

Serial-Omics and Untargeted Fluxomics: From Tumors to Bodily Fluids to Dried Blood Spots

- **3-Omics from one sample prep**
 - Value for reproducibility and precious sampling
 - Interesting applications – blood spots, cells, breast tumors, etc.

- **Untargeted metabolite and lipid $^{13}\text{C}/^{15}\text{N}$ Fluxomics**
 - Beyond just what we know
 - If we can identify it with high resolution, we can flux it

John M. Asara, Ph.D.

Core B: Mass Spectrometry

Beth Israel Deaconess Medical Center

Harvard Medical School



John M. Asara, Ph.D. – Director, Associate Professor of Medicine

Min Yuan, B.S. – Sr. Research Associate, Lab Manager

He Huang, Ph.D. – Research Fellow, Research Associate

Contact and Info

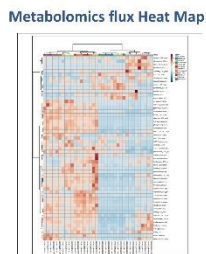
- Contact John Asara, jasara@bidmc.harvard.edu for project discussion
- Per unique sample for BIDMC & DF/HCC affiliates;
- Prices and more info available at [www.bidmcmasspec.org](http://www.bidmcmassspec.org)

Metabolomics

- Targeted Metabolic Profiling (~300 targets) by SRM
 - Central carbon metabolism
 - Glycolysis
 - TCA cycle
 - Pentose phosphate pathway
 - Amino acids
 - Nucleotides
 - Reactive oxygen species
 - Methionine metabolism, etc.
- ¹³C/¹⁵N Metabolic Flux Tracing (~150 targets)
- ¹³C/¹⁵N Isotopomer tracing of central carbon metabolites (~70 targets)
- Untargeted Metabolic Profiling by High Resolution LC-MS/MS (>450 molecules)
- Absolute quantitation with calibration curve or labeled internal standard

Metabolomics Instrumentation

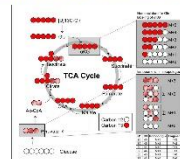
Thermo QExactive Orbitrap HF coupled to Agilent 1200 HPLC



AB/SCIEX QTRAP 6500 triple quadrupole coupled to Shimadzu UF-HPLC



Example of ¹³C isotopomer Flux



Lipidomics

- Untargeted Lipidomic Profiling (>1,200 molecules) by High Resolution LC-MS/MS
 - 20 main classes, >90 sub-class of lipids
 - Phospholipids, triglycerides, ceramides, etc.
 - Individual lipid molecule ID and quantification
 - Lipid class and fatty acid profiles
- ¹³C/¹⁵N Isotopomer tracing
 - All identified lipid ions
- Targeted Lipid Profiling
 - Coming soon

Multi-Omics Overlay Plot

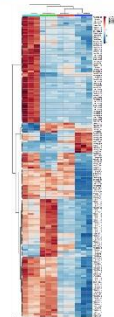


Lipidomics Instrumentation

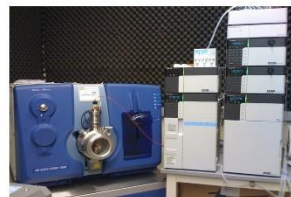
Thermo QExactive Orbitrap Plus coupled to Agilent 1200 HPLC



Lipidomics Heat Map



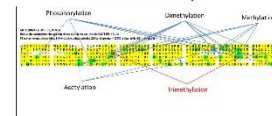
AB/SCIEX QTRAP 5500 triple quadrupole coupled to Shimadzu UF-HPLC



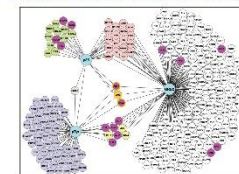
Proteomics (PTM-omics)

- Protein Identification from Gels and Solution
 - SDS-PAGE gel slices or small regions
 - On-bead digestion
 - Solution based or protein pellets
- Phosphoproteomics
 - pTyr enrichment
 - IMAC/TiO2 global Phospho enrichment
 - Phospho-specific Ab
- Post-Translation Modification Mapping of Proteins
 - Ubiquitination, acetylation, methylation, phosphorylation, etc.
- Quantitation with SILAC and TMT labeling
- Protein Complex analysis
 - Immunoprecipitations
 - Label-free Quantitation

