RFMF 2016
Montpellier, May 30 2016

Workshop ^{13}C-fluxomics

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MetaToul – Toulouse Metabolomics and Fluxomics Platform

RFMF Workshop - ^{13}C-fluxomics
Introduction

Résultats sondage
Merci de votre participation

Vos attentes vis-à-vis du contenu de l'atelier:					Motivation pour l'atelier:		
1. concepts généraux en 13C-fluxomique	2. aspects expérimentaux (expériences de marquage, échantillonage, etc)	3. Mesures isotopiques (spectrométrie de masse, RMN)	4. Calcul de flux à partir de données isotopiques	5. l'ensemble des 4 aspects précédents	je ne connais pas la 13C-fluxomique et je recherche une première initiation	je connais certains aspects de la 13C-fluxomique et je souhaite mieux connaitre les autres aspects	je suis un expert de la 13C-fluxomique et je souhaite échanger sur des points particuliers
9	12	13	13	20	**12**	18	0

Pendant l'atelier:
Ne pas hésiter à intervenir à tout moment pour des questions, des commentaires, des compléments, etc

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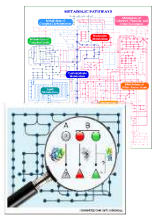
Introduction

Fluxomics: a tool for the functional analysis of metabolic systems



- **Basic concepts**
  - Why measuring fluxes
  - What is a flux and how to measure fluxes
  - Benefit of isotopic tracers
- **^{13}C-labelling experiment**
  - Design of label input
  - ^{13}C-labelling experiment
  - Sampling
- **Isotopic profiling**
  - NMR-based isotopic profiling
  - MS-based isotopic profiling
- **Flux calcul.**
  - requirements
  - Flux calculation
  - Sensitivity analysis

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Introduction

Part I - Basic concepts

- **Basic concepts**
  - Why measuring fluxes
  - What is a flux
  - Benefit of isotopic tracers
- **13C labelling experiment**
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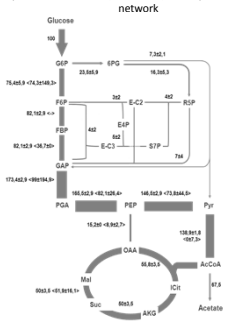
Why measuring fluxes ?

The outcome of fluxomics: the flux map

- Distribution of intracellular carbon (& energy) fluxes *in vivo*
- Represents the actual (contextual) activity of the metabolic network



ISOTOPIC PROFILING

Metabolic network topology
- Identification of (novel) pathways, enzymes
- Linear pathways, cycles, reaction reversibilty
- Compartmentation, channeling

« TRUE » FLUXOMICS

Quantification of pathway activity
- Carbon fluxes
- Energy fluxes (ATP, redox)
- Response to environmental / genetic modifications

Applications
- Microrganisms, plant cells & tissues, animal cells
- Systems biology
- Biotechnology
- Synthetic biology
- Pharmacology, drug targeting



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What is a flux?

What is a flux ?

A flux is a flow = volume of fluid or amount of matter which passes per time unit


Internet flow


water flow


cash flow !!!

Often assimilated to rate or speed (velocity) although the definitions are different: Speed can be thought of as the rate at which an object covers distance.

Biochemistry:
Flux: flow of matter passing though a biochemical conversion. Linked to reactions.
Rate: used to characterize enzyme velocity. Related to catalysis, linked to enzymes.

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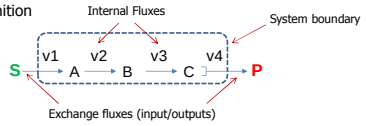
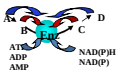
What is a flux?

Step 1: System definition



There is no direct method to measure intracellular fluxes

Mass balances
1. Stoechiometric approaches
2. Isotopic approaches



Kinetics
1. Formal kinetics
2. Simplified kinetics,LinLog

$$C_E^i = \frac{\partial J}{\partial E} \quad \varepsilon_X^E = \frac{\partial E}{\partial X}$$

Sensitivity analysis
1. Metabolic Control
2. Biochemical Systems
3. Hierarchical Control

***in silico* Analysis**
Constraint-based models

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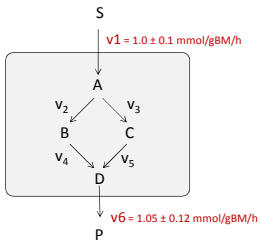
Stoichiometric & isotopic modeling

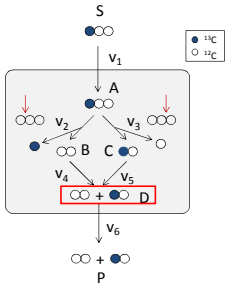
Stoichiometric modeling



$$dM/dt = S \cdot v$$

$$\begin{bmatrix} dA/dt \\ dB/dt \\ dC/dt \end{bmatrix} = \begin{bmatrix} 1 & -1 & 0 & 0 \\ 0 & 1 & -1 & 0 \\ 0 & 0 & 1 & -2 \end{bmatrix} \cdot \begin{bmatrix} v1 \\ v2 \\ v3 \\ v4 \end{bmatrix}$$

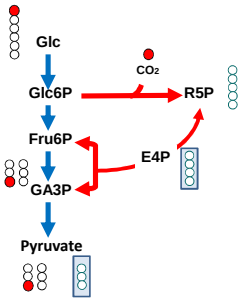
^{13}C-metabolic flux analysis



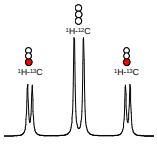
$D_{01}/(D_{00}+D_{10}) = v_5/(v_4+v_5)$

^{13}C-Fluxomics – basic concepts
Isotopic Profiling

Isotopic Profiling : identification of active pathways



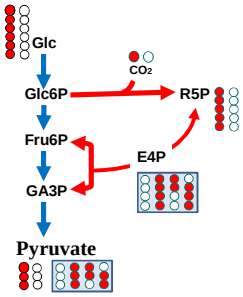
1H-NMR: specific enrichment



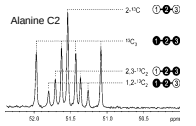
- % of carbon-13 in a specific carbon position (varies from 1,1 % to 100%)
- detection by NMR only (^{13}C / ^{1}H)

^{13}C-Fluxomics – basic concepts
Isotopic Profiling

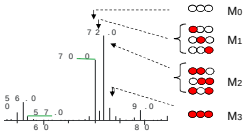
Isotopic Profiling : identification of active pathways



NMR: positional isotopomers



MS: isotopologues



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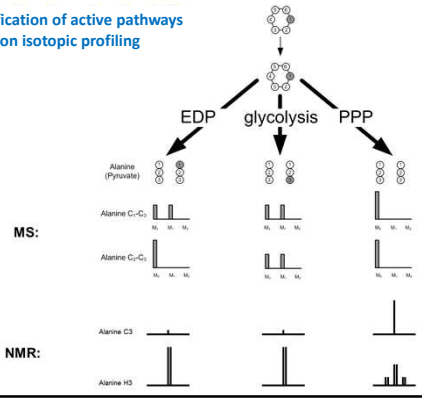
^{13}C-Fluxomics – basic concepts : Isotopic Profiling

Identification of active pathways based on isotopic profiling



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Isotopic species

Isotopic species

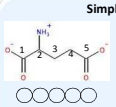
Focus on carbon isotopes : ^{12}C and ^{13}C (^{14}C & ^{11}C not significant)

Glutamate

Molecular Formula: $C_5H_9NO_4$

Molecular Weight: 147.12926 g/mol

Simplified representation



Numbering of carbon atom positions according to chemistry rules

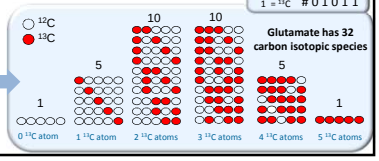
Simplified representation of the carbon backbone (ordered from left to right)

Alternative notation:
0 = ^{12}C
1 = ^{13}C
#01011

How many carbon isotopic species for glutamate ?

