Lipidomics Workshop
8:00AM-1:00 PM, Saturday, April 28, 2007
Grand Ballroom South of the Renaissance Hotel
999 Ninth Street NW, Washington, DC
Al Merrill cell phone: 678-895-7029
8:00  Welcome and overview of the Lipid Maps Consortium and world-wide lipidomics initiatives. Edward Dennis

8:15  Analytical technologies panel. Al Merrill, Moderator

8:20  Brief introduction to lipid analysis by mass spectrometry and challenges, as illustrated with neutral and phospho-glycero lipids. Robert Murphy

9:10  Sphingolipids (and precursor fatty acyl-CoA’s). M. Cameron Sullards

9:35  Sterols. Jeffrey McDonald

10:00  Coffee break

10:15  Eicosanoids. Rick Harkewicz

10:40  Novel lipid analysis. Teresa Garrett

11:05  Internal standards for lipidomic analysis. Walt Shaw

11:30  Data handling and bioinformatics, pathway visualization & interpretation. Eoin Fahy

11:55  Close of general session, follow-up open discussions over snacks

1:00  End of session

Support for this workshop has been provided by the American Society for Nutrition, the LIPID MAPS Glue grant (NIH U54 GM069338), Avanti Polar Lipids, Advion Biosystems and Applied Biosystems.
Sphingolipids (and precursor fatty acyl-CoA’s)
M. Cameron Sullards

Schools of Chemistry, Biochemistry and Biology & Petit Institute for Bioengineering and Bioscience Georgia Institute of Technology

Other LIPID MAPS Sphingolipid Core members:

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<th>Field</th>
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<tr>
<td>Mass spectrometry</td>
<td>Al Merrill</td>
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<td>Cell biology</td>
<td>Jeremy Allegood</td>
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Outline:
A. Brief introduction to the lipid class: nomenclature & range of compounds to analyze
B. Sample preparation issues: solvents, chromatography, recovery, reproducibility
C. Compound identification: Characteristic fragmentations; MS/MS and MS$^n$ (LC for isomers and isomers, etc.)
D. Quantitation: Internal standards, etc.
E. Data analysis/visualization: LIMS, Website, Marker View, other.
F. Comparison of Lipid MAPS methods with others in the literature
G. Remaining challenges and opportunities
H. Discoveries from sphingolipidomic analysis thus far
A. Brief introduction to the lipid class: nomenclature & range of compounds to analyze

**Backbone variation**

**Sphingoid base:**
- Sphinganine (d18:0)
- 4-Hydroxysphinganine (phytosphingosine) (t18:0)
- D-erythro-sphingosine (d18:1)

**Ceramide:**
- Shown: N-palmitoylsphingosine (d18:1/16:0)
- Other fatty acids—typically C16-C26
- 0-1 double bond
- sometimes α-hydroxy

**Headgroup variation**

**Phosphosphingolipids:**
- \(-\text{OP(O}_2^-\text{)}\text{O-}\text{choline, Sphingomyelin}\)

**Glycosphingolipids:**
- Glc, Gal, Lac, Sulfatides…>400; see www.lipidmap.org; www.sphingomap.org

**Lactosylceramide (LacCer)**

**GalNAc III**

**Gal IV**

**Gal II**

**Glc I**

**NeuAc**

**Neu5Acα2-3(Galβ1-3GalNAcβ1-4)Galβ1-4Glcβ1Cer**

**II^3Neu5AcGg₄Cer**

**Neu5Ac**

**GM1**

**GM3**
SphinGOMAP (www.sphingomap.org)
B. Sample preparation issues: solvents, chromatography, recovery, reproducibility:

A (Polar):
- 30-100 mm Petri dish w/cells
- PBS scrape pellet
- + CH$_3$OH + CHCl$_3$ + Internal Stds (Avanti)
- Sonicate 48 °C ~12 h
- 0.1 M KOH 37 °C ~2 h
- Centrifuge & transfer to autoinjector vial
- Reverse phase LC-MS$^n$ >80%

B (Non-polar):
- + HOAc + CHCl$_3$ + H$_2$O
- Centrifuge & recover lower phase
- Dry, Redissolve in LC mobile phase
- Centrifuge & transfer to autoinjector vial
- Normal phase LC MS$^n$ 80-90%
C. Characteristic fragmentations of sphingolipids

R = n-alkyl chain, R' = H, Glu/Gal, Lac, and R'' = H, PO₃
C. Sphingolipid analysis by LC MS/MS

Species analyzed to date (in most mammalian sphingoid base & FA variants):

✓ SM’s, GlcCer, GalCer, LacCer, Sulfatide (quantitative analysis of globosides and gangliosides)
✓ Cer & Cer-P
✓ So, Sa and 4-hydroxy-Sa
✓ So-P, Sa-P and other derivatives (lysoSM, psychosine, N-methyl-)
✓ Plus metabolites labeled with stable isotope precursors

