10.555 Bioinformatics

Lecture 8

Developing metrics of cell physiology. Flux determination

Gregory Stephanopoulos
MIT
Probing cellular function

Environment

DNA microarrays

DNA

mRNA

Fluxes

Proteins

Proteomics

Signal Transduction

Metabolic Flux Analysis

Lecture 8

Metrics of physiology. Flux determination
Case study: Aminoacid biosynthesis in *Corynebacterium glutamicum*

- **GLC** → Trehalose
- **GLC6P** → Ribu5P
- **Fru6P** → E4P, Sed7P
- **GAP** → Ribu5P
- **G3P** → Ribu5P

**CO₂**

- **PEP** → PEP
- **PYR** → AcCoA, Ala, Val, Lac
- **AcCoA** → Acetate

- **Asp** → PYR
- **OAA** → Isocit
- **Isocit** → aKG
- **aKG** → Glt, Glum

- **NH₃** → H4D, Suc, Meso-DAP
- **Suc** → SucCoA
- **SucCoA** → Lysine

- **CO₂**

**Lecture 8 Determination**
Definitions

**Metabolic Pathway:** A sequence of *feasible* and *observable* bioreaction steps connecting sets of input and output metabolites.

**Metabolic flux:** The rate of material processing through a metabolic pathway
Flux as fundamental determinant of cell physiology

• Along with metabolite concentrations, fluxes define a minimum set of information needed to describe cell physiology
• Fluxes determine the degree of engagement of various enzymes in a conversion pathway
• Fluxes elucidate pathway flux control
• Useful for:
  - Calculating theoretical yields
  - Determining non-measured metabolite rates
  - Observing pathway function in vivo
NORMAL INSULIN RESPONSE TO INCREASED PLASMA GLUCOSE

DECREASED GLUCONEOGENESIS VIA DECREASED PEPCK GENE EXPRESSION

INCREASED GLUCOSE OXIDATION VIA INCREASED ACETYL COA CARBOXYLASE & MALONYL COA

HEPATIC INSULIN RESISTANCE, POOR RESPONSE TO INCREASED PLASMA GLUCOSE

HPERGLYCEMIA VIA INCREASED PEPCK GENE EXPRESSION

HPERGLYCEMIA VIA DECREASED ACETYL COA CARBOXYLASE & MALONYL COA
In-vivo flux determination

- **Prior research:** Based on isolating pathway of interest from rest of metabolism
  - Limited success. Results of questionable value
- **This research:** Has yielded validated estimates of *in-vivo* metabolic fluxes. Approach is based on:
  - Material balances
  - Measurement of $^{13}$C label enrichment in selected (secreted) metabolites
  - Careful analysis of NMR spectra *fine structure*
  - Isotopomer MW distribution measured by GC-MS
How do we measure fluxes?

GLC → GLC6P → Fru1,6b P

O2

NH3

Biomass

Products

CO2
Metabolite Balancing: Linear Systems

- Include only branch points in the network (PSSH)

\[
\begin{align*}
A & \xrightarrow{v_1} X \xrightarrow{v_2} B \\
A & \xrightarrow{J = v_1 = v_2} B \\
A & \xrightarrow{J = v_1 = v_2 = r_A = r_B} B
\end{align*}
\]

- Degrees of freedom

Measurement of \( r_A \) or \( r_B \) suffices to determine flux \( J \)

Measurement of both \( r_A \) and \( r_B \) yields over-determined system
Metabolite Balancing: Branched Pathways

\[ \begin{align*}
    & A: \quad v_1 + v_2 = r_A \\
    & B: \quad v_1 - v_5 = 0 \\
    & C: \quad v_5 - v_6 = r_C \\
    & D: \quad v_2 - v_3 - v_4 = 0 \\
    & E: \quad v_3 = r_E \\
    & G: \quad v_4 = r_G \\
    & F: \quad v_6 = r_F \\
\end{align*} \]

6 unknown fluxes \( v_1 - v_6 \)

