LUNCHEON SEMINAR
METABOLOMICS AND BEYOND: SOLUTIONS FOR UNDERSTANDING METABOLISM

- dMRM Database and Method for the analysis of central carbon metabolites
- VistaFlux for qualitative flux analysis
- Seahorse XF metabolic analyzers for in vivo cellular metabolism
Metabolomics and Beyond: Solutions for Understanding Metabolism

Christine Miller
Norton Kitagawa
Alex Liversage

Metabolomics Society 2016
Agilent – THE Leader in Metabolomics!

Agilent Innovations for Metabolomics

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Enabling Metabolomics Research

Agilent Technologies is the leading company with platforms and software that support metabolomics research

• Team of knowledgeable scientists dedicated to metabolomics

• Broad instrument portfolio (LC/MS, CE/MS, GC/MS, SFC/MS)

• Common MS raw data processing software

• Single statistics analysis software — use the same visualization and statistical analysis tools for your metabolomics (and multi-omics!) study

• New MassHunter VistaFlux for qualitative flux analysis

• Seahorse technology for cellular metabolic analysis
Most Extensive Software Portfolio for Metabolomics Data Analysis

- Find features across many complex data files
- Align extraction results from many files
- Analyze extracted data for statistically different features
- Annotate and identify interesting features
- Search and map annotated metabolites to find interesting pathways
- Visualize results on pathways
New Features in MassHunter Profinder B.08.00

Targeted isotopologue feature extraction for LC/MS data using a target list with retention times

• Single file export (Profinder Archive .pfa) for project data to Omix Premium

Untargeted and recursive feature extraction support for both GC/Q-TOF and GC/MSD

All Ions fragment confirmation support for both LC/TOF and GC/TOF data files

• Targeted feature extraction with MS/MS confirmation using PCDL libraries

Faster data processing for large MassHunter Profinder projects

• All feature extraction algorithms are multithreaded
• Memory consumption cut in half
Metabolomics dMRM Database & Method

Targeted Analysis for Central Carbon Metabolism
Metabolomics dMRM Database and Method
*Integrated with Agilent’s Metabolomics Workflows*

Database covers >215 central carbon metabolites

Designed for 1290 Flex pump and 6460/6470 QQQ LC/MS Systems

Provides an optimized method and database with stable, robust chromatography
Metabolomics dMRM Database and Method

**LC/QQQ System**

Agilent 1290 Infinity II LC system:
- Infinity II Flex pump
- Multisampler with chiller
- Multicolumn Thermostat with 2-position/6-port ultra high pressure valve

Agilent 6460 or 6470 Triple Quadrupole LC/MS system
Curated MRM Transitions

The compound database with MRM transitions was developed together with the Rosebrock Lab.

Compound and MRM transitions are extensively curated.

<table>
<thead>
<tr>
<th>Curation verifies these entries for correctness:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Database</strong></td>
</tr>
<tr>
<td>• Compound common name</td>
</tr>
<tr>
<td>• MRM transitions (precursor and product m/z)</td>
</tr>
<tr>
<td>• Fragmentor voltage</td>
</tr>
<tr>
<td>• Collision energy</td>
</tr>
<tr>
<td>• Retention time and retention time window</td>
</tr>
<tr>
<td>• Unit mass of neutral molecule</td>
</tr>
<tr>
<td>• Molecular formula</td>
</tr>
<tr>
<td>• CAS number of the native compound</td>
</tr>
</tbody>
</table>

The curated database eliminates the need to develop MRM transitions and retention times for a large numbers of compounds.
Chromatographic Separation of Acidic Metabolites

Negative Mode Ionization

- Mobile phase: solvent A (97:3 water/methanol with 10 mM tributylamine + 15 mM acetic acid) and solvent B (97:3 acetonitrile/methanol with 10 mM tributylamine + 15 mM acetic acid).
- Columns: Zorbax RRHD Extend-C18 2.1 x 150 mm column and guard column
Why Reverse Phase Ion Pair Chromatography?

- Better separation for acidic compounds
- Better protection for sugar phosphates and nucleotides
- Excellent retention time reproducibility across large sample sets

However when switching polarities to positive mode:

- A cleaning procedure must be followed to remove ion pair reagents, which stick to LC parts and the MS source.
Ion Pair Method Separates Biologically Relevant Isomers

- Isoleucine
- Leucine

- Glucose-6-phosphate
- Glucose-1-phosphate
Demonstrated Robustness of Reverse Phase Ion Pair Chromatography

RT Deviation for 62 different samples over 3 days

RT Reproducibility After 7 Months (> 4000 sample injections!)

