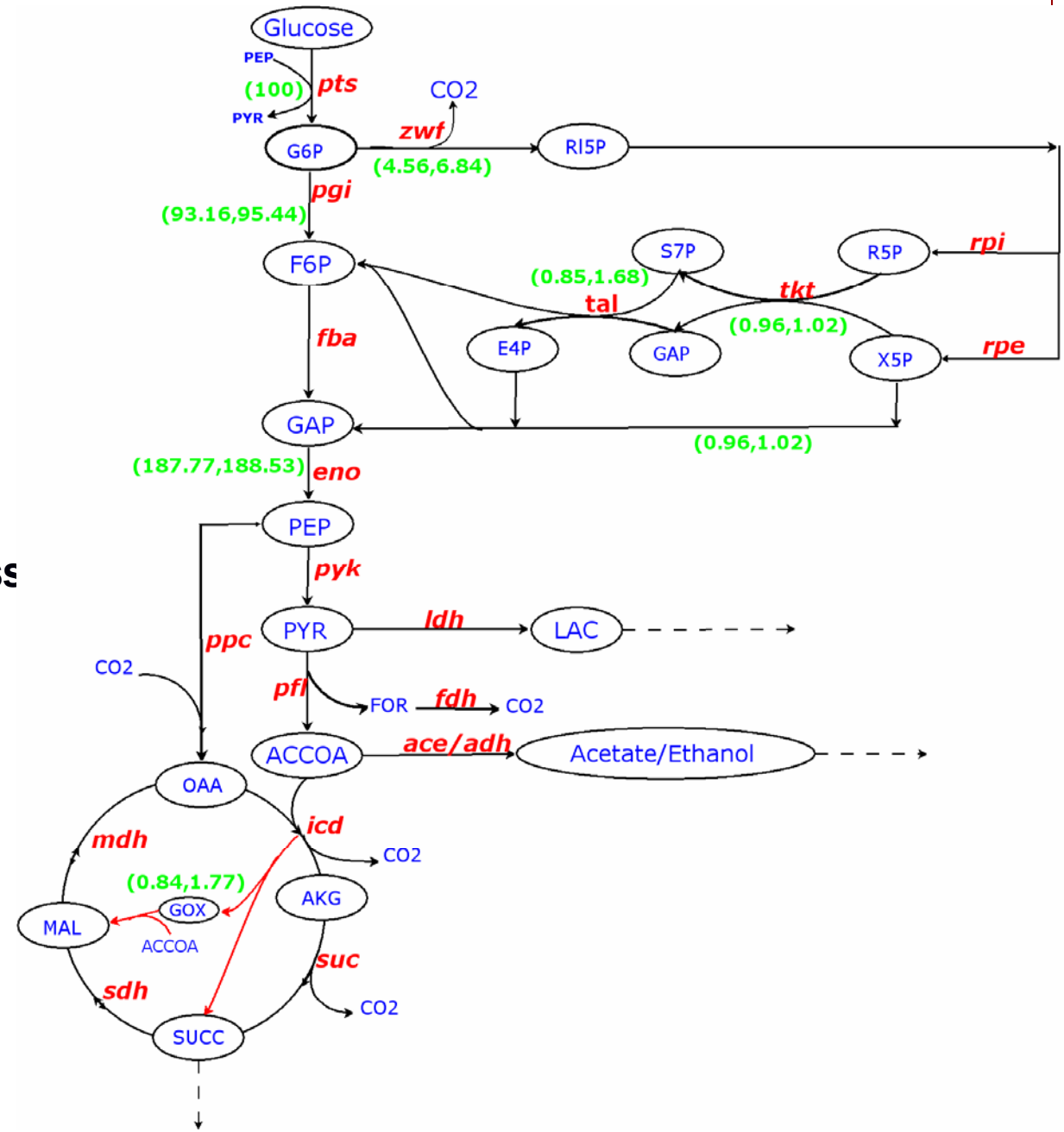
Anaerobic Metabolism

<u>Challenges:</u>

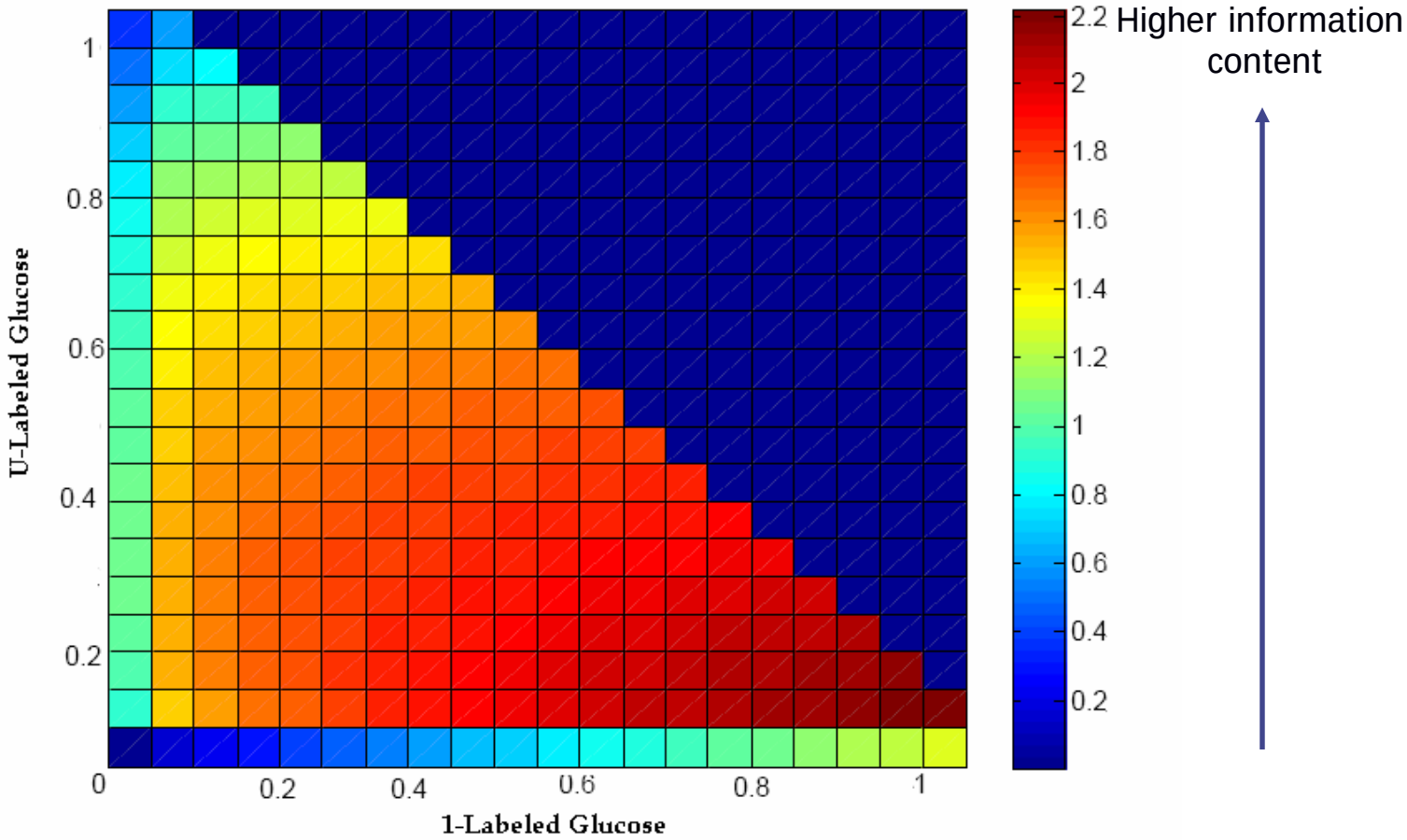
Smaller flux to biomass

Many products

Less rearrangement – TCA cycle incomplete.