Lecture 8

Metrics of physiology. Flux determination
Metabolite Balancing (cont’d)

\[
\begin{bmatrix}
1 & 1 & 1 & 1 & -1 \\
1 & -1 & -1 & -1 & 1 \\
0 & 0 & 0 & 0 & 1
\end{bmatrix}
\begin{bmatrix}
v_1 \\
v_2 \\
v_3 \\
v_4 \\
v_5 \\
v_6 \end{bmatrix}
= \begin{bmatrix}
r_A \\
r_C \\
r_E \\
r_G \\
r_F \end{bmatrix}
= 0
\]

unknown \( v_c \)
measured \( v_m \)

\[
G_C^T \cdot v_c + G_m^T \cdot v_m = 0
\]
**Metabolite Balancing (cont’d)**

\[
\begin{align*}
\min ( \overline{v}_m - \mathbf{v}_m )^T \cdot \text{Cov}_{\mathbf{v}_m}^{-1} \cdot ( \overline{v}_m - \mathbf{v}_m ) \\
\text{subject to:} \\
\mathbf{G}_c^T \cdot \mathbf{v}_c + \mathbf{G}_m^T \cdot \mathbf{v}_m = \mathbf{0}
\end{align*}
\]

- \( \mathbf{v}_m \): the ‘true’ (i.e. adjusted to the measurements) value of the vector of measured fluxes
- \( \overline{v}_m \): the measured value of the vector of measured fluxes
- \( \text{Cov}_{\mathbf{v}_m} \): covariance matrix of the measurements (diagonal in the case of independent measurements)
- \( \mathbf{G}_c, \mathbf{G}_m \): from: measurement distribution / equipment specs / experience
Ensure that $G_C^T$ is **fully-ranked**

- $G_C^T$ is **singular** (i.e. not fully-ranked), when:
  - # balances < # of unknown fluxes
  - # balances $\geq$ # of unknowns, **but**
    
    *linear dependencies* are present in $G_C^T$

  because...

- Parallel pathways are present in the network (i.e. *linearly dependent* reactions called also *structurally singular groups*)

  **Examples**: anaplerotic pathways, nitrogen assimilation, trans-hydro-dehydrogenase, isoenzymes

- Redundant measurements are present (see example 8.5)
Flux determination by metabolite balancing

- Based on the balance for the conc’n of each metabolite $X$:
  \[
  \frac{dX}{dt} = r_{\text{met}} - \mu X_{\text{met}} = 0
  \]

  - The term $\mu X_{\text{met}}$ (dilution effect by growth) is small relatively to turnover rates (small pools)
  - $dX/dt = 0$ by Pseudo Steady State Hypothesis
    \[
    r_{\text{met}} = G^T v = 0
    \]
    (G is rxm matrix of stoichiometry)
  
  - Partition $G^T$ such that $0 = G^T v = G^T_m v_m + G^T_c v_c$
    \[
    v_c = -(G^T_c)^{-1} G^T_m v_m
    \]

  Ensure that $G_c$ is of full rank (i.e., do not include linearly dependent reactions or redundant measurements)
Flux determination by metabolite balancing

- Redundancy is important. See notes for general procedure to:
  - Test consistency
  - Reconcile measurements
  - Determine new estimates that satisfy all balances (including the redundant)
Metabolite Balancing (cont’d)

Degree of observability (D.O) = # of fluxes determinable (observable) from the measurements

\[ \text{D.O.} = \text{rank of } G_c^T \]

Degree of redundancy (D.R) = # of redundant measurements = # measurements that can also be determined from the balances and the rest of the measurements

\[ \text{D.R.} = \# \text{ measurements} - \text{D.O.} \]

- If # unknown fluxes > D.O. \( \rightarrow \) underdetermined systems: Linear Programming
- If D.R. > 0 \( \rightarrow \) overdetermined (for the observable fluxes) systems:

Solve by least squares - Gross error determination
Gross Error Determination (1)

Algorithm (refer to slide 12 on Metabolite Balancing):