5 carbon positins. Each carbon position can contain either a 12C or a 13C atom:
5 positions → 2^5 = 32 carbon isotopic species

General case: n carbons
→ 2^n carbon isotopic species



Glutamate has 32 carbon isotopic species

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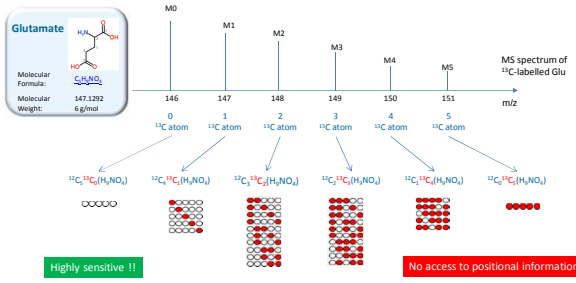
Isotopic profiling

Isotopic measurement by MS: isotopologues

MS discriminates between Isotopologues ('isotopic homologues')

IUPAC definition : Isotopologues refer to **molecular entities** that differ only in their **isotopic composition** (number of isotopic substitutions), e.g. CH_4, CH_3D, CH_2D_2.



Highly sensitive !!

No access to positional information !!

EC21

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Isotopic profiling

How to measure isotopic species

Isotopic measurement by NMR

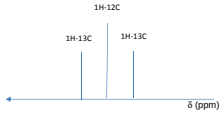
Separation according to chemical environment
Access to positional information
2 main nuclei of interest: ^{13}C and ^{1}H

^{13}C-NMR spectrum

Direct observation of ^{13}C nuclei

^{13}C isotopomers

^{1}H-NMR spectrum



Observation of ^{13}C (and ^{12}C) nuclei via attached protons

^{13}C enrichments

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Isotopic species

Definitions

Isotopologue = 'isotopic homologue'

IUPAC definition : Isotopologues refer to **molecular entities** that differ only in their **isotopic composition** (number of isotopic substitutions), according to its IUPAC definition e.g. CH_4, CH_3D, CH_2D_2. "

Isotopomer = 'isotopic isomer'

IUPAC definition : Isomers having the same number of each isotopic atom but differing in their positions.
→ **same chemical formula** (not the same molecular entity!)
→ different position of isotopes

Usual definition in ^{13}C-fluxomics: molecules of the **same chemical entity** but with a different isotopic composition.

Diapositive 14

EC21 Changement direction ppm
Edern Cahoreau; 30/05/2016

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Isotopic species

Exercices !

	Isotopologue	Isotopomer IUPAC	Isotopomer 13C-fluxomics
CD_3-COOH and CD_2H-COOH			
$C_2O_2D_3H$ and CD_2H-COOD ?			
$^{13}CH_3$-$^{13}CH_2$-COOH and $^{13}CH_3$-CH_2-^{13}COOH			
How many for alanine ?			

n carbon atoms: n+1 isotopologues
2^n isotopomers (13C fluxomics)

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Part II - ^{13}C-labelling experiments

- Basic concepts
  - Why measuring fluxes
  - What is a flux and how to measure fluxes
  - Benefit of isotopic tracers
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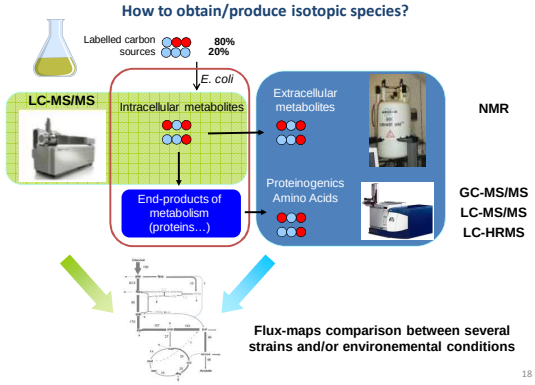
^{13}C-Labelling Experiments

How to obtain/produce isotopic species?



Flux-maps comparison between several strains and/or environemental conditions

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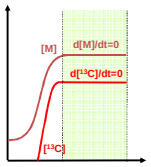
^{13}C-Labelling Experiments

The level of information depends on the type of experimental approaches

1. Metabolic & isotopic stationnary states

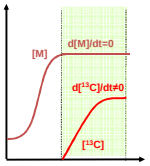


- Net & exchanges Fluxes
- Stoechiometric & isotopic equations
- Mathematical symplifications
- Analysis of metabolism products

- Does not exist in nature
- Biological and methodological limitations

Currently used

2. Metabolic stationary state & isotpopic dynamic state

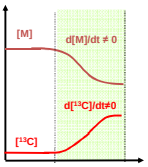


- Net & exchanges Fluxes
- Access to a higher number of fluxes
- Analysis of intermediates of metabolism
- Identification of metabolic pathway (networks)
- Resolution of metabolic pathway

- Needs of sensitive analytical tools
- Suitable quenching & sampling of metabolism

few examples

3. Metabolic & isotopic dynamic states



- Enzyme kinetics
- Dynamic of metabolic responses
- Redistribution of fluxes (net, energetic…)
- Access to regulation networks (hierarchical and/or metabolic control)

- ODE matrixes
- Needs of metabolomic & isotopic datas

Few examples

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^{13}C-Labelling Experiments

The choice of labelled substrate

- Specifically labelled substrates (e.g. [1-^{13}C]glucose): investigation of specific pathways
- Uniformly labelled substrates ([U-^{13}C]glucose): Overall view on the main pathways of central carbon metabolism
- Combination of both

IsoDesign

Millard P. & al 2013

Costs

[1-^{13}C]glucose 70€/g
[U-^{13}C]glucose 80€/g
[1,2-^{13}C]glucose 780€/g
[U-^{13}C]glutamine 5560€/g

Link between metabolites turn-over & labelling incorporation

→ Labelled substrate = ^{13}C glucose
- Isotopic Steady state of glycolytic intermediates: range of seconds – minutes
- Isotopic Steady state of TCA intermediates: more than 30min
- Isotopic Steady state of protein-bound AA: several hours

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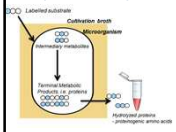
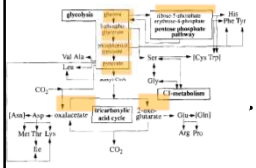
^{13}C-Labelling Experiments

Isotopic stationary ^{13}C-MFA: analysis of proteinogenic amino acids



- Proteins are abundant, stable end-products of metabolism
- Provide abundant material (half of CDW is protein) for isotopic profiling
- 16 out of 20 amino acids can be analyzed (by LC-MS) after HCl hydrolysis of biomass
  - cysteine, tryptophan – oxidized during hydrolysis;
  - glutamine and asparagine deaminated into glutamate and aspartate
- The labelling of proteinogenic amino acids give information on 8 precursor metabolites located across central carbon metabolism



Neidhardt, 1987

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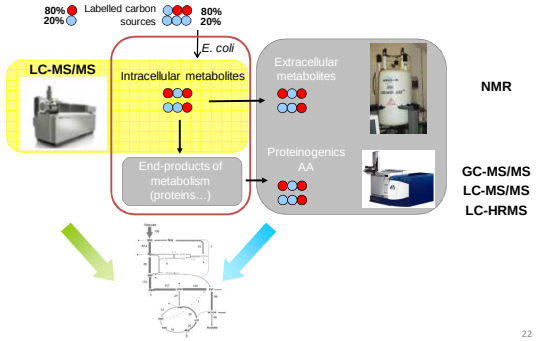
^{13}C-Labelling Experiments

Experimental Strategy – intracellular metabolites & dynamic….