PTM Site Map

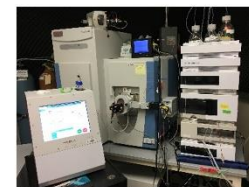


Protein-Protein Interaction Network



Proteomics Instrumentation

Thermo QExactive Orbitrap HF coupled to Proxeon EASY-nLCII nano-HPLC



Scaffold Quantitative Protein ID

Protein ID	Protein Name	Accession	Score	Label
1	Actin	P12345	1000	Green
2	Albumin	P67890	950	Green
3	Myoglobin	P12345	900	Green
4	Insulin	P67890	850	Green
5	Cytochrome c	P12345	800	Green
6	Hemoglobin	P67890	750	Green
7	Albumin	P67890	700	Green
8	Actin	P12345	650	Green
9	Myoglobin	P12345	600	Green
10	Insulin	P67890	550	Green
11	Cytochrome c	P12345	500	Green
12	Hemoglobin	P67890	450	Green
13	Albumin	P67890	400	Green
14	Actin	P12345	350	Green
15	Myoglobin	P12345	300	Green
16	Insulin	P67890	250	Green
17	Cytochrome c	P12345	200	Green
18	Hemoglobin	P67890	150	Green
19	Albumin	P67890	100	Green
20	Actin	P12345	50	Green

Thermo hybrid Orbitrap Elite coupled to Proxeon EASY-nLCII nano-HPLC



Phosphorylation Site Maps



PPG integral in the development of many of these services

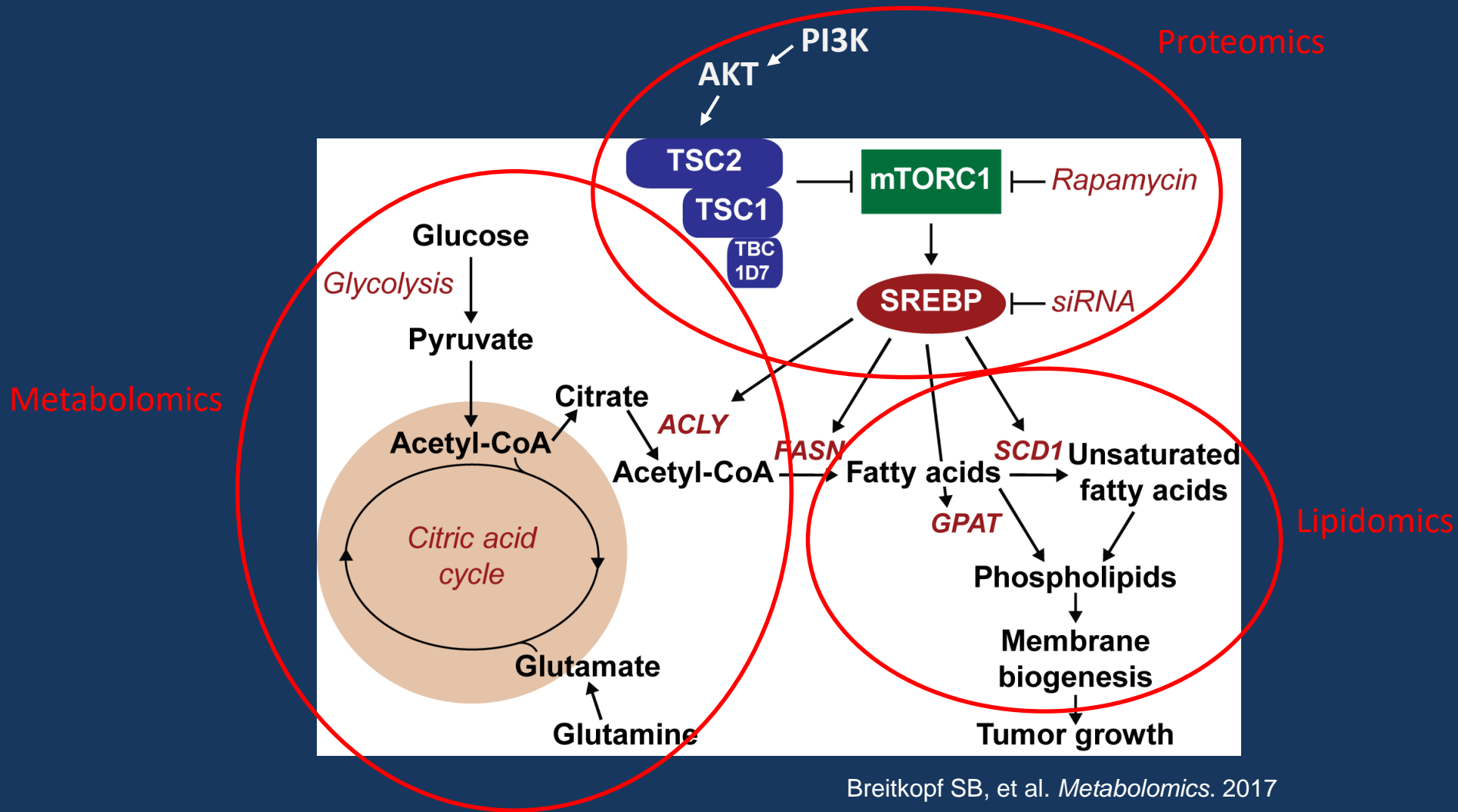
Coming soon...