Sullards, Merrill & coworkers
STKE (2001),
Methods (2005),
Meth. Enzymol. (in press) &
www.sphingomap.org
Summary of conditions used for Liquid Chromatography

Reversed Phase

- LCB analysis: So, Sa, phyto So, So-1-P, Sa-1-P, and standards
- 2.1 x 50mm Supelco Discovery C18, 5 µm, 120 Å
- A: 74:25:1 CH₃OH/H₂O/HCOOH
  B: 99:1 CH₃OH/HCOOH
- Flow rate 1 mL/min, 0.6 min. 80:20 A/B, 1.8 min. to 100% B, 0.6 min. hold 100% B

Normal Phase

- Complex SL's: Cer, GlcCer, GalCer, LacCer, SM, and standards
- 2.1 x 50 mm Supelco NH₂, 3 µm, 120 Å
- A: 97:2:1 CH₃CN/CH₃OH/CH₃COOH
  B: 99:1 CH₃OH/CH₃COOH
  both 5mM Ammonium Acetate
- Flow rate 1.5 mL/min, 0.5 min. 100% A, 0.2 min. gradient to 90:10 A/B, 0.5 min. hold, 0.4 min. to 82:18 A/B, 0.6 min hold, 0.4 min. to 100% B

Number of molecules per RAW cell (million)

Serine + Palmitoyl-CoA → 3-Ketosphinganine <0.01 → Sphinganine (d18:0) → Sphinganine 1-phosphate 1.3 (d18:0-P)

Ceramide synthases + Fatty acyl-CoA → 3-Ketosphinganine

All subspecies have been quantified for these chain lengths, too (only a few are shown: all are at www.lipidmaps.org)
Summary of compounds analyzed to date by “Inside-out” sphingolipidomics

Acidic sphingolipids
Infusion 0.6 mL/h (MeOH)
Prec 290 sCan [MCA 60 Scans (3s/scans)]
DP = -100 CE = -75 IS = -4500

Sphinganine 1-phosphate (d18:0-P)

Ceramide synthases
+ Fatty acyl-CoA

Raw cells +KdO₂
Lipid A

Variation in backbone, acyl chain & headgroup specificity

Summary of compounds analyzed to date by “Inside-out” sphingolipidomics

Sphinganine 1-phosphate (d18:0-P)

Ceramide synthases
+ Fatty acyl-CoA

Raw cells +KdO₂
Lipid A

Variation in backbone, acyl chain & headgroup specificity
Work-flow for analysis of new samples using this LC-MS/MS Methodology

1. Identify structure specific dissociations unique to various classes (e.g., SM, GlcCer, GalCer, LacCer, etc.)

2. Utilize precursor ion and neutral loss scans to identify individual headgroup, sphingoid base, and fatty acid combinations.

3. Optimize ionization and dissociation conditions for all species.

4. Optimize LC as required to minimize ionization suppression effects, and interferences arising from isobaric, isotopic, and isomeric species (repeat #3 if necessary).

5. Optimize conditions for quantitation via ratio of peak areas vs validated internal standards for all of the species present.
Example: Identification of sphingolipid subspecies via Neutral Loss or Precursor Ion Scans
Comparison of ion abundance for ceramides of varying chain length when analyzed under single ionization and dissociation conditions vs optimized MRM.
Criteria for selection of internal standards

1. Must have the same chemical and physical properties as the analyte of interest, ideally stable isotope labeled analogs.
2. Should be practical for “omic” analysis--i.e., cover as many subspecies as possible because adding an internal standard for every analyte would require 100s to 1000s of molecules to be synthesized, added and analyzed, which is too expensive, time consuming and possibly analytically impossible.

LIPID MAPS Sphingolipidomics cocktail (available from Avanti Polar Lipids): 10 uncommon sphingolipid species that are used to spike samples prior to extraction
For sphingoid bases: odd chain length variants that elute under similar conditions so there is little ionization or dissociation effects and precursor and product ion masses are slightly shifted.

- d17:1 “sphingosine” and “sphingosine 1-phosphate” homologs
- d17:0 “sphinganine” and “sphinganine 1-phosphate” homologs

For complex sphingolipids: shorter fatty acid chain length variants (C12:0) that co-elute with analytes of interest so there are no ionization effects, and have different precursor ion masses but similar fragmentation when optimized.