Identifiability of fluxes with two labelled compounds



Summary

- Implemented a carbon labeling experiment to evaluate compartmented fluxes
- Developed a computational framework to aid in flux evaluation
  - Computer software (NMR2flux) to evaluate fluxes, reversibilities, and confidence intervals

Larger Networks

- Network Topology
- Extracellular, biomass, composition measurements
- Metabolite profiling/retrobiosynthetic approach
- Effective methods for analyzing isotopomer abundances
- Choice of label for identifiability of fluxes
- Uncertainty or range in flux estimates

Acknowledgments

Flux Map Creation in Plants

- Ganesh Sriram (ChE)
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- Prof. Marty Spalding (GDCB)
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- Dr. Bruce Fulton (NMR Facility)

Metabolic Engineering of E. coli Sugar-Utilization Regulatory Systems

- Prof Ramon Gonzalez, Rice
- Prof. Ka-Yiu San, Rice
- Madhuresh Choudhury (ChE)

Funding: Interagency Program in Metabolic Engineering (NSF,USDA,DOE,EPA)

Funding: NSF, Plant Sciences Institute, Iowa Soybean Board, Cargill

Isotopomer balance

$$0 = \left\{ \begin{array}{l} v_{tal_f^p} \cdot \mathrm{S7P^p}[\mathbf{12}3\mathrm{xxxx}] \cdot \mathrm{T3P^p}[\mathbf{12}3] + v_{hxi_f^p} \cdot \mathrm{G6P^p}[\mathbf{12345}6] \\ + v_{f16bp^p} \cdot \mathrm{T3P^p}[\mathbf{12}3] \cdot \mathrm{T3P^p}[1\mathbf{23}] + v_{tktB_f^p} \cdot \mathrm{P5P^p}[\mathbf{12}\mathrm{xxx}] \cdot \mathrm{E4P^p}[1\mathbf{23}4] \end{array} \right\}$$
$$- \left\{ v_{pfk_b^p} \cdot \mathrm{F6P^p}[\mathbf{12345}6] + v_{hxi_b^p} \cdot \mathrm{F6P^p}[\mathbf{12345}6] + v_{tal_b^p} \cdot \mathrm{F6P^p}[\mathbf{12345}6] + v_{tktB_b^p} \cdot \mathrm{F6P^p}[\mathbf{12345}6] \right\}$$

- Our network had 564 balances, and 33 unknowns.
- However, balances for all isotopomers (whether measured or not) in the metabolic network must be written and solved to calculate fluxes.

Boolean function mapping

- **What:** A method to convert reactant isotopomers to product isotopomers automatically (Sriram G., and J. V. Shanks, 2004, *Metab. Eng*).
- **How:** Metabolic reactions can be represented by the following schema:

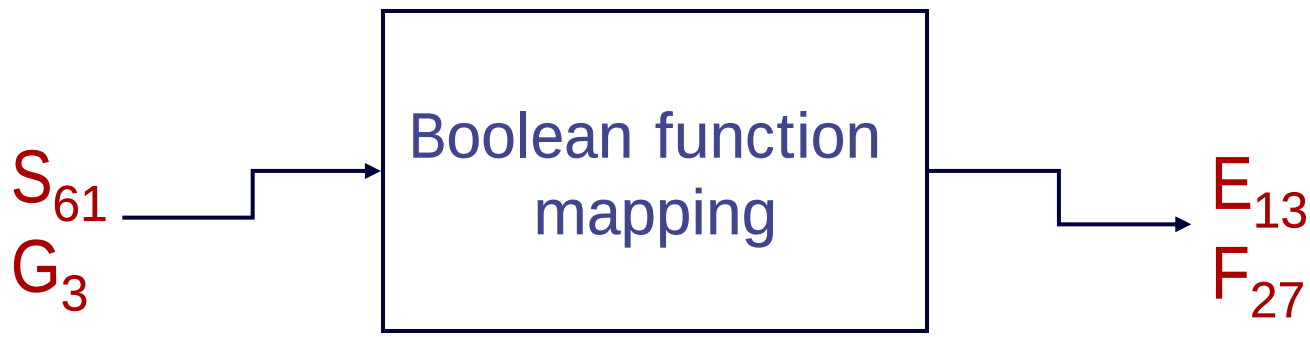
  $R1 + R2 \rightarrow P1 + P2$ (overall reaction)

  $R1^L\text{-}R1^R + R2^L\text{-}R2^R \rightarrow P1^L\text{-}P1^R + P2^L\text{-}P2^R$ (typical carbon skeleton rearrangement)

- We recognize four moves in this schema, which are represented as Boolean functions:
  - Fragmentation $R1 \rightarrow R1^L\text{-}R1^R$
  - Reversal $R1^R \rightarrow \underline{R1^R}$ (occasional, e.g. F6P $\rightarrow$ T3P reaction, fumarase reaction)
  - Transposition $R1^R \rightarrow P2^L$
  - Condensation $P2^L\text{-}P2^R \rightarrow P2$

Example: transaldolase reaction

- Assume reactant isotopomers are S_{61} (0111101) and G_3 (011)
- Manual isotopomer enumeration shows that **E_{13} (1101)** and **F_{27} (011011)** should result.