If we have more equations than unknowns, use some of these equations to eliminate all unknown fluxes $v_c$ to get:

$$A \cdot v_m = 0$$

Note: # rows of A = # of redundant measurements

Due to the presence of measurement noise:

$$A \cdot v_m = \varepsilon$$

$\varepsilon$ : the residual of the constraints
**Gross Error Determination (2)**

\[ \min \left( \vec{v}_m - \bar{v}_m \right)^T \cdot \text{Cov}^{-1}_{\vec{v}_m} \cdot \left( \vec{v}_m - \bar{v}_m \right) \]

subject to:

\[ A \cdot \vec{v}_m = 0 \]

**solution:**

\[ \vec{v}_m = (I - \text{Cov}_{\vec{v}_m} \cdot A^T \cdot (A \cdot \text{Cov}_{\vec{v}_m} \cdot A^T)^{-1} \cdot A) \cdot \bar{v}_m \]

**Consistency index \( h \):**

\[ h = \left( \vec{v}_m - \bar{v}_m \right)^T \cdot \text{Cov}^{-1}_{\vec{v}_m} \cdot \left( \vec{v}_m - \bar{v}_m \right) \]

\[ h = (\vec{v}_m)^T \cdot A^T \cdot (A \cdot \text{Cov}_{\vec{v}_m} \cdot A^T)^{-1} \cdot A \cdot \bar{v}_m \]
Gross Error Determination (3)

If:

\[ h \geq \chi^2_{1-\theta}(m) \]

Then:

*reject the hypothesis* that measurement errors are insignificant

with confidence level of 1-\(\theta\)

m = degrees of freedom = number of redundant measurements
### Probability Points of the $x^2$ Distribution with $m$ Degrees of Freedom

<table>
<thead>
<tr>
<th>Degrees of freedom</th>
<th>$\theta$ (tail area probability)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>$m = 1$</td>
<td>0.455</td>
</tr>
<tr>
<td>$m = 2$</td>
<td>1.39</td>
</tr>
<tr>
<td>$m = 3$</td>
<td>2.37</td>
</tr>
<tr>
<td>$m = 4$</td>
<td>3.36</td>
</tr>
</tbody>
</table>
### Gross Error Determination - Example 1

After deletion of the measurement of Glucose-Limited Continuous Culture of *S. cerevisiae* CBS 426 with Ethanol and Glycerol Production under Anaerobic Conditions

<table>
<thead>
<tr>
<th>D (h⁻¹)</th>
<th>No deletion</th>
<th>Glucose</th>
<th>Biomass</th>
<th>CO₂</th>
<th>Ethanol</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.012</td>
<td>2.02</td>
<td>0.04</td>
<td>0.00</td>
<td>1.90</td>
<td>1.37</td>
<td>0.28</td>
</tr>
<tr>
<td>0.014</td>
<td>3.32</td>
<td>1.27</td>
<td>1.64</td>
<td>0.63</td>
<td>3.31</td>
<td>2.56</td>
</tr>
<tr>
<td>0.014</td>
<td><strong>10.99</strong></td>
<td>9.53</td>
<td>10.19</td>
<td><strong>0.00</strong></td>
<td>9.05</td>
<td><strong>10.99</strong></td>
</tr>
<tr>
<td>0.017</td>
<td>0.27</td>
<td>0.13</td>
<td>0.16</td>
<td>0.04</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td>0.027</td>
<td>1.52</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>0.030</td>
<td>0.33</td>
<td>0.18</td>
<td>0.13</td>
<td>0.28</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>0.034</td>
<td>1.52</td>
<td>0.32</td>
<td>0.52</td>
<td>0.56</td>
<td>1.52</td>
<td>1.09</td>
</tr>
<tr>
<td>0.051</td>
<td><strong>7.17</strong></td>
<td>3.37</td>
<td>4.50</td>
<td><strong>0.74</strong></td>
<td>5.98</td>
<td>6.79</td>
</tr>
<tr>
<td>0.053</td>
<td>0.57</td>
<td>0.26</td>
<td>0.18</td>
<td>0.51</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>0.056</td>
<td>0.88</td>
<td>0.61</td>
<td>0.72</td>
<td>0.01</td>
<td>0.64</td>
<td>0.88</td>
</tr>
<tr>
<td>0.058</td>
<td>1.66</td>
<td>1.14</td>
<td>1.34</td>
<td>0.02</td>
<td>1.18</td>
<td>1.65</td>
</tr>
<tr>
<td>0.058</td>
<td><strong>10.20</strong></td>
<td>8.69</td>
<td>7.81</td>
<td>8.24</td>
<td><strong>0.00</strong></td>
<td>4.56</td>
</tr>
<tr>
<td>0.062</td>
<td>2.61</td>
<td>2.46</td>
<td>2.58</td>
<td>0.23</td>
<td>0.94</td>
<td>2.43</td>
</tr>
<tr>
<td>0.067</td>
<td>0.79</td>
<td>0.78</td>
<td>0.74</td>
<td>0.20</td>
<td>0.15</td>
<td>0.57</td>
</tr>
</tbody>
</table>