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Easy Transfer From Database to Method in MassHunter
Simple Quantitative Batch Review and Export to MPP

MassHunter Quant Batch Review

Export to MPP

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Agilent Technologies
Metabolomics is uniquely suited for pre-built methods: Compounds of interest are shared across samples

The same metabolites and pathways are present across many organisms, allowing use of one method for many biological samples and questions.

Data from Adam Rosebrock Lab, University of Toronto
Summary

Agilent offers an comprehensive metabolomics portfolio that includes sample preparation, data acquisition and data analysis.

Metabolomics dMRM Database and Method provides an optimized, robust analytical method for routine targeted analysis of more than 215 central carbon metabolites.
Acknowledgements

- Adam Rosebrock
  • Soumaya Zlitni
- Amy Caudy

- Eric Brown
MassHunter VistaFlux

Qualitative Flux Analysis
Stable Isotope Tracing Using MassHunter VistaFlux

**Qualitative Flux Analysis**

- Metabolomics provides static information on cellular molecular composition
- Qualitative flux analysis reveals *in vivo* pathway activity
- Qualitative flux analysis tracks the flow of metabolites through a pathway

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VistaFlux Stable Isotope Tracing

Isotopologue Tracking

Use of stable isotope labels (\(^{13}\mathrm{C},\ ^{15}\mathrm{N},\ \text{and}\ ^{2}\mathrm{H}\))

Monitor stable isotope incorporation

Fumarate \(\mathrm{C}_4\mathrm{H}_4\mathrm{O}_4\)

**Isotopologues**

- **M+0**: \(^{13}\mathrm{C}_0\mathrm{C}_4\mathrm{H}_4\mathrm{O}_4\)
- **M+1**: \(^{13}\mathrm{C}_1\mathrm{C}_3\mathrm{H}_4\mathrm{O}_4\)
- **M+2**: \(^{13}\mathrm{C}_2\mathrm{C}_2\mathrm{H}_4\mathrm{O}_4\)
- **M+3**: \(^{13}\mathrm{C}_3\mathrm{C}_1\mathrm{H}_4\mathrm{O}_4\)
- **M+4**: \(^{13}\mathrm{C}_4\mathrm{C}_0\mathrm{H}_4\mathrm{O}_4\)

Counts vs. Mass-to-Charge (m/z)

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4 M+1 Isotopomers
Manual Qualitative Flux Analysis Workflow

Current Flux Data Analysis

- Create target metabolite list
- Determine $m/z$ for all possible isotopologues
- Extract isotopologues for targets
- Integrate peaks and copy results to Excel
- Correct for natural isotope abundance
- Manually visualize on pathways

- Tedious, error-prone and time-consuming
- Limits the number of compounds analyzed
Batch Qualitative Flux Analysis Workflow

**Agilent’s VistaFlux Workflow**

- **Create Target Metabolite List**
- **Acquire Data**
  - MassHunter Acquisition
  - LC/TOF or LC/Q-TOF
- **Extract Features**
- **Profinder**
- **Visualize on Pathways**
  - Omix Premium

**Pathways to PCDL**
**PCDL Manager**

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Target List Creation Based on Pathways of Interest

Pathways to PCDL

Select pathway(s) of interest
- Supports BioCyc, KEGG and WikiPathways

Select a subset metabolite list from METLIN PCDL
- Includes all metabolites (eliminates duplicates)
- Includes name, structure, empirical formula and identifiers
- Includes MS/MS spectra
Acquire Agilent LC/MS Data for Targeted Metabolites

*TOF/Q-TOF LC/MS Analysis Considerations*

**LC**
- Separate isomeric metabolites to eliminate isotopologue interference
- Separate metabolites with overlapping isotopologues

**MS**
- High mass accuracy and resolution to confidently track isotopologues
- Good isotopic fidelity to provide accurate ratios to track incorporation levels
- Wide dynamic range for detection of low and high intensity isotopologues in a single experiment
Targeted Data Mining

MassHunter Profinder Batch Isotopologue Extraction

Compound Table

Data File Details

Compound EICs

Compound MS

Isotopologues

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VistaFlux Demo
Visualizing Qualitative Flux Results