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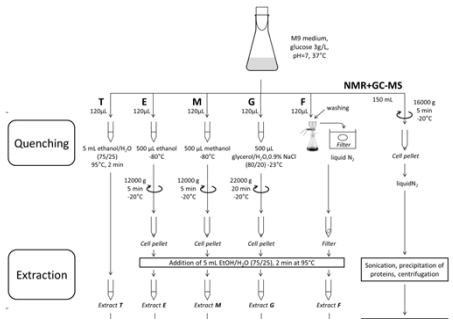
^{13}C-Labelling Experiments

Evaluation of Sampling methods in 13C-MFA for intracellular metabolites



Millard P., Anal.Biochem, 2014

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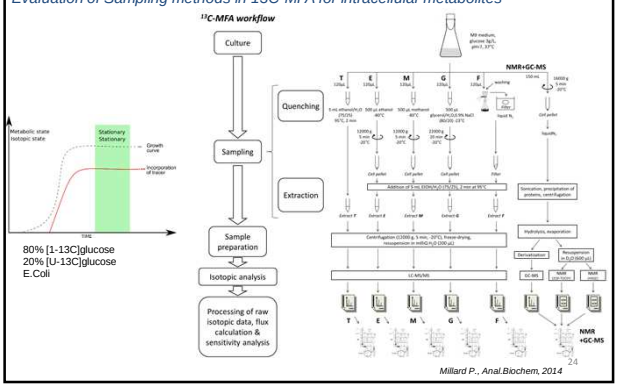
^{13}C-Labelling Experiments

Evaluation of Sampling methods in 13C-MFA for intracellular metabolites



Millard P., Anal.Biochem, 2014

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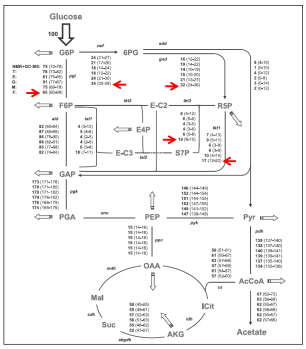
^{13}C-Labelling Experiments

Evaluation of Sampling methods in 13C-MFA

Efficiency of 5 sampling methods

Culture *E.coli* in metabolic and isotopic steady-state.

Comparison of obtained flux maps to flux map from proteinogenic Amino Acids (GC-MS, NMR)



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Part III - NMR-based isotopic profiling

- Basic concepts
  - Why measuring fluxes
  - What is a flux and how to measure fluxes
  - Benefit of isotopic tracers
- ^{13}C-labelling experiment
  - Design of label input
  - 13C-labelling experiment
  - Sampling
- Isotopic profiling
  - NMR-based isotopic profiling
  - MS-based isotopic profiling
- Flux calculation
  - requirements
  - Flux calculation
  - Sensitivity analysis

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NMR-based isotopic profiling

NMR & METABOLISM

Advantages
- Analysis of complex mixtures without separation
- Detailed structural information
- Multi-nuclear: ^{1}H, ^{13}C, ^{31}P, ^{15}N, etc
- Isotopic analysis (stable isotopes)
- in vivo /in situ analysis (non invasif et non destructif)
- Can be coupled to LC
- Robust, reliable (HT analysis)

Drawbacks
- Limited sensitivity (micro to nano-moles): major metabolites
- Cost of equipments (analysis not necessarily expensive)
- Dynamic range

Frequently observed nucleus: ^{1}H, ^{13}C, ^{31}P, ^{19}F, ^{15}N

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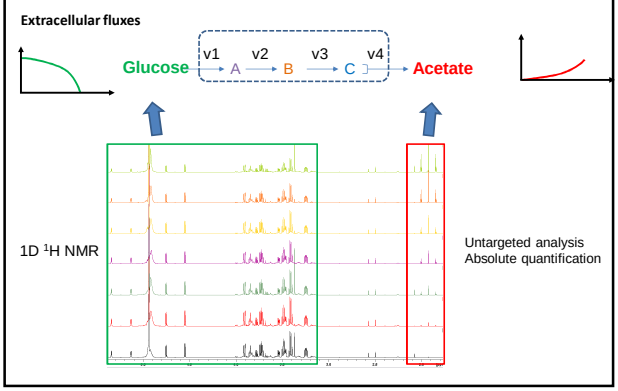
Measurement of input/output fluxes



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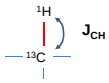
NMR-Based ^{13}C-Fluxomics

Exploitation of Scalar coulping (J)

Homonuclear ^{13}C – ^{13}C coupling $\mathbf{J_{CC}}$



Heteronuclear ^{1}H – ^{13}C coupling $\mathbf{J_{CH}}$

13C-NMR
- Spin 1/2
- Natural Abundancy : 1.07%
- Relative sensitivity to ^{1}H: 1,6 %
- Spectral range : 220 ppm

Limitations
- Sensitivity

1H-NMR
- Spin= 1/2
- Natural abundancy : 99,98%
- Relative sensitivity to ^{1}H: 100 %
- Spectrale range : ~ 0 – 12 ppm
- Always used in metabolism

Limitations
- narrow spectral width

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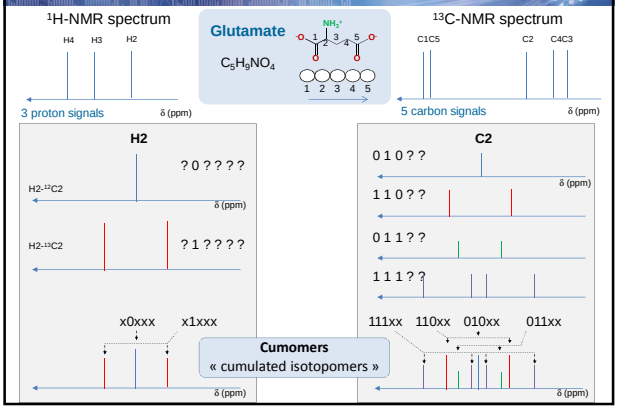
^{13}C Labeling patterns in NRM : Positional Isotopomers and Cumomers



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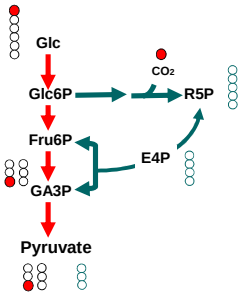
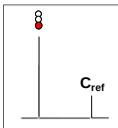
Isotopic profiling & pathway identification

• Specific enrichment = % of ^{13}C in a specific carbon position (varies from 1,1 % to 100%)