- C12-Cer, C25-Cer, C12-Cer-1-P, C12-GlcCer, C12-LacCer, C12 SM

Also available: C12-Sulfatide
Under development: C12-GM1 and other complex glycosphingolipids
C17:1 sphingosylphosphocholine; N-methyl-sphingoid bases
Analyzed by LC MS/MS using an ABI 4000 QTRap

Comparison of MRM for ceramides and dihydroceramides of varying chain length compared to C12-Cer internal standard
E. Data analysis/visualization:

Example data from Lipid MAPS experiments:
Changes in Cer in RAW 267 cells treated with Kdo₂ Lipid A
(see www.lipidmaps.org)

Basic LIPID MAPS Protocol

Plate cells → Add agent (LPS, Kdo2 Lipid A) → Grow → Incubation → Analyze

Pre-treatments (if any)

N-acyl chain subspecies

Data analysis/visualization:
**CORE I: Sphingolipids (only C16 shown)**

**Biological Replicates +/- SE**

- **KDO**: 3-Ketosphinganine (below LOD)
- **Control**: Serine + Palmitoyl-CoA
- **Sphinganine**: Sphinganine
- **Sphinganine 1-P**: Sphingamine 1-P
- **Sphingosine**: Sphingosine
- **Sphingosine 1-P**: Sphingosine 1-P
- **C16Cer**: C16Cer
- **C16DHCer**: C16DHCer
- **C16DHSM**: C16DHSM
- **C16DHGlcCer**: C16DHGlcCer
- **C16SM**: C16SM
- **C16GlcCer**: C16GlcCer
F. Comparison of these methods with other sphingolipidomic techniques in the literature

"Shotgun" techniques: Use the same precursor ion / neutral loss scans - Great for profiling, not quantitation, suffer from ionization suppression, isotopic, isobaric, and isomeric interferences especially without hydrolysis and extraction.

Nanospray ionization: Greatly improved sensitivity and reduced chemical noise: allows detection of low abundance species, detailed structural analyses on numerous species, chip-based systems can be coupled to LC, and fraction collection.

Ultra high resolution mass analysis: allows differentiation of isobaric / isotopic interferences and alternative fragmentation techniques.
G. Remaining challenges (and opportunities)

Discovery of new subspecies (and new functions for known subspecies)--such as N-methylsphingoid bases
Obtain standards for glycosphingolipids (and new sphingolipids).
Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.
Develop methods to differentiate appearance/disappearance of particular subspecies via de novo biosynthesis vs turnover.
Develop methods to determine which sphingolipids are associated with different cells in a tissue (and eventually, subcellular localization).
Example of Lipid MAPS timecourse data set for sphingolipids (see www.lipidmaps.org)
Ser + PalCoA
\[ \downarrow \]
3-KetoSa
\[ \downarrow \]
Sa
\[ \downarrow \]
GlcDH Cer \[ \leftarrow \] DHCer \[ \rightarrow \] DHSM

Fenretinide
\[ \downarrow \]
GlcCer \[ \leftarrow \] Cer \[ \rightarrow \] SM

Fold change

[Color scale from 0.1 to 10]
G. Remaining challenges (and opportunities)

Discovery of new subspecies (and new functions for known subspecies).
Obtain standards for glycosphingolipids.
Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.
Develop methods to differentiate appearance/disappearance of particular subspecies via \textit{de novo} biosynthesis vs turnover.
Develop methods to determine which sphingolipids are associated with different cells in a tissue (and eventually, subcellular localization).
Analysis of sphingolipid biosynthesis using stable isotope labeled precursors

RAW 10% time course w/[\textsuperscript{13}C]palmitate

0.1 mM [\textsuperscript{13}C]palmitate

\[ \begin{align*} 
\text{Ser + PalCoA} \\
\text{3-KetoSa} \\
\text{Sa} \\
\text{GlcDH Cer} \\
\text{DHCer} \\
\text{DHS M} \\
\text{GlcCer} \\
\text{Cer} \\
\text{SM} 
\end{align*} \]
To understand sphingolipid biosynthesis one must also know the availability of the co-substrate fatty acyl-CoA’s.

C16:0-CoA (10 pmol/µL in methanol 10 mM triethylammonium acetate) was infused at 5 µL/min. 4000QTrap parameters: IS = 5400 V, Gs1 = 12 psi, CUR = 10 psi, DP = 180 V, CE = 52 V, CXP = 14.3 V, CAD = Med
G. Remaining challenges (and opportunities)

Discovery of new subspecies (and new functions for known subspecies).
Obtain standards for glycosphingolipids.
Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.
Develop methods to differentiate appearance/disappearance of particular subspecies via de novo biosynthesis vs turnover.

Develop methods to determine which sphingolipids are associated with different cells in a tissue (and eventually, subcellular localization)--Murphy
H. Examples of discoveries from sphingolipidomic analysis thus far

*de novo* sphingolipid biosynthesis is induced by Kdo<sub>2</sub> Lipid A (has been correlated with gene array data showing increases in SPT1 and SPT2 mRNA)

Basic LIPID MAPS Protocol

- Plate cells
- Add agent (LPS, Kdo2 Lipid A)
- Grow
- Incubation
- Analyze

N-acyl chain subspecies

Examples of discoveries from sphingolipidomic analysis thus far
Number of molecules per RAW cell (million)

[Diagram of lipid metabolism showing various substrates, enzymes, and products with numbers indicating their concentrations in million molecules per RAW cell.]