$h \ [ \chi^2 (90\%, 2) = 4.6 \ ]$
**Gross Error Determination - Example 2**

After deletion of the measurement of

\[ h \quad [\chi^2 (90\%, 2) = 4.6] \]

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>No deletion</th>
<th>Substrate</th>
<th>( \text{O}_2 )</th>
<th>Biomass</th>
<th>( \text{CO}_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>109.94</td>
<td>76.04</td>
<td>36.74</td>
<td>46.30</td>
<td>5.03</td>
</tr>
<tr>
<td>2</td>
<td>26.08</td>
<td>24.29</td>
<td>4.06</td>
<td>16.00</td>
<td>1.90</td>
</tr>
<tr>
<td>4</td>
<td>21.54</td>
<td>17.24</td>
<td>7.07</td>
<td>7.69</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>8.35</td>
<td>5.63</td>
<td>4.37</td>
<td>1.12</td>
<td>0.78</td>
</tr>
<tr>
<td>10</td>
<td>4.47</td>
<td>1.72</td>
<td>3.65</td>
<td>0.01</td>
<td>1.68</td>
</tr>
<tr>
<td>12</td>
<td>0.92</td>
<td>0.83</td>
<td>0.28</td>
<td>0.40</td>
<td>0.00</td>
</tr>
<tr>
<td>14</td>
<td>1.05</td>
<td>1.04</td>
<td>0.03</td>
<td>0.89</td>
<td>0.18</td>
</tr>
<tr>
<td>16</td>
<td>3.36</td>
<td>3.19</td>
<td>0.00</td>
<td>3.20</td>
<td>1.23</td>
</tr>
<tr>
<td>18</td>
<td>2.68</td>
<td>2.66</td>
<td>0.08</td>
<td>2.36</td>
<td>0.65</td>
</tr>
<tr>
<td>19.5</td>
<td>2.60</td>
<td>2.60</td>
<td>0.18</td>
<td>2.14</td>
<td>0.46</td>
</tr>
<tr>
<td>21.5</td>
<td>1.97</td>
<td>1.69</td>
<td>0.71</td>
<td>0.85</td>
<td>0.00</td>
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<tr>
<td>23.5</td>
<td>3.68</td>
<td>3.67</td>
<td>0.29</td>
<td>3.07</td>
<td>0.74</td>
</tr>
<tr>
<td>25.5</td>
<td>5.95</td>
<td>5.21</td>
<td>2.06</td>
<td>3.41</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Lecture 8

Metrics of physiology. Flux determination
### Gross Error Determination - Example 3