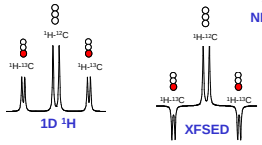
^{13}C-NMR



+ : Large spectral window
Access to all carbons
- : Low sensitivity
Need for quantitative reference
Indirect measurement
(metabolite pool known)

1D ^{13}C

^{1}H-NMR

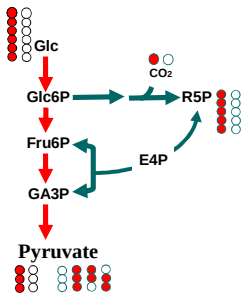


1D ^{1}H — XFSED

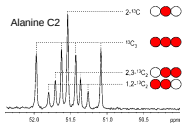
+ : High sensitivity
No calibration: direct measurement
- : Reduced spectral window
Access to protonated carbons only

RFMF Workshop - ^{13}C-fluxomics
Isotopic profiling & pathway identification

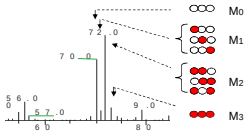
• Isotopomers = isotopic isomers
• detection by NMR or MS



NMR: positional isotopomers



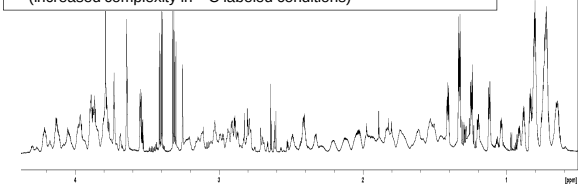
MS: isotopologues



RFMF Workshop - ^{13}C-fluxomics
Limits of 1D NMR

Only 1D NMR? A bit complex…

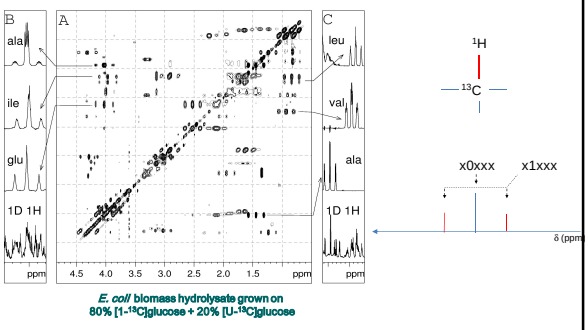
- 1D limitations (overlaps)
- Problem for resolution of mixture
- Particularly usefull for **isotopic analyses** in complex mixture (increased complexity in ^{13}C labeled conditions)



Need of a better resolution → **nD NMR**

RFMF Workshop - ^{13}C-fluxomics
2D NMR for ^{13}C labeling measurements

2D ZQF-TOCSY → ^{13}C Specific Enrichment

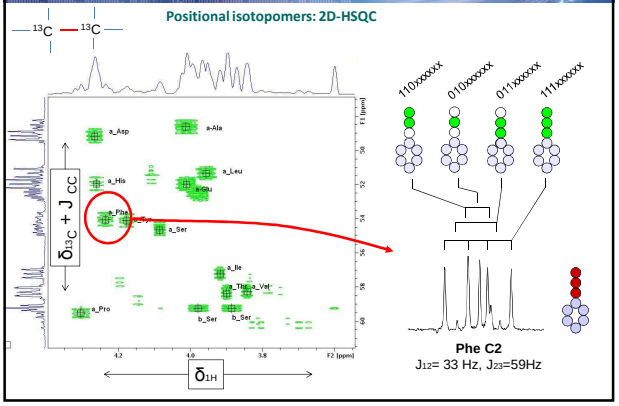


E. coli biomass hydrolysate grown on
80% [1-^{13}C]glucose + 20% [U-^{13}C]glucose

Massou et al. 2007

RFMF Workshop - ^{13}C-fluxomics
2D NMR for ^{13}C labeling measurements

Positional isotopomers: 2D-HSQC



Phe C2
J_{12}= 33 Hz, J_{23}=59Hz

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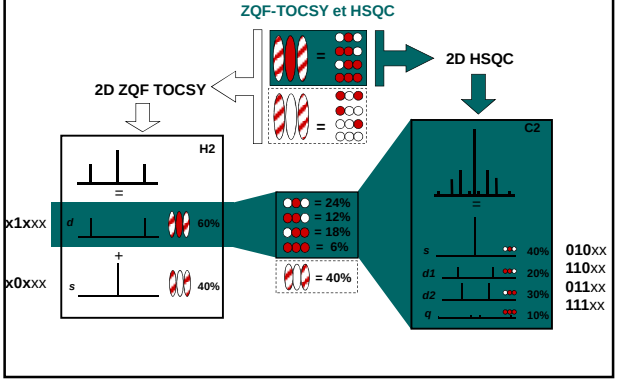
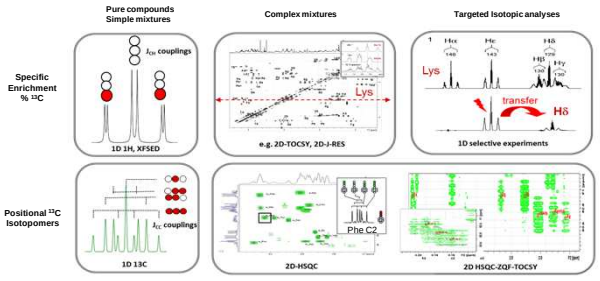
2D NMR for ^{13}C labeling measurements

ZQF-TOCSY et HSQC



RFMF Workshop - ^{13}C-fluxomics
2D NMR for ^{13}C labeling measurements



Szyperski, 1995 – Massou et al. 2007 – Nicolas et al., 2007 – Cahoreau et al., 2011

P65

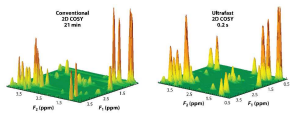
NMR-based strategies for high-throughput fluxomics

Edern Cahoreau[1,2], Lindsay Peyriga[1,2], Cécilia Bergès[1,2], Stephanie Heux[1], Jean-Charles Portais[1,2,3]

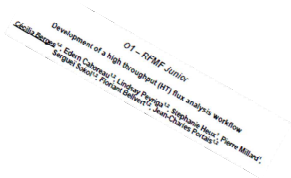
RFMF Workshop - ^{13}C-fluxomics
Perspectives & developments

NMR For ^{13}C-MFA

- Long acquisition time → development of high throughput 2D-NMR (fast and ultrafast…)
  H-Jres, Ultrafast-H-Jres, Ultrafast cosy



Giraudeau P, Frydman L. 2014. Annu. Rev. Anal. Chem. 7:129–61



- Not easily quantitative, **Validation of NMR methods is the key**
- Still some labeled informations difficult to reach
  - Unprotonated ^{13}C with heteronuclear 2Ds (COOH, C-OH, C=O, …)
  - Remaining signal overlaps in 2D

  → Development of new approaches
  (Adding a 3rd dimension: ^{15}N labeling for example…)
- Need to adapt methods and gain in sensitivity to measure **new targets (central metabolites, free Amino acids…)**