Quick Summary of Isotopologue Results on Network

- Metabolite node coloring gives quick summary of results on network
- Fold-change (left), label incorporation (middle) and fractional labeling (right) are shown here
Visualizing Qualitative Flux Results

Quilt Plot Display of Isotopologue Results

- Quilt plot displays all time points and isotopologue results
- Statistically significant label incorporation shown with *
Visualizing Qualitative Flux Results

Label Incorporation Display of Isotopologue Results

- Can display metabolite abundance, label incorporation, or fractional labeling
- Percent label incorporation shown here with unlabeled and labeled for each metabolite

Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center
Comprehensive Network View of Isotopologue Results

Cancer Cell Line with Enzyme Mutation (IDH2)

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Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center
Summary

Agilent offers an comprehensive metabolomics portfolio that includes sample preparation, data acquisition and data analysis. The new Agilent VistaFlux workflow easily extracts isotopologue information and visualizes results on pathways.

- Manual EIC extraction for each isotopologue and natural isotope correction for 5 metabolites at all time points (15 data files) required 2 weeks.
- The VistaFlux workflow performed the same analysis for 17 metabolites in about 2 minutes.
Acknowledgements

- Justin Cross
- Hui Liu
- Weige Qin
- Hardik Shah
- Bhavapiya Vaitheesvaran
- Vladimir Yong
- Lucia Salamanca-Cardona
- Kayvan R. Keshari
Metabolic Analyzers

Alex Liversage
UK & IRE Seahorse Instrument Specialist
Seahorse is the market leader in cellular energy metabolism

>1,300 instruments
>10,000 users

>2,400 publications (Jan 2016)

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No matter what you call it … energy metabolism affects every therapeutic area

**Cancer Letters**

*Metabolic reprogramming supports the invasive phenotype in malignant melanoma*

*Bettum et al*

**Nature**

*Metabolic rescue in pluripotent cells from patients with mtDNA disease*

*Ma et al*

**Retrovirology**

*HIV-1 pathogenicity and virion production are dependent on the metabolic phenotype of activated CD4+ T cells*

*Hegedus et al*

**Cell Reports**

*Article*

*Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research*

*Moulian et al*

**PNAS**

*CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability*

*W. Van der Windt et al*

**Research Article**

*Systemic Lupus Erythematosus*

*Normalization of CD4+ T cell metabolism reverses lupus*

*Yin et al*

**Nature Cell Biology**

*PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis*

*LeBleu et al*

**Nature Cell Biology**

*Ablation of LGR4 promotes energy expenditure by driving white-to-brown fat switch*

*Wang et al*
Metabolism – a lot you can look at

Many don’t know where to start
But if something changed the cells energy metabolism

...Seahorse sees it! ...in real time!!
Cells Generate Energy via Two Metabolic Pathways

Glycolysis

- Fermentation of:  
  - Glucose

[ pH ]

Respiration

- Oxidation of:  
  - Glucose  
  - Fat  
  - Amino Acids

Oxygen

Metabolism in XF assays is measured via the *rates of change* in pH and oxygen concentration.
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Cellular energy metabolism for any researcher, regardless of training

Kits (Reagents) ➔ Instrument ➔ Cells

No Sample Prep
[Just add cells]

Kits
[Open and go]

Fast Results
[In <90 minutes]

Analysis Tools
[Guides understanding]
The most rapid, user friendly measure of energy metabolism: The XF Cell Energy Phenotype Test

Resuspend kit Reagents (oligomycin & FCCP)

Pair sensor cartridge with miniPlate containing your cells

Run assay on your XFp

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Define metabolic phenotypes

XF Energy Map

- Aerobic
- Energetic
- Neurons
- Activated T cells
- Memory T Cells
- Tumor Cells
- Glycolytic
- Quiescent

Glycolysis

Extracellular Acidification Rate (ECAR)

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Measure metabolic potential

XF Energy Map

- Aerobic
- Stressed Neurons
- Quiescent
- Glycolytic
- Mitochondrial Respiration
- Oxygen Consumption Rate (OCR)
- Extracellular Acidification Rate (ECAR)

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Measure metabolic potential

XF Energy Map

- Aerobic
- Energetic
- Stressed
- Impaired Neurons
- Quiescent
- Glycolytic
- Glycolysis
- Extracellular Acidification Rate (ECAR)