Given: $\chi^2 (90\%, 2) = 4.6$

After deletion of the measurement of $0.050 \, \text{D(h}^{-1})$

<table>
<thead>
<tr>
<th>$h , [\chi^2 (90%, 2) = 4.6]$</th>
<th>No deletion</th>
<th>Glycerol</th>
<th>$O_2$</th>
<th>Biomass</th>
<th>$CO_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>0.06</td>
<td>0.06</td>
<td>0.01</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>0.115</td>
<td>0.20</td>
<td>0.14</td>
<td>0.01</td>
<td>0.16</td>
<td>0.12</td>
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<tr>
<td>0.125</td>
<td>0.24</td>
<td>0.14</td>
<td>0.17</td>
<td>0.11</td>
<td>0.03</td>
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<td>0.250</td>
<td>0.26</td>
<td>0.24</td>
<td>0.00</td>
<td>0.26</td>
<td>0.07</td>
</tr>
<tr>
<td>0.350</td>
<td>0.32</td>
<td>0.28</td>
<td>0.12</td>
<td>0.24</td>
<td>0.01</td>
</tr>
<tr>
<td>0.485</td>
<td>0.40</td>
<td>0.31</td>
<td>0.19</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>0.510</td>
<td>0.33</td>
<td>0.33</td>
<td>0.06</td>
<td>0.30</td>
<td>0.00</td>
</tr>
<tr>
<td>0.625</td>
<td>0.39</td>
<td>0.34</td>
<td>0.14</td>
<td>0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>0.75</td>
<td>0.50</td>
<td>0.36</td>
<td>0.27</td>
<td>0.28</td>
<td>0.05</td>
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<tr>
<td>0.850</td>
<td>0.40</td>
<td>0.37</td>
<td>0.10</td>
<td>0.33</td>
<td>0.00</td>
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<td>0.910</td>
<td>0.38</td>
<td>0.37</td>
<td>0.00</td>
<td>0.38</td>
<td>0.06</td>
</tr>
<tr>
<td>0.935</td>
<td>0.46</td>
<td>0.37</td>
<td>0.20</td>
<td>0.30</td>
<td>0.03</td>
</tr>
<tr>
<td>0.980</td>
<td>1.48</td>
<td>0.38</td>
<td>0.75</td>
<td>0.64</td>
<td>1.31</td>
</tr>
<tr>
<td>1.010</td>
<td>5.55</td>
<td>0.37</td>
<td>5.54</td>
<td>0.07</td>
<td>4.26</td>
</tr>
</tbody>
</table>

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Glycerol-limited continuous culture of 
Aerobacter aerogens
In-vivo flux determination: RESULTS

- Flux distributions at G6P in *C. glutamicum*
  - Flexible node
  - Trans-hydrogenase activity discovered
- Unequivocal evidence of PYR carboxylation
- Complete kinetic analysis of the H₄D node
- Mammalian cell culture:
  - Glycosylation strongly depends on G6P fluxes
  - Strong correlation between glycolytic fluxes and apoptotic death
Fluxes provide invaluable perspectives of physiological state and metabolic control.
Flux determination from $^{13}$C label enrichment of (secreted) metabolites
RESULTS (cont’d)

• Determination of Group Control Coefficients (GCC) from flux estimates
• GCC are measures of the control exercised by a group of reactions on a flux
• See notes
Group Control Coefficients:

Application to lysine biosynthesis

- Perturbations
  - Glucose concentration
  - GPI mutant
  - Gluconate addition
  - Fluoropyruvate
  - Threonine inhibition

<table>
<thead>
<tr>
<th>Perturbed branch</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.07</td>
<td>0.51</td>
<td>0.42</td>
</tr>
<tr>
<td>B</td>
<td>0.09</td>
<td>1.22</td>
<td>0.31</td>
</tr>
<tr>
<td>C</td>
<td>0.02</td>
<td>-0.34</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Lecture 8

Metrics of physiology. Flux determination


RESULTS (cont’d)

• Cell death in culture (CHO, Hybridomas)
  ✫ Strongly correlated to culture energetics
  ✫ Cells with high mitochondrial membrane potential (MMP) are resistant to apoptosis inducers
Growth and death rates vs. dilution rate

- Growth Rate
- Death Rate

% of death rate due to apoptosis

96%
71%
49%
35%
Steady state multiplicity

Cell Density and Viability vs. Dilution Rate
Multiple Steady States

- Cell Density (cell/mL)
- Viability (%)
- Dilution Rate at Steady State (hr⁻¹)

- Viable Cells
- Total Cells
- Viability

Lecture 8
Metabolic flux analysis

Figure 7.4a Estimated intracellular fluxes at DO=60. The numbers indicate the fluxes in units of mmole/cell/hr * 10^-10.
ATP generation and pyruvate flux distribution
Steady state multiplicity

Cell Density and Viability vs. Dilution Rate
Multiple Steady States

- Viable Cells
- Total Cells
- Viability

Dilution Rate at Steady State (hr⁻¹)