RFMF Workshop - ^{13}C-fluxomics

EC14

Part IV - MS-based Isotopic profiling

- Basic concepts
  - Why measuring fluxes
  - What is a flux and how to measure fluxes
  - Benefit of isotopic tracers
- Design of labelling experiment
  - Design of label input
  - 13C-labelling experiment
  - Sampling
- Isotopic profiling
  - NMR-based isotopic profiling
  - MS-based isotopic profiling
- Flux calcul
  - requirements
  - Flux calculation
  - Sensitivity analysis

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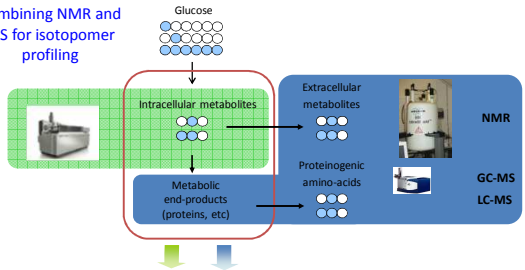
Diapositive 39

EC14 C'est bien la partie IV et non V?
Edern Cahoreau; 28/05/2016

RFMF Workshop - ^{13}C-fluxomics
MS-based isotopic profiling

Combining NMR and MS for isotopomer profiling



Increasing number and quality of isotopic data

e.g. *E. coli* grown on glucose: 253 isotopic data
- 57 LC-MS/MS data
- 63 GC-MSdata
- 133 NMR data

Valuable for pathway/network identification & highly-resolved flux maps

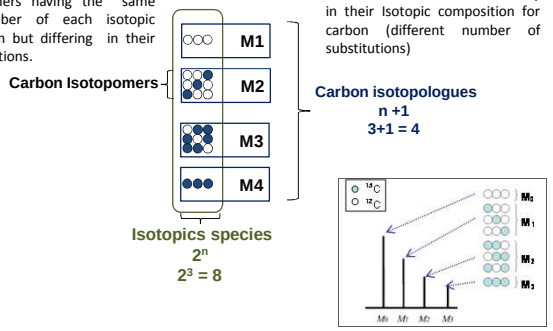
Expanding the range of applications (metabolic complexity)

Experimental design becomes critical

RFMF Workshop - ^{13}C-fluxomics
Definitions

Carbon isotopomer:
Isomers having the same number of each isotopic atom but differing in their positions.

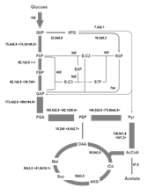
Carbon isotopologue:
Molecular entities that differ only in their Isotopic composition for carbon (different number of substitutions)



RFMF Workshop - ^{13}C-fluxomics
Approaches for Isotopic profile measurement by MS

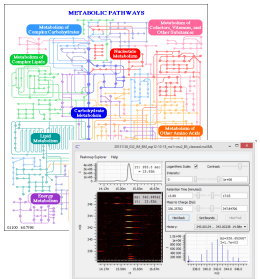
Two different approaches can be used:

targeted approaches:



- MS low resolution
- MS high resolution

untargeted approaches:

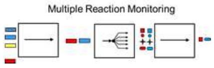


- MS high resolution

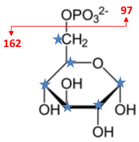
eMZed Kiefer et al. Bioinformatics. 2013 29:963-4

RFMF Workshop - ^{13}C-fluxomics
Isotopic profile measurement : Targeted approach with low resolution

low resolution MS: MRM mode



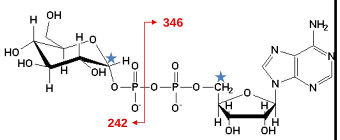
N+1 transitions when fragment without C
Ex: Glucose6P



M0: 259/97
M1: 260/97
…
M6: 265/97

➔7 transitions

Increase of transitions when fragment with C
Ex: ADP-glucose



M0: 588/346
M1-02: 589/346 M1-02: 589/347
M2-01: 590/346 M2-02: 590/347 M2-03: 590/349
…
M16: 604/346

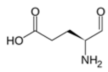
➔77 transitions

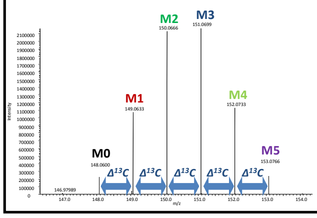
RFMF Workshop - ^{13}C-fluxomics
Isotopic profile measurement : Targeted approach with high resolution

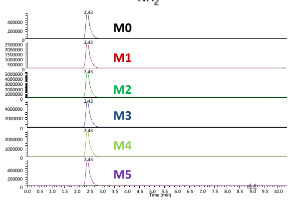
HRMS: strategy in mode MS Fullscan

Extraction of data (a posteriori)
- Calculation of the exact mass of [M+H]+ from formula
- Addition of $\Delta^{13}C$=1,0034 (in function of the number of C in molecule)
- Extraction of each isotopologue mass
- Integration of each peak

- *[M+H]⁺ Glutamate =148.0600 m/z*





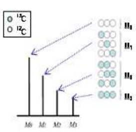


RFMF Workshop - ^{13}C-fluxomics
MS Carbon Isotopologue Measurement

<u>**Carbon Isotopologue Abundance (CIA)**</u> are given with a relative unit Mk : $(0 \leq Mk \leq 1)$:

$$Mk = \frac{Area\ Mk}{\sum_{i=0}^{n} Mi}$$

$$\text{Ex: } M0 = \frac{Area\ M0}{Area\ M0 + Area\ M1 + Area\ M2 + Area\ M3}$$



<u>**Carbon Isotopologue Distribution (CID)**</u> Is defined as the relative distribution of all carbon isotopologues existing for a given compound. (CID is a vector of CIA)

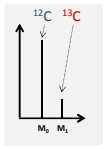
<u>**^{13}C enrichment:**</u>
^{13}C enrichment is the global ^{13}C percentage (or ratio) of a molecule.
^{13}C enrichment is given with a relative unit : $(0 \leq \%13C \leq 100)$:

$$\%\,^{13}C_M = \frac{0 * Area\ M0 + 1 * Area\ M1 + 2 * Area\ M2 \ldots + n * Area\ Mn}{n}$$

Correction of the natural abundance of the other elements presents in the molecule

RFMF Workshop - ^{13}C-fluxomics
Natural abundance correction

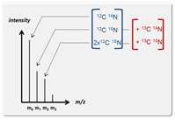
Low resolution vs High resolution



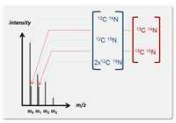
Example: CN

Contribution of naturally occurring isotopes

Element	Mostly occurring mass (M_0)	M_0	M_1	M_2	M_4
H	1	0.999885	0.000115		
N	14	0.99632	0.00368		
O	16	0.99757	0.00038	0.00205	
Si	28	0.922297	0.046832	0.030872	
P	31	1			
S	32	0.9493	0.0076	0.0429	0.0002



Low resolution : *Correction of naturally occurring isotopes*

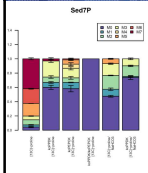


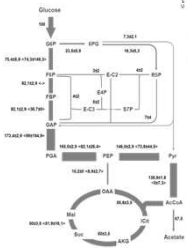
High resolution : *Correction depend of resolution for example : R 60 000: correction of O and H (R>22 000 for ^{15}N-^{13}C differentiation)*

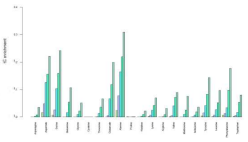
➔ Natural abundance correction with IsoCor software (Millard *et al.*, 2012)

IsoCor

RFMF Workshop - ^{13}C-fluxomics
Validation of isotopic profile measurements ?