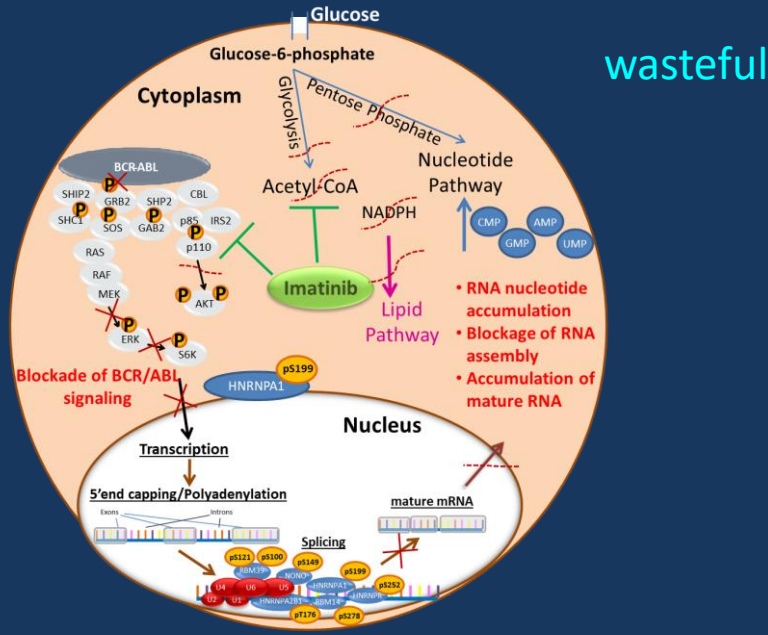
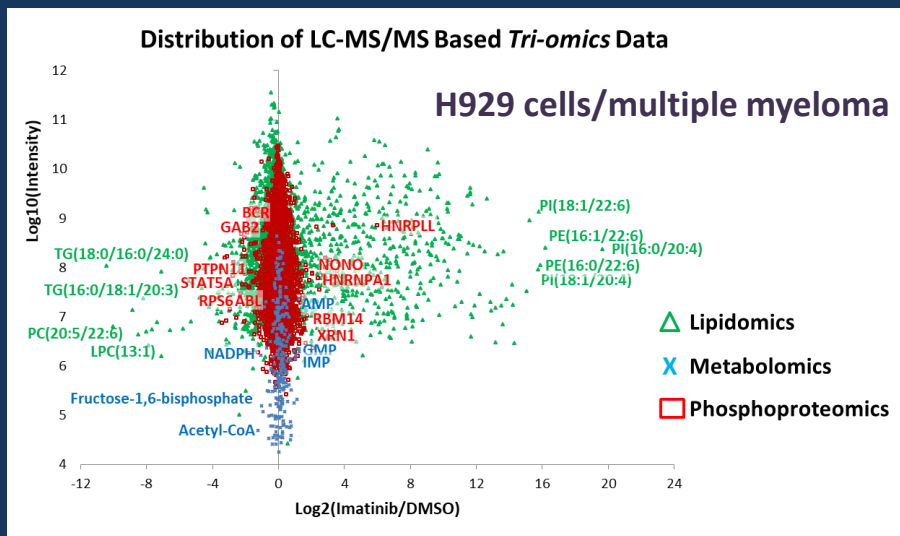
Thermo Fusion Lumos Tribrid MS
PO held up by pandemic 🙄