All subspecies have been quantified for these chain lengths, too (only a few are shown; all are at www.lipidmaps.org)
H. Examples of additional discoveries from sphingolipidomic analysis thus far


**Ceramide kinase utilizes ceramide provided by ceramide transport protein. Localization to subcellular compartments of eicosanoid synthesis.** Lamour NF, Stahelin RV, Wijesinghe DS, Maceyka M, Wang E, Allegood JC, Merrill AH Jr, Cho W, Chalfant CE. J Lipid Res. 2007 Mar 27; [Epub ahead of print]

The LIPID MAPS Consortium (www.lipidmaps.org) is conducting an open Workshop on “Lipidomics” at Experimental Biology 2007 under the auspices of the American Society for Nutrition. A panel of eight experts will discuss methods that have been developed under an NIH (NIGMS) “Glue-grant” for analysis of neutral glycerolipids, phospholipids, eicosanoids, sterols, sphingolipids, prenols and various novel lipids by mass spectrometry as well as related issues such as sample extraction, internal standards, data handling and display, and nomenclature. In addition to reviewing the LIPID MAPS protocols, the panel will invite questions and discussion regarding these issues and the directions of future lipidomics research and developments. The workshop will be held in the Grand Ballroom South of the Renaissance Hotel, 999 Ninth Street NW, Washington, DC, from 8 am to 1 pm on Saturday, April 28, 2007, including an hour for open discussion over snacks. Attendance is free (registration for FASEB Experimental Biology is not required), but persons who are interested in attending are encouraged to contact Al Merrill (al.merrill@biology.gatech.edu), although “walk ins” are also welcome. Funding for the workshop has been provided by the LIPID MAPS Glue grant (U54 GM069338), Avanti Polar Lipids, Applied Biosystems and Advion Biosystems.

8:00 Welcome and overview of the Lipid Maps Consortium and world-wide lipidomics initiatives. Edward Dennis
8:15 Analytical technologies panel: Each speaker will define the Lipid Maps protocols that are being used for sample handling, extraction, and analysis, followed by open discussion of methodologic issues of interest to the audience (different approaches, problems, etc.). Al Merrill, moderator
  8:20 Brief introduction to lipid analysis by mass spectrometry and challenges, as illustrated with neutral and phospho-glycerolipids. Robert Murphy
  8:50 Discussion
9:10 Sphingolipids (and precursor fatty acyl-CoA’s). Cameron Sullards
  9:25 Discussion
9:35 Sterols. Jeffrey McDonald
  9:50 Discussion
10:00 Coffee break
10:15 Eicosanoids. Rick Harkewicz
  10:30 Discussion
10:40 Novel lipid analysis. Teresa Garrett
  10:55 Discussion
11:05 Internal standards for lipidomic analysis. Walt Shaw
  11:20 Discussion
11:30 Data handling and bioinformatics, pathway visualization and interpretation. Eoin Fahy
  11:45 Discussion
11:55 Closing of general session
12:00 Follow-up open discussions over snacks
1:00 End of session
You are invited to attend a

LIPIDOMICS WORKSHOP

at Experimental Biology 2007, Washington, DC
on Saturday, April 28, 2007 from 8 AM – Noon

Sponsored by: the American Society for Nutrition,
the LIPID MAPS Consortium, and Avanti Polar Lipids, Inc.

The Workshop will provide an overview of protocols that have been developed by the LIPID MAPS Consortium for Lipidomic analysis using mass spectrometry as well as related issues such as sample handling and extraction, internal standards for quantitative analysis, data management and display, lipid nomenclature, and an overview of global lipidomic initiatives.

The Panel

• Edward Dennis
  University of California, San Diego
• Eoin Fahy
  University of California, San Diego
• Teresa Garrett
  Duke University Medical Center
• Rick Harkewicz
  University of California, San Diego
• Jeffrey McDonald
  University of Texas Southwestern Medical Center
• Al Merrill
  Georgia Institute of Technology
• Robert Murphy
  University of Colorado Health Sciences Center
• Walt Shaw
  Avanti Polar Lipids
• Cameron Sullards
  Georgia Institute of Technology

To Attend

This Workshop will be conducted as a satellite session at Experimental Biology 2007 under the sponsorship of the American Society for Nutrition (ASN). If you plan to attend please Email: al.merrill@biology.gatech.edu

For information about EB 2007 - registration, lodging, etc. visit: www.eb2007.org

Can you identify (and quantify) these lipids* in a complex mixture?

If not, this workshop will teach you how - for these and thousands of other lipids

*S-1-P
Cholesterol
PC
GlcCer

www.lipidmaps.org