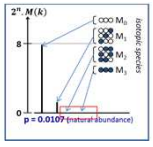


how to be sure about the quality of measures of isotopologues by mass spectrometry ?

RFMF Workshop - ^{13}C-fluxomics
Validation of isotopic profile measurements ?

- **Classical issues of MS-based quantification *(linearity, LOQ, precision, trueness…)***

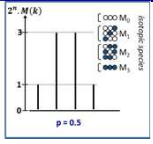
➔ **BUT apply to all isotopologues of a molecule**
- increased risk of contamination and overlap
- Validation for each individual isotopic species
- Linearity needed within the isotopic cluster (dynamic range ≠ between isotopologues)



$$M(k) = \binom{n}{k} . p^k . (1-p)^{n-k}$$

p=enrichment
n=number of ^{12}C+^{13}C
k= number of ^{13}C

All isotopic species in same proportion

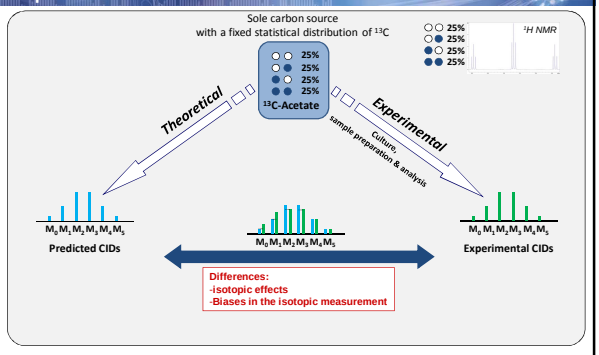


(Millard et al, Anal Chem 2014)

- **Standard sample for validation of isotopic measurements**
  - Commercial standards: expensive, not always available
  - Natural abundance: Not access to heavy isotopologues

- **Production of a labeled standard sample (p=0,5): predictable CIDs**
  ➔ labeling independant of the metabolic network

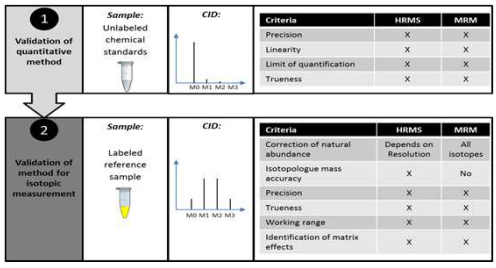
Reference material for validation of isotopic measurement



Validation in MRM for central metabolism compounds of *E. coli*

(Millard et al, Anal Chem 2014)

RFMF Workshop - ^{13}C-fluxomics
Validation of method for isotopic measurement



Criteria	HRMS	MRM
Precision	X	X
Linearity	X	X
Limit of quantification	X	X
Trueness	X	X

Criteria	HRMS	MRM
Correction of natural abundance	Depends on Resolution	All isotopes
Isotopologue mass accuracy	X	No
Precision	X	X
Trueness	X	X
Working range	X	X
Identification of matrix effects	X	X

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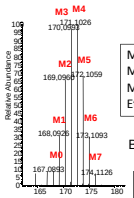
A workflow for the assessment of the quality of isotopologue distribution measurements by mass spectrometry

Maud Heuillet[1,2], Florian Bellvert[1,2], Edern Cahoreau[1,2], Fabien Letisse[1], Pierre Millard[1], Jean-Charles Portais[1,2]

RFMF Workshop - ^{13}C-fluxomics
Validation of method for isotopic measurement

HR mass spectrometer accuracy

Isotopologue mass accuracy : Agreement between the exact mass of the ^{13}C isotope (+1.0034 m/z) and the mass differences between the carbon isotopologue peaks in the isotopic cluster.

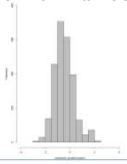


M1-M0= $\Delta^{13}C_{measured}$
M2-M1= $\Delta^{13}C_{measured}$
M3-M2= $\Delta^{13}C_{measured}$
Etc....

Bias=$\Delta^{13}C_{measured}$ - $\Delta^{13}C_{theorétical}$

Data amino acids:
$\Delta^{13}C_{measured}$ =1,00333 ± 0,0004 m/z
Bias= -2,4 x 10^{-5} m/z

HR mass spectrometer trueness : closeness of agreement between the exact mass measured for each carbon isotopologue of a metabolite with that of the exact mass predicted for this isotopologue from its elemental formula.



Ex: Alanine
$[M+H]^+$: $C_3H_8NO_2$

Alanine M0 $m/z_{predicted}$: 90.05496
Alanine M0 $m/z_{measured}$: 90.05485

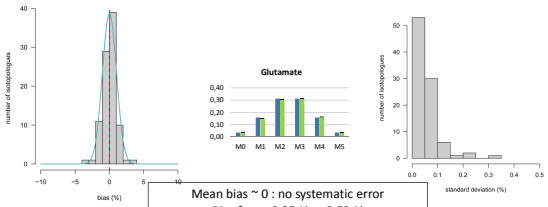
Δ measured-predicted: -1.22 ppm

RFMF Workshop - ^{13}C-fluxomics
Validation of method for isotopic measurement

CID measurement accuracy

CID Trueness: closeness of agreement between the measured CIDs and the CIDs predicted for the reference material. It is expressed as bias.

CID Precision: spread of measured CID values calculated from replicate measurements of the reference material. It is expressed as standard deviation.

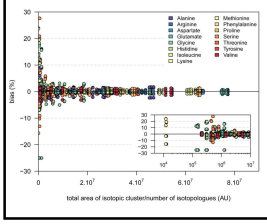


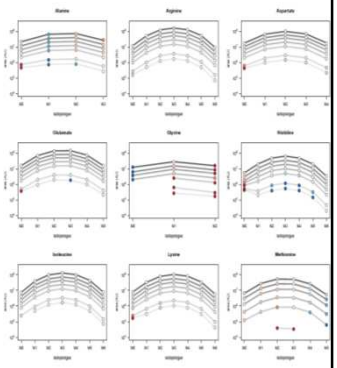
Mean bias ~ 0 : no systematic error
Bias from -2.97 % to 2.72 %
SD: 0.8%

RFMF Workshop - ^{13}C-fluxomics
Validation of method for isotopic measurement

Working range of isotopic measurement: Trueness measured for each isotopologue for various amounts of reference material.

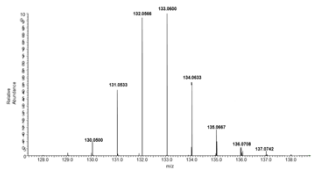
The LOQ is best determined from the ratio "total isotopic cluster area/ number of isotopologues " is below 5% of bias .

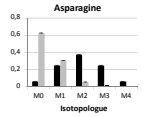




Validation of method for isotopic measurement

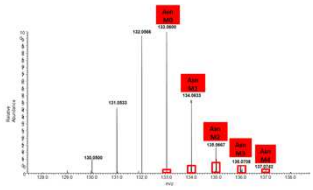
Identification of potential analytical issues: Check that there was no overlap of isotopic clusters on the whole chromatogram indicating, indicating a co-elution, occurrence of (unlabeled) contaminants, etc.