Integrating Different –Omics Approaches

Cell signaling to central carbon and fatty acid metabolism



Separate Omics experiments (Old)



Breitkopf et. al., 2015, *Anal. Chem.*

wasteful

Integrated (Serial) -Omics experiments (New)

Our current approach

methy-tert-butyl ether (MTBE)

Non-polar Lipid
Thermo QExactive Plus

Targeted Polar Metabolites
AB/SCIEX 5500 QTRAP

Untargeted Polar Metabolites
Thermo QExactive HF

Phosphopeptides & Global Proteins
Thermo QExactive HF

SDS-PAGE fxn



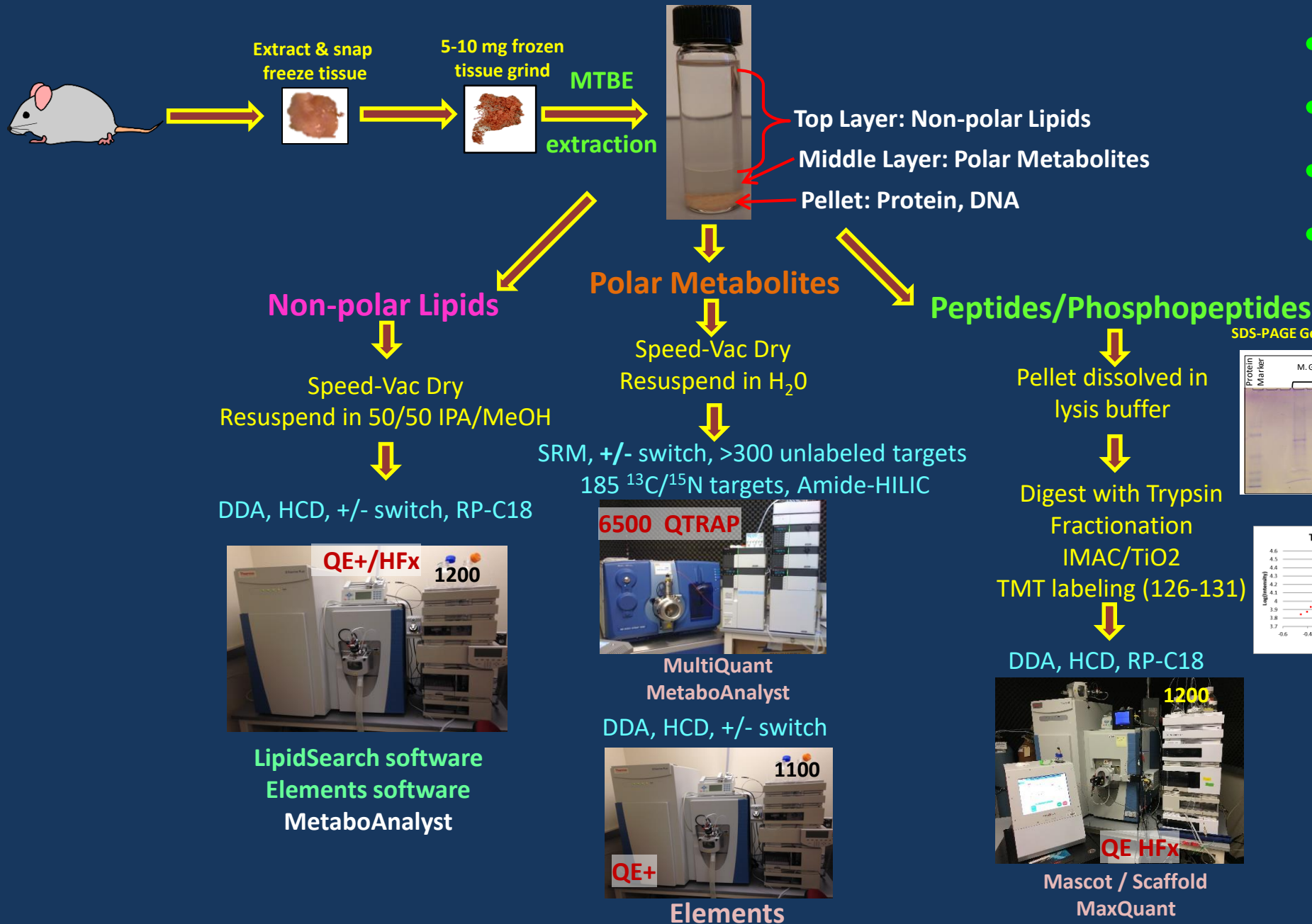
Breitkopf et. al, 2017, *Sci Reports*

Willmitzer lab, Max-Planck Institute, Germany -Plants

Ahrends lab, Univ. of Vienna-stem cells

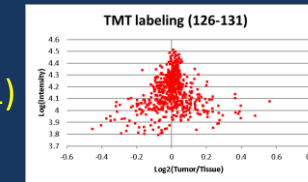
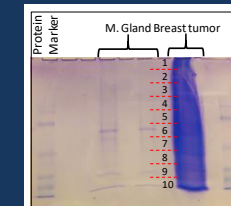