- 67% time reduction compared to existing methods (Wiechert *et al.*, 2001).
- Time/iteration (soybean network) = 0.48 s on a 3 GHz pentium.

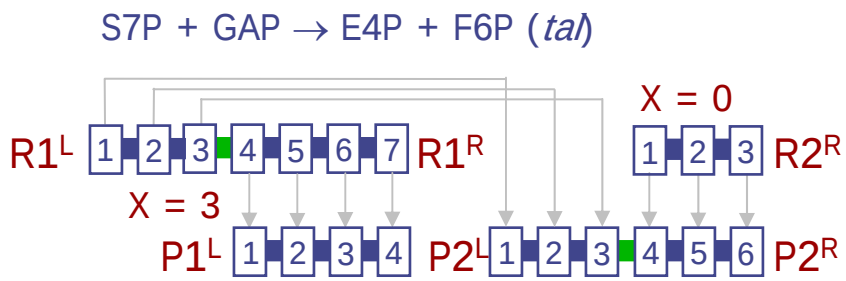
Sriram, G., and Shanks, J. V., 'Improvements in metabolic flux analysis using carbon labeling experiments: bondomer balancing and Boolean function mapping.' Metab. Eng., 2004.

return

Boolean function mapping

Sriram, G., and Shanks, J. V., 'Improvements in metabolic flux analysis using carbon labeling experiments: bondomer balancing and Boolean function mapping.' Metab. Eng., 2004

- In the *tal* reaction, let us assume we have S_{61} (0111101) and G_3 (011) as the reactants. From inspection, we should expect to get **E_7 (1101)** and **F_{27} (011011)**.



- When the Boolean/arithmetic functions are executed, we get:

  Fragmentation: $R1^L$ = 61 / 2^4 = 3 (011), $R1^R$ = 61 % 2^4 = 13 (1101);

  $R2^L$ = NULL, $R2^R$ = 3 (011)

  Transposition: $R2^R$ = 3 (011) becomes $P2^R$ = 24 (00001); 3 (<<) 3 = 24

  Note: Now $P1^L$ = $R1^R$ = 13, $P1^R$ = 0; $P2^L$ = $R1^L$ = 3, $P2^R$ = 24.

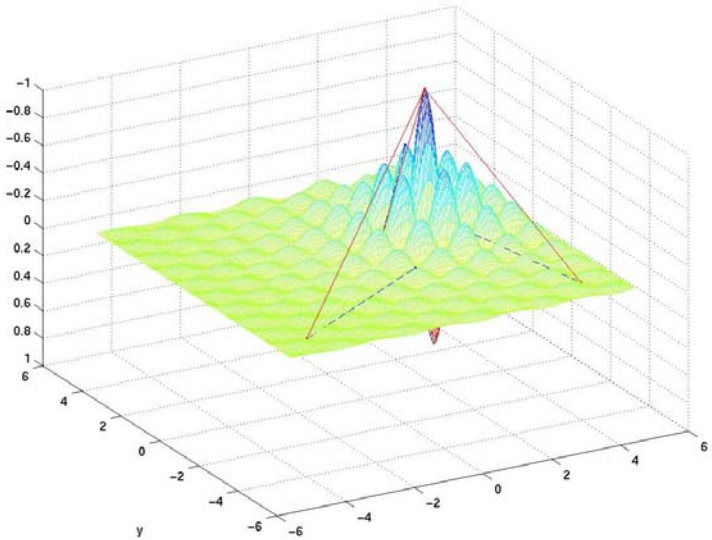
  Condensation: P1 = $P1^L$ + $P1^R$ = 13 + 0 = 13 **(E_{13} = 1101)**;

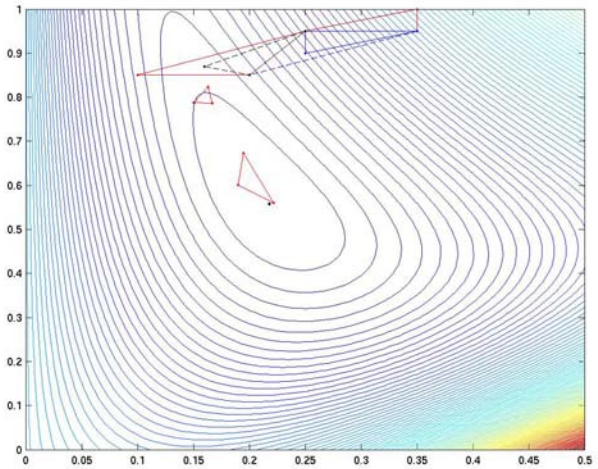
  P2 = $P2^L$ + $P2^R$ = 24 + 3 = 27 **(F_{27} = 011011)**.

return

Optimization module

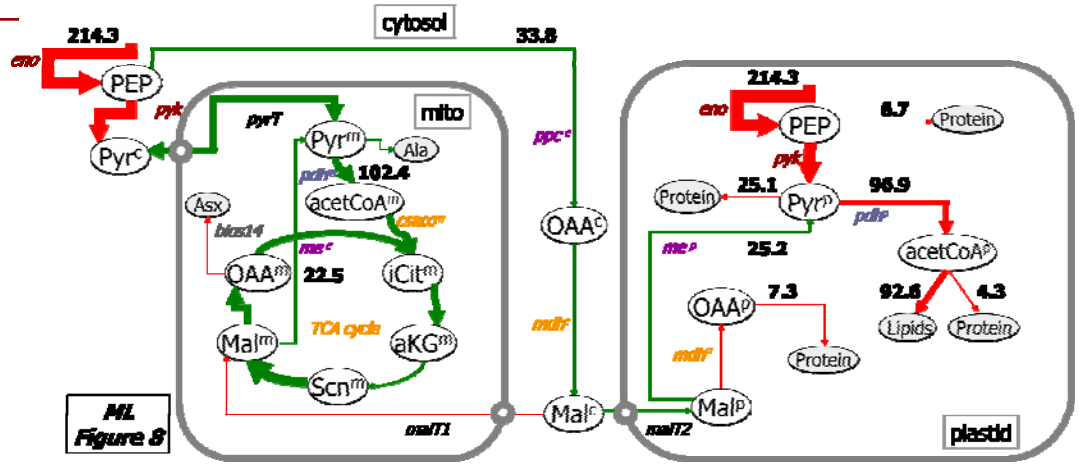
- Minimization of error between experimental and simulated intensities.
- Simulated annealing routine was developed (global optimization).
- Downhill simplex algorithm was used near the optimum (local optimization).



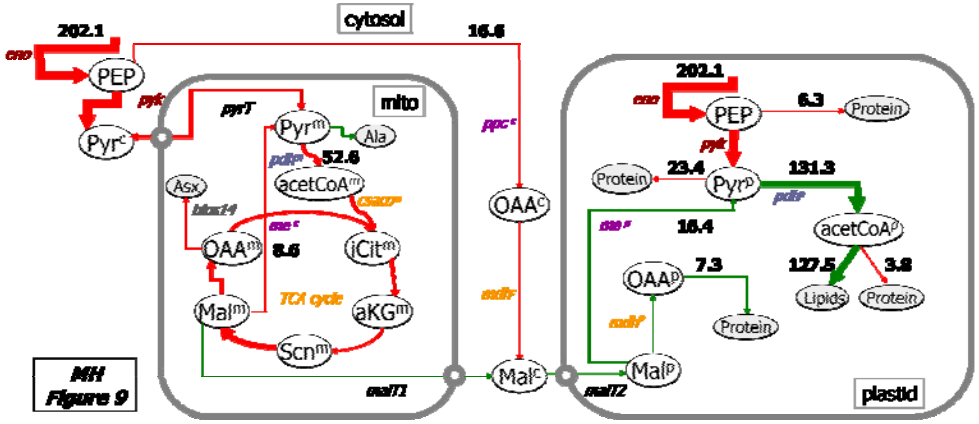


return

Plastidic Pyruvate Node



Pyr crossroad for ML temp. condition



Pyr crossroad for MH temp. condition