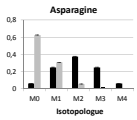




RFMF Workshop - ^{13}C-fluxomics
Validation of method for isotopic measurement

Identification of potential analytical issues: Check that there was no overlap of isotopic clusters on the whole chromatogram indicating, indicating a co-elution, occurrence of (unlabeled) contaminants, etc.





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Validation of method for isotopic measurement

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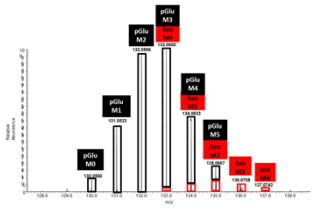
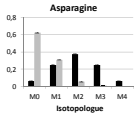
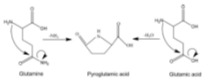




In source cyclisation of Glutamic acid and Glutamine on Pytoglutamate



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Part V – Flux calculation

- Basic concepts
  - Why measuring fluxes
  - What is a flux and how to measure fluxes
  - Benefit of isotopic tracers
- labelling experiment
  - Design of label input
  - 13C-labelling experiment
  - Sampling
- Isotopic profiling
  - NMR-based isotopic profiling
  - MS-based isotopic profiling
- Flux calcul
  - requirements
  - Flux calculation
  - Sensitivity analysis

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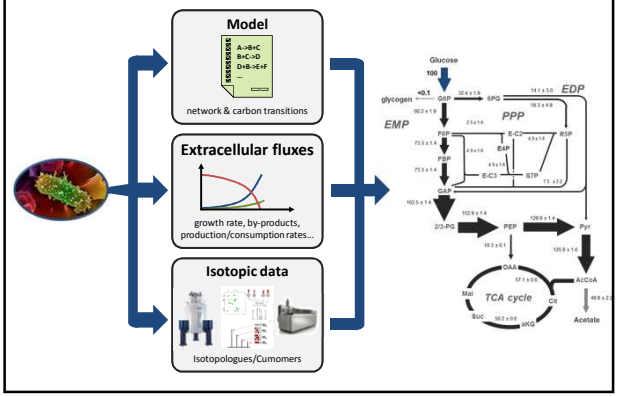
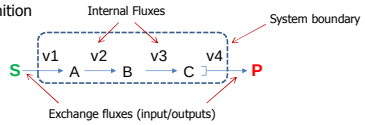
Flux calculation: required information



Module n° 1 – Flux theory
Metabolic Flux Analyses: Stoichiometric modeling

Step 1: System definition



Step 2: Mathematical formalism

$$\begin{aligned} dA/dt &= v1 - v2 \\ dB/dt &= v2 - v3 \\ dC/dt &= v3 - 2*v4 \end{aligned}$$

$$\begin{bmatrix} dA/dt \\ dB/dt \\ dC/dt \end{bmatrix} = \begin{bmatrix} 1 & -1 & 0 & 0 \\ 0 & 1 & -1 & 0 \\ 0 & 0 & 1 & -2 \end{bmatrix} . \begin{bmatrix} v1 \\ v2 \\ v3 \\ v4 \end{bmatrix}$$

Mass balance

metabolite vector — flux vector

$$dM/dt = S \cdot v$$

stoichiometric matrix

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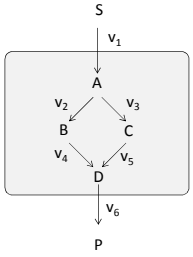
Flux calculation: system definition

vS = 1.0 ± 0.1 mmol/gBM/h



vP = 1.05 ± 0.12 mmol/gBM/h

At metabolic steady-state:

$$dM/dt = 0$$
$$S \cdot v = 0$$

Network topology

$$\begin{bmatrix} dA/dt \\ dB/dt \\ dC/dt \\ dD/dt \end{bmatrix} = \begin{bmatrix} 1 & -1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & -1 & 0 & 0 \\ 0 & 0 & 1 & 0 & -1 & 0 \\ 0 & 0 & 0 & 1 & 1 & -1 \end{bmatrix} . \begin{matrix} v1 \\ v2 \\ v3 \\ v4 \\ v5 \\ v6 \end{matrix}$$

degrees of freedom: F=2

+ Measurement of extracellular fluxes ($v_{1,6}$)

degrees of freedom: F=1

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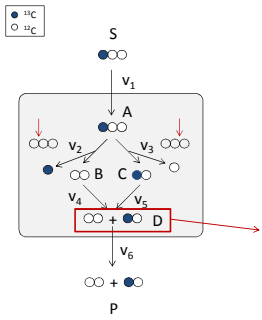
Flux calculation: system definition



Network topology

$$\begin{bmatrix} dA/dt \\ dB/dt \\ dC/dt \\ dD/dt \end{bmatrix} = \begin{bmatrix} 1 & -1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & -1 & 0 & 0 \\ 0 & 0 & 1 & 0 & -1 & 0 \\ 0 & 0 & 0 & 1 & 1 & -1 \end{bmatrix} \cdot \begin{matrix} v1 \\ v2 \\ v3 \\ v4 \\ v5 \\ v6 \end{matrix}$$

degrees of freedom: F=2

+ Measurement of extracellular fluxes ($v_{1,6}$)

degrees of freedom: F=1

+ Measurement of labeling (D or P)

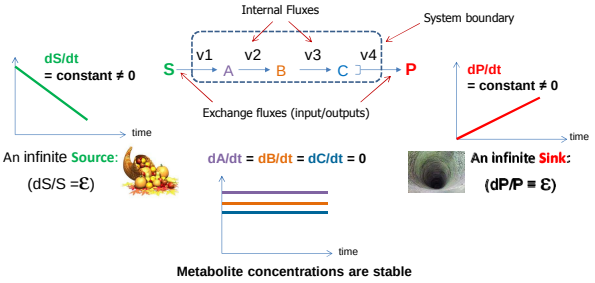
Eq 1: $D_{00}/(D_{00}+D_{10}) = v_4/(v_4+v_5)$

Eq 2: $D_{01}/(D_{00}+D_{10}) = v_5/(v_4+v_5)$

Eq 1 / Eq 2: $D_{00}/D_{01}=v_4/v_5$

degrees of freedom: F=0

Module n° 1 – Flux theory
Metabolic Flux Analyses: Metabolic steady-state



An infinite **Source**:
(dS/S =Ɛ)

dA/dt = dB/dt = dC/dt = 0

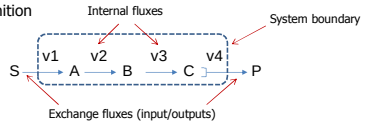
An infinite **Sink**:
(dP/P ≡ Ɛ)

Metabolite concentrations are stable

$$\boldsymbol{dM/dt = 0}$$
$$\boldsymbol{S \cdot v = 0}$$

Module n° 1 – Flux theory
Metabolic Flux Analyses: System determination

Step 1: System definition



Step 2: Mathematical formalism

Can we find v such that S.v=0 ?

m : Number of equations (=internal metabolites)

$$\begin{bmatrix} dA/dt \\ dB/dt \\ dC/dt \end{bmatrix} = \begin{bmatrix} 1 & -1 & 0 & 0 \\ 0 & 1 & -1 & 0 \\ 0 & 0 & 1 & -2 \end{bmatrix} \cdot \begin{bmatrix} v1 \\ v2 \\ v3 \\ v4 \end{bmatrix}$$

n : Number of variables (=fluxes)