Integrated Serial-Omics Platform Workflow

Applications

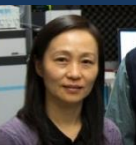


- Tumors/Cell lines
- Dried Blood
- Urine/Serum
- Hair

SDS-PAGE Gel Fractionation



Min Yuan



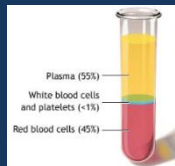
Susanne Breitkopf



Platform for *Untargeted* Lipidomics/Metabolomics/Proteomics

Breitkopf SB, et al. *Metabolomics*. 20

Blood plasma



Cancer cells



Tumor tissue



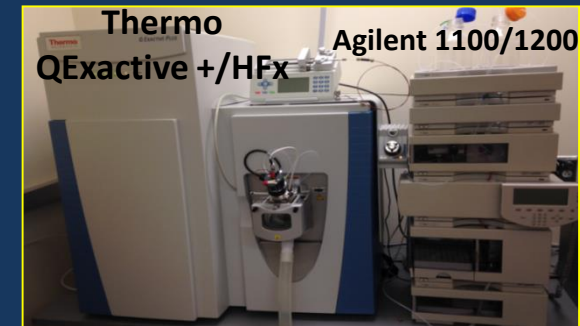
MTBE



Lipid layer

Aqueous layer

Solid pellet

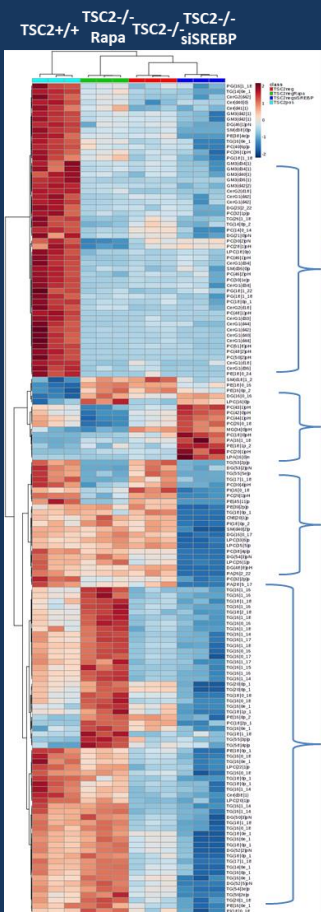


• identification based on *high mass accuracy MS and MS2 data*

2.1 mm x 10 cm C₁₈ (lipidomics); 75 μm x 10 cm C₁₈ (proteomics)
4.6 mm x 10 cm amide HILIC (metabolomics)

Pos/Neg polarity switching (~10 points/peak)