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Measure metabolic potential

**XF Energy Map**

- **Aerobic**
  - Stressed
  - At risk Neurons
  - Quiescent

- **Glycolytic**

- **Energetic**

**Glycolysis**

- Extracellular Acidification Rate (ECAR)

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Watch cells switch metabolic pathways

**XF Energy Map**

- **Mitochondrial Respiration**
  - Aerobic
  - Energetic

- **Oxygen Consumption Rate (OCR)**

- **Glycolysis**
  - Quiescent
  - Glycolytic

- **Extracellular Acidification Rate (ECAR)**

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Watch cells switch metabolic pathways

XF Energy Map

Oxygen Consumption Rate (OCR)
Mitochondrial Respiration

Aerobic
Stressed
p53 Arrested tumor Cell

Glycolytic
Stressed
Tumor Cells

Energetic

Quiescent

Glycolysis

Extracellular Acidification Rate (ECAR)

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Watch cells switch metabolic pathways

If you see a change in a Seahorse assay you know it's important to the cell.

You can't be as confident with other non-live cell techniques.

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Metabolic programs control immune cell differentiation and function

Naive T cell
Activate T cell
Effector T cell
Memory T cell

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Seahorse standardized assays address the most common questions.

Metabolic Phenotype

Glycolysis

Mitochondria

Glucose, Glutamine, Fatty Acids

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Exponential increase in publications combining cellular metabolism and molecular metabolism

Cumulative XF + MS Publication Count thru End 2015

- Total number of publications citing XF data: ~2400
- ~400 publications citing XF data and Mass Spec data
What is the basis of the synergy between XF and MS?

Seahorse XF Assays

1. Cellular metabolic rates
2. Measure rate of mitochondrial respiration and glycolysis (big picture)
3. Quantify rate of substrate/fuel pathways (e.g. glucose, glutamine, etc.)

Agilent Metabolomics/Flux Analyses

1. Detailed molecular pathways
2. Map glycolytic and mitochondrial pathways (detailed view)
3. Trace the flow of substrates through metabolic pathways

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XF assays target and guide metabolomic/flux studies (experimental design, time course assessment, culture conditions, etc.)

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**Rate**

**XF substrate assay points to palmitate as key nutrient for unique class of lymphoma**

U\(^{13}\)C-Palmitate tracer assay explains role of fatty acids in tumor growth and survival

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Seahorse energy map proves that cells can respire without a functional TCA cycle

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\(^{13}\)C-tracer assay maps the survival pathway in cells with a dysfunctional mitochondria

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Seahorse XF assay confirms glycolytic shift required for Dendritic cell maturation

Dendritic cell genomic screen hits multiple glycolytic genes

Seahorse XF assay confirms glycolytic shift required for Dendritic cell maturation


Seahorse energy map shows novel kinase controls macrophage activation through glycolytic switch


Proteomics screen identifies novel kinase regulating macrophage activation

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Metabolomics and $^{13}$C flux data enhanced and extended by Seahorse XF assays

Metabolomics identified novel gene that regulates entry of pyruvate to mitochondria

Seahorse provided function confirmation

$^{13}$C flux shows two possible pathways for glutamine in cancer cells

Seahorse determines mito respiration rate in 2D and 3D tumors to establish relevant flux
Novel cancer pathway: glutamine running in ‘reverse’

Required combination of Agilent and Seahorse instruments to reveal

“We had extensively characterized intermediary metabolism using isotope tracing and mass spectrometry, but we needed precise measurements of oxygen consumption to complete the analysis.”

Ralph Deberardinis, M.D., Ph.D.

“Altogether, I feel this is a prime example of how mass spectrometry and Seahorse can be integrated to understand metabolic phenotypes.”
An instrument for every need and purpose

XFe96 Analyzer
Most versatile
3D biology
(96 well)

XFp Analyzer
Routine and
precious samples
(8 well)

XFe24 Analyzer
Moderate throughput
Large well volume
(24 well)
Where to find out more and learn

Products and Technology

- Product Brochures
- Product Flyers
- Research Specific Brochures
- Pre-sales slide deck
- Technical Literature
  - Application Notes
  - Technical Briefs
  - Assay Protocols
  - Technical Tips and Hints
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- Live Chat

Available on website, [www.seahorsebio.com](http://www.seahorsebio.com) until June 6

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Understand Cellular and Molecular Metabolism

Agilent Seahorse and Mass Spectrometry Together

Cellular Metabolism

Molecular Metabolism

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