Degrees of freedom: F=n-m

F=0	System determined
F<0	System overdetermined
F>0	System undetermined

Module n° 1 – Flux theory
Metabolic Flux Analyses: How to calculate fluxes

Case 1: the system is determined from the measurement of extracellular fluxes

solve $S \cdot v = 0$

Case 2: the system is undetermined — Need additional constraints/equations

2 different yet complementary approaches

Flux balance analysis

In silico analysis 

Find a set of fluxes that are optimal according to a particular objective

^{13}C-metabolic flux analysis

In vivo analysis

Find a set of fluxes that explains experimental data

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Flux calculation: available software

- Many software are available
- Differ in the type of isotopic data that can be included, level of details, mathematical formalism, calculation times, robustness, accuracy…
- A few ones (not exhaustive!):

Influx_s (Sokol *et al.*, 2012)	13CFlux (Wiechert *et al.*, 2001)	OpenFlux (Quek *et al.*, 2009)	FiatFlux (Zamboni *et al.*, 2005)
NMR and MS		MS only	
Flux distribution			Local flux ratios
Rapid, stable	Slow calculation and/or need repetitions (low stability)		
Non-linear statistics	Linear statistics	Non-linear statistics	

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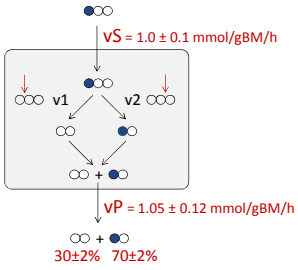
Flux calculation: dealing with experimental errors

Experimental data (in/out fluxes & metabolite labeling) contain errors



No exact solution, we are looking for an optimal solution

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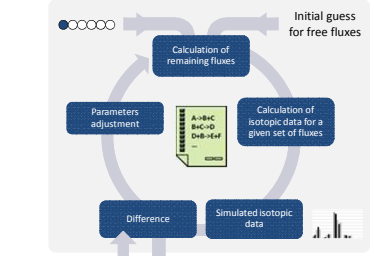
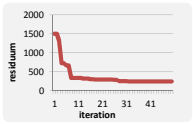
Flux calculation: principle

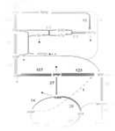
Fitting procedure



$$residuum = \sum_i \left(\frac{sim_i - meas_i}{sd_i}\right)^2$$

An iterative approach

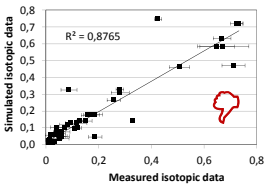




Optimal flux distribution

Module n° 5 – Flux calculation
Metabolic Flux Analyses: Evaluation of fitting quality

Case 1

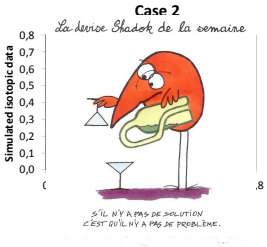


65 data
residuum = 164.2
khi2 = 5.4

At level of 95% confidence, model 1 does not fit the data good enough with respect to the provided measurement SD

Rule of thumb: residuum < data ?
Statistical evaluation : khi-2 test

Case 2



residuum = 17.3
khi2 = 0.5

At level of 95% confidence, model 2 fits the data good enough with respect to the provided measurement SD

Module n° 5 – Flux calculation
Metabolic Flux Analyses: Sensitivity analysis

Many phenomenon cause uncertainty in measurements

Extracellular fluxes

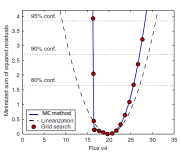
growth rate, by-products, production/consumption rates...

Isotopic data

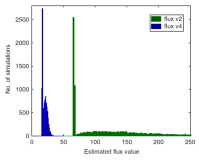
Isotopologues/Cumomers

Impact of measurements uncertainty on the calculated fluxes?

Linear vs Non linear statistics



Monte carlo (nonlinear) statistics



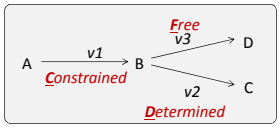
Module n° 5 – Flux calculation
Metabolic Flux Analyses: FTBL model

PROJECT : **metadata** (date, version, model name…)

NETWORK : list of all reactions (stoichiometry + carbon atom transitions)

Flux_name	*EDUCT_1*	*EDUCT_2*	*PROD_1*	*PROD_2*
ta	Ery4P	Fru6P	GA3P	Sed7P
	#ABCD	#abcdef	#def	#abcABCD

FLUXES: NET and ***XCH***



EQUALITIES and ***INEQUALITIES***

e.g., for an irreversible reaction: XCH: C, 0 ; and NET > 0

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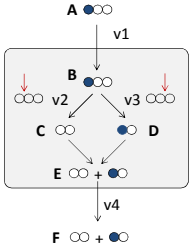
Experimental design



A	F through v2	F through v3	v2 and v3 determined by
#100	#00	#10	positional data, CID
#010	#10	#01	positional data only
#001	#01	#00	positional data, CID
#111	#11	#11	no
…	…	…	…

Identifiability and precision on the fluxes = f(- label input - accessible isotopic data - precision on the measurements)

➡ **Needs to maximize information on fluxes with minimal efforts**

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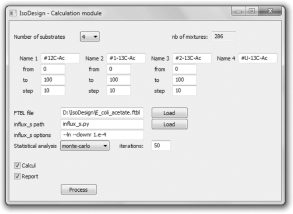
Experimental design

IsoDesign: A software for optimizing the design of ^{13}C-metabolic flux analysis experiments

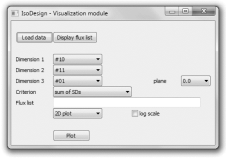
Millard et al, 2014, Biotech Bioeng 111:1,202

A

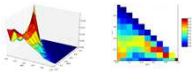


Calculation module:
- Label input up to 6 isotopic forms
- All kind of data (MS, MS/MS, ^{1}H NMR, ^{13}C NMR…)
- Non linear methods

B



Visualization module:
- Many scoring criteria
- Sensitivity landscape



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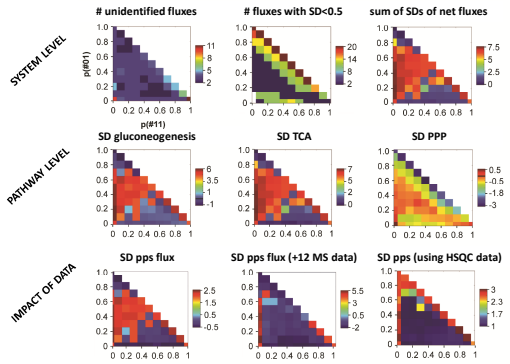
Experimental design



The optimal label input depends on the biological question

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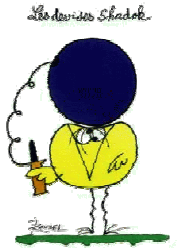
Take home message

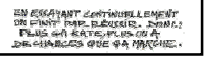
Fluxes can only be calculated provided:

- biochemical knowledge is sufficient (no *ab initio* approach to date)
- experiment has been correctly DESIGNED
- experimental data are RELIABLE

Check extensively the results!



^{13}C-flux calculation is sometimes a long and painfull process…



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Acknowledgements

The documents presented in this workshop come from a fluxomics training currently in preparation at MetaboHUB-MetaToul.

The following people have contributed to these documents (listed by alphabetical order):

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^{13}C-Fluxomics – Flux map