HCD-DDA (Top 8) pos and neg mode



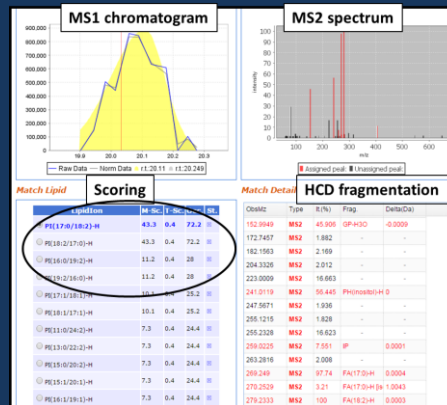
Ceramide
Ganglioside

PC, LPA

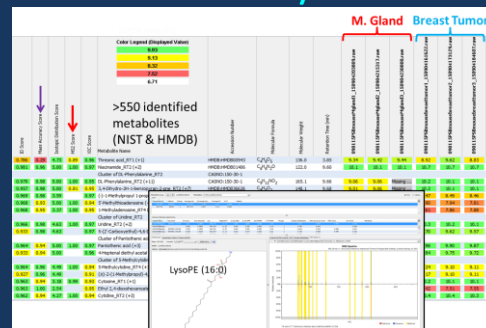
DG, LPC

Triglycerides

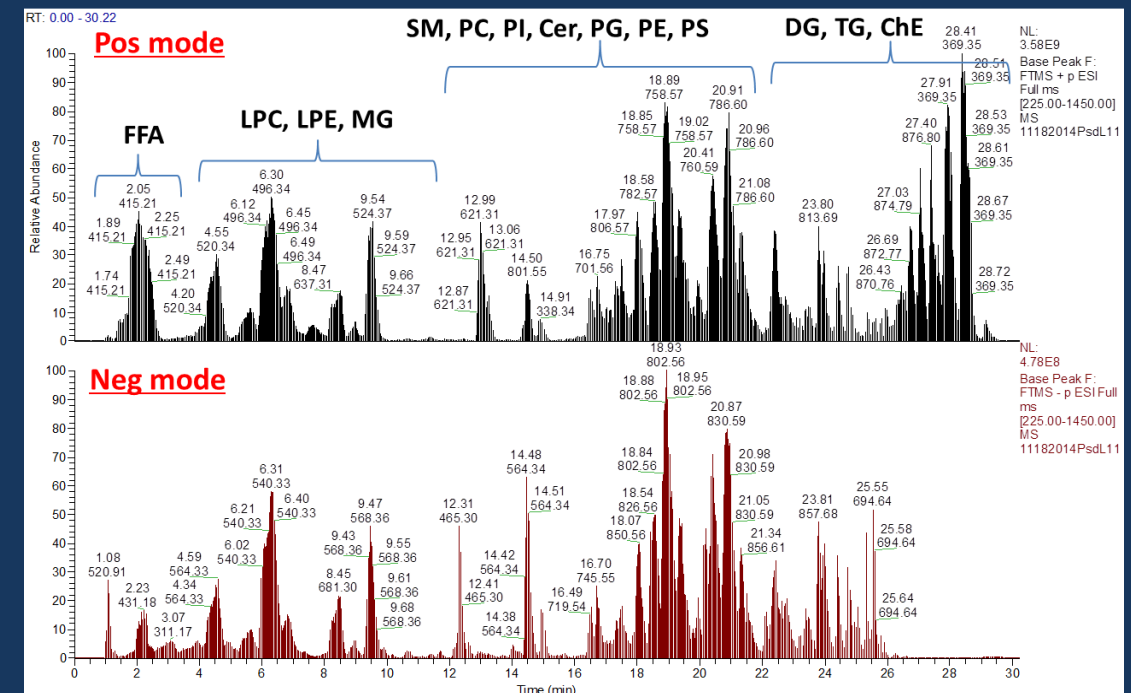
LipidSearch



20 main lipid classes and 81 subclasses
Elements 2.2/Scaffold

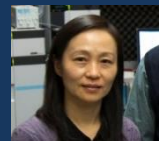


NIST 2017: 652,475 MS/MS spectra; Protein DBs

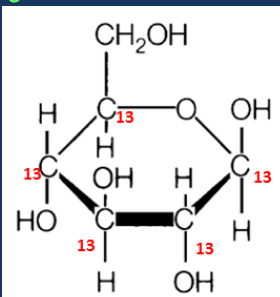


Targeted Metabolic Flux Analysis (SRM)

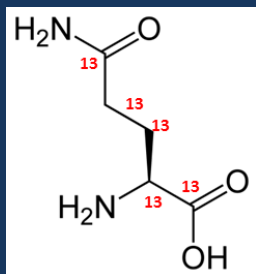
Yuan M, et al. *Nat Protoc.* 2019.
Yuan M, et al. *Nat Protoc.* 2012.