Viability (%)
Hybridoma physiology at steady states

Rhodamine Staining of Hybridomas at Steady State

- Mean
- Std. Deviation

Fluorescence Absorbance Units

<table>
<thead>
<tr>
<th>D (1/H)</th>
<th>Absorbance Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>D=0.03 1/H</td>
</tr>
<tr>
<td>0.02</td>
<td>D=0.02 1/H</td>
</tr>
<tr>
<td>0.01</td>
<td>D=0.01 1/H</td>
</tr>
<tr>
<td>0.04</td>
<td>D=0.04 1/H (New)</td>
</tr>
</tbody>
</table>

Lecture 8
Metrics of physiology. Flux determination
Conclusions

- Cell survival in nutrient-limited environment depends on mitochondria membrane potential.
- Hybridomas with high metabolic activity can be selected in chemostats, (or by sorting).
- Hyperactive cells are very efficient in nutrient utilization (minimal lactate production). They retain the hypermetabolic state upon return to nutrient-rich environment.
**Important issues in flux determination**

- Measurement redundancy allows validation of measurements and pathway biochemistry
- Pathway modification and discovery are part of the flux determination process
- Metabolic and isotopic steady states are strict requirements for isotopic label analysis
- Analogy to material structure determination
Using only extracellular accumulation rates:

- only net fluxes can be observed

\[ v_{\text{net}} = v_f - v_b \]
\[ v_{\text{exch}} = \min(\|v_f\|, \|v_b\|) \]

- net fluxes of pathways that branch apart and rejoin later cannot be differentiated
Singular net flux groups in lysine biosynthesis network

Lecture 8 Metrics of physiology. Flux determination

- **GLC** → **GLC6P**
- **CO2** → **Ribu5P**
- **Fru1,6bP** → **FRU6P**
- **GAP** → **G3P**
- **PEP** → **PYR**
- **PYR** → **AcCoA**
- **AcCoA** → **OAA**
- **OAA** → **Isocit**
- **Isocit** → **aKG**
- **aKG** → **Glt**
- **Glt** → **Gln**
- **H4D** → **meso-DAP**
- **meso-DAP** → **Lysine**
- **Lysine** → **Threonicine**
- **Threonicine** → **Methionine**
- **Methionine** → **Homoserine**
- **Homoserine** → **Aspartatic semialdehyde**
- **Aspartatic semialdehyde** → **Asp**
- **Asp** → **PYR**
- **PYR** → **ALA, VAL, LAC**
- **ALA, VAL, LAC** → **Acetate**
- **Acetate** → **GLC**
- **GLC** → **CO2**
Use of tracers in MFA

- Use of radioactive tracers
- Local network results (see example of H₄D branch point, example 9.1)
- Global network analysis. Methods based on balances of:
  - Metabolites
  - Label vs. non-label
  - Enrichment
  - Isotopomers
- Approach depends on measurements available
Use of radiolabeled substrates for flux determination

- At steady state, a pulse of a radiolabeled metabolite ($M^*$) can be used to determine the flux ($J$) from a metabolite pool ($M$) (single compartment model)

\[
\frac{dM^*}{dt} = -\left(\frac{M^*}{M}\right)J
\]

\[
\ln\left(\frac{M^*(t)}{M^*(0)}\right) = -\left(\frac{J}{M}\right)t
\]

- Radiolabeled metabolites are used for greater measurement sensitivity and minimal perturbation of the steady state.
**Considering transport**

Steady state material balance equations on labeled metabolite $M (M^*)$

![Diagram with fluxes and metabolite concentrations]

\[
\frac{dM_{in}^*}{dt} = -\left(\frac{J}{M_{in}}\right)M_{in}^* - J_{exc}^M \frac{1}{M_{in}} M_{in}^* + J_{up}^M \frac{1}{M_{ex}} M_{ex}^*
\]

\[
\frac{dM_{ex}^*}{dt} = J_{exc}^M \frac{1}{M_{in}} M_{in}^* - J_{up}^M \frac{1}{M_{ex}} M_{ex}^*
\]

\[
\frac{dM_{tot}^*}{dt} = -\left(\frac{J}{M_{in}}\right)M_{in}^*
\]