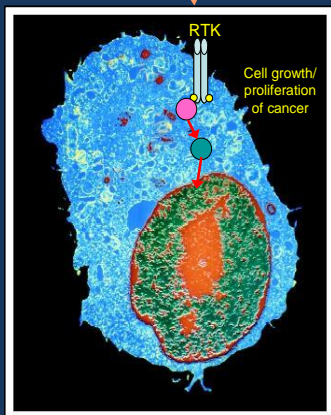
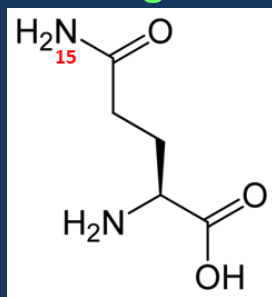
¹³C₆-labeled glucose



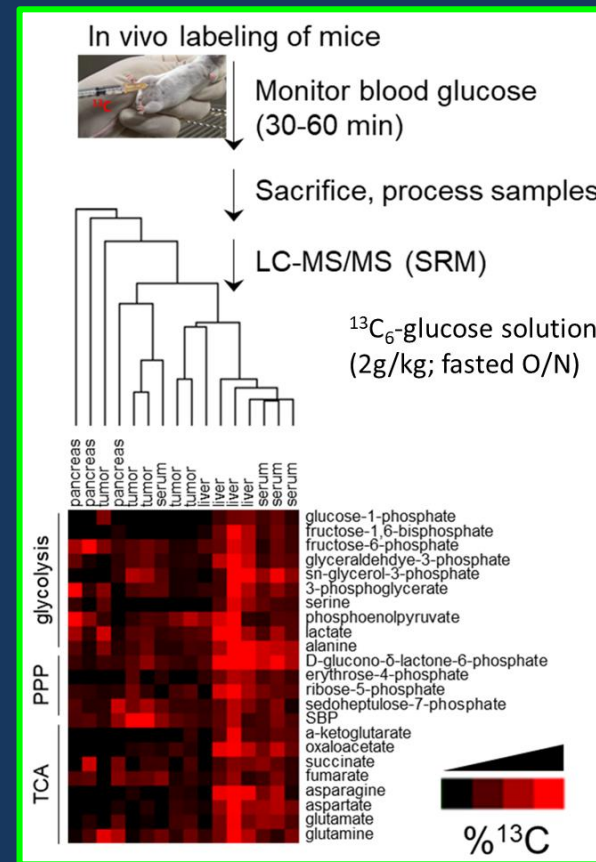
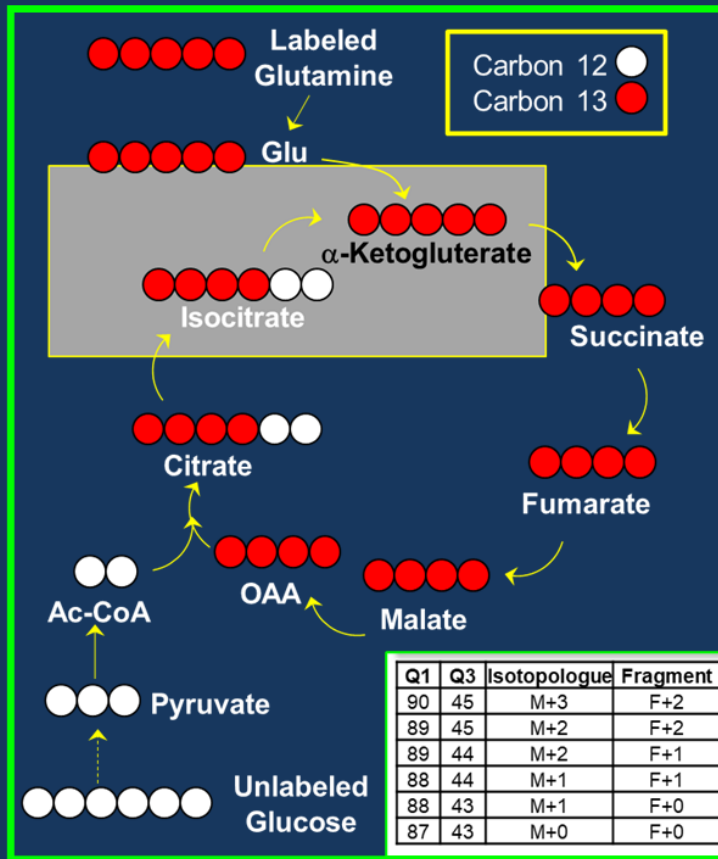
¹³C₅-labeled glutamine



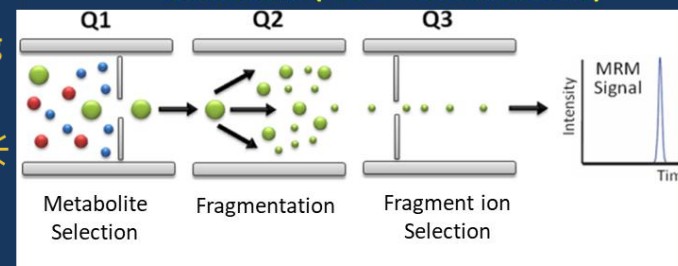
¹⁵N-labeled glutamine



Cancer cells



Selected Reaction Monitoring (SRM) ~300 metabolite transitions (~150 ¹³C metabolites)



+/- switching

Amide HILIC

4.6 mm x 10cm

pH=9.0, NH₄⁺

400 μL/min

- We have developed methods for >20 labeling sources
- Mostly PPG induced development

IsoSearch: Untargeted $^{13}\text{C}/^{15}\text{N}$ Flux-Omics

^{13}C glucose/ ^{15}N glutamine, etc.



Elements/LipidSearch/Mascot

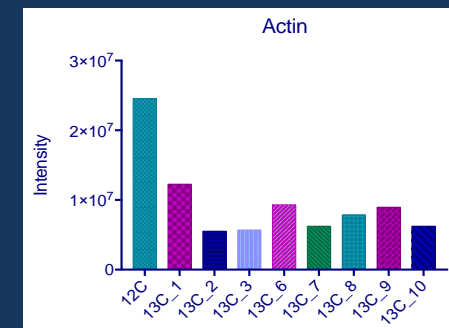
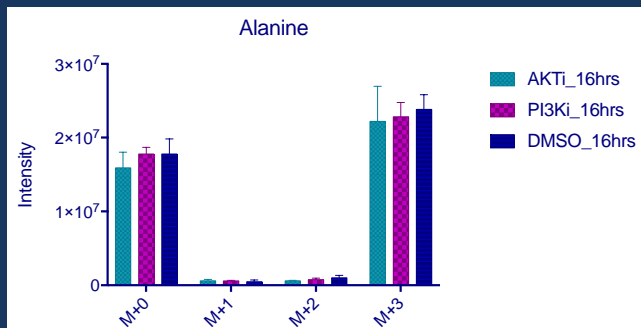
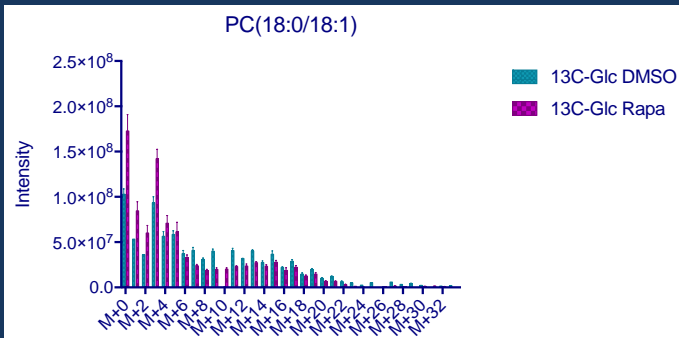
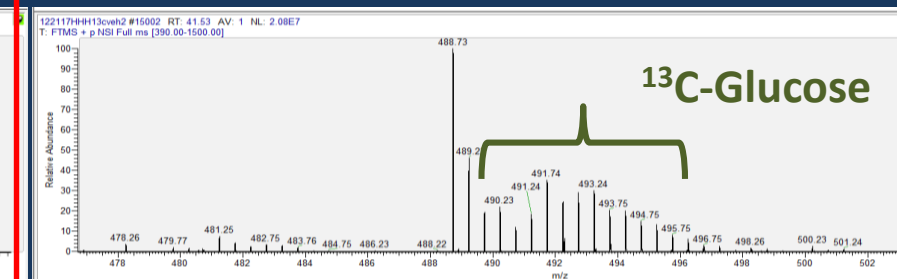