Thus, we need to measure $M_{in}$, $M_{in}^*$ and $M_{tot}^*$
Flux determination with radioactive tracers

Addition of radiolabeled $M^*$

- Flux in $J$
- Pool of metabolite $M$
- Flux out $J$

Assuming rapid eqlm. between intracellular and extracellular $M$:

\[
\frac{dM^*_\text{tot}}{dt} = -\left( \frac{J}{M_{\text{in}}} X \right) M^*_{\text{in}}
\]

\[
\frac{dM^*_\text{tot}}{dt} = -\left( \frac{J}{M_{\text{tot}}} X \right) M^*_\text{tot}
\]

\[
\ln\left( \frac{M^*_\text{tot}(t)}{M^*_\text{tot}(0)} \right) = -\left( \frac{J}{M_{\text{tot}}} X \right) t
\]
Methods depend on available measurements

A. Well defined systems

Lecture 8

Metrics of physiology. Flux determination 44
Use of the chemostat for flux measurements

- Assume pseudo-steady state conditions maintained in shake flask

Lecture 8

Metrics of physiology. Flux determination
$^{14}$C-Tracer Experiments

$^{14}$C Profiles: $^{14}$C-Indene Oxide Feed

$^{14}$C Profiles: $^{14}$C-cis-(2R)-Indandiol Feed
**Flux Determination**

\[
\ln \left( \frac{M_{tot}^* (t)}{M_{tot}^* (0)} \right) = - \left( \frac{J}{M_{tot}} X \right) t = -kt
\]

**Indene Oxide Hydrolysis Rate Constant Determination**

\[ y = -0.0089x \quad R^2 = 0.9006 \]

\[ k_{H/X} = 0.54 \pm 0.06 \text{ h}^{-1} \]

**cis(2R)-Dehydrogenase Flux Determination**

\[ y = -0.0031x \quad R^2 = 0.9775 \]

\[ v_{RDH} = 18 \pm 2 \text{ µmol/h/g DCW} \]
Use of tracers in MFA

- Use of radioactive tracers
- Local network results (see example of H₄D branch point, example 9.1)
- Global network analysis. Methods based on balances of:
  - Metabolites
  - Label vs. non-label
  - Enrichment
  - Isotopomers
- Atom mapping matrices
Flux determination from $^{13}$C label enrichment of (secreted) metabolites

![Diagram showing flux determination through metabolic pathways](image-url)
Use of tracers in MFA

• Use of radioactive tracers
• Local network results (see example of H_4D branch point, example 9.1)
• **Global network analysis. Methods based on balances of:**
  - Metabolites
  - Label vs. non-label
  - Enrichment
  - Isotopomers
• Atom mapping matrices
Isotopomers

- Molecules of the same metabolite labeled differently upon introduction of a labeled substrate
Use of isotopic tracers

GLC

$^{13}$C or $^{14}$C

O$_2$

NH$_3$

Biomass

Products

CO$_2$

GLC

GLC6P

FRU6P

Ribu5P

Ribu5P

E4P

Sed7P

Fru1.6b

GAP

G3P

PEP

PYR

PYR

AcCoA

OAA

Isocit

Acetate

ALA, VAL, LAC

CO$_2$

CO$_2$

Asp

H4D

meso-DAP

Lysine

aKG

Glt

Gln

Suc

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13C-NMR Spectroscopy

- Determines the label enrichment of a particular carbon atom in a molecule

\[ \begin{align*}
\text{C-1} &= 1 + 0 + 0 + 0 \\
\text{C-2} &= 0 + 1 + 0 + 0 \\
\text{C-3} &= 0 + 0 + 1 + 0
\end{align*} \]
Mass Spectrometry

- Differentiates ions based on mass/charge ratio

Mass Spectrometer

\[
M+0 = \bullet \bullet \bullet \\
M+1 = \bullet \bullet + \bullet \bullet + \bullet \bullet \bullet \\
M+2 = \bullet \bullet + \bullet \bullet + \bullet \bullet \bullet \\
M+3 = \bullet \bullet \bullet
\]
Detailed accounting of metabolite isotopomers allows:

- **Accurate prediction of $^{13}\text{C}$ enrichment:**
  \[ ^{13}\text{C}_1 = L_{15} + L_{16} + L_{146} + L_{136} \]
  \[ ^{13}\text{C}_2 = L_2 + L_{24} + L_{25} + L_{26} + L_{246} \]
  \[ ^{13}\text{C}_3 = L_3 + L_{136} \]

- **Prediction of iso-topomer MW distr’n**
  \[ L_{M+1} = L_2 + L_3 \]
  \[ L_{M+2} = L_{15} + L_{16} + L_{24} + L_{25} + L_{26} + L_{46} \]
  \[ L_{M+3} = L_{136} + L_{146} + L_{246} \]
Isotopomer balance around D:

\[
\begin{align*}
\text{v2} & \quad + \quad \text{v5}^- \\
\end{align*}
\]

\[
= (rD + v5^+) \]

Lecture 8

Metrics of physiology. Flux determination
**Isotopomers**: metabolite molecules with different labeling patterns

### Carbon enrichment distribution

\[ C-1 = [b] + [e] + [f] + [h] \]
\[ C-2 = [c] + [e] + [g] + [h] \]
\[ C-3 = [d] + [f] + [g] + [h] \]

### Molecular weight distribution

\[ M = [a] \]
\[ M+2 = [e] + [f] + [g] \]
\[ M+1 = [b] + [c] + [d] \]
\[ M+3 = [d] + [f] + [g] + [h] \]
\[ M_1 = [a] + [d] \]
\[ M_1 + 1 = [b] + [c] + [f] + [g] \]
\[ M_1 + 2 = [e] + [h] \]
\[ M_2 = [a] + [b] + [c] + [e] \]
\[ M_2 + 1 = [d] + [f] + [g] + [h] \]
Derivatization of Amino Acids with TBDMS

- Derivatives can be volatilized for GC separation

TBDMS-derivative of Valine:

- To obtain the mass isotopomer distribution of the aminoacid, we need to correct the measurements for the natural abundance of the rest of the atoms in the derivatives
Fragmentation of TBDMS-derivatives

- Ionization prior to MS generates fragments of the derivatives

Positional information about the isotopic tracer can be obtained

\[
\begin{align*}
260+0 &= \text{Fragment} 1 \\
260+1 &= \text{Fragment 2} \\
260+2 &= \text{Fragment 3} \\
260+3 &= \text{Fragment 4}
\end{align*}
\]

\[
\begin{align*}
158+0 &= \text{Fragment 5} \\
158+1 &= \text{Fragment 6} \\
158+2 &= \text{Fragment 7}
\end{align*}
\]

Metrics of physiology. Flux determination
ISOTOPOMER DISTRIBUTION ANALYSIS

\[
g_i : \vec{v} \cdot A_i \cdot \vec{X}_{iso} + \vec{X}_{iso}^T \cdot Q_i (\vec{v}) \cdot \vec{X}_{iso} = \vec{c}
\]

\(i = 1, 2, \ldots, N\) (number of isotopomers)

\(\vec{X}_{iso}\) : vector of relative populations of isotopomers

\(\vec{v}\) : vector of intracellular fluxes

**Carbon enrichments**

\[
\vec{M}_{enr} = \vec{K} \cdot \vec{X}_{iso}
\]

**Molecular weight distribution**

\[
\vec{M}_{MW} = \vec{L} \cdot \vec{X}_{iso}
\]

**Fine structure of NMR spectra**

\[
\vec{M}_{NMR,\text{fine}} = \vec{U} \cdot \vec{X}_{iso}
\]

\(\vec{K}, \vec{L}, \vec{U}\) are known
Metabolic Flux Determination

- Chemostat
- TBDMS-Derivatization
- Hydrolysis of biomass
- GC-MS
- Mass isotopomer fractions
- Extracellular metabolite rates
- HPLC
- Flux Map

Lecture 8
Mass spectrometric measurements (1)

![Graph showing isotopomer fraction over time after introduction of labeled substrate](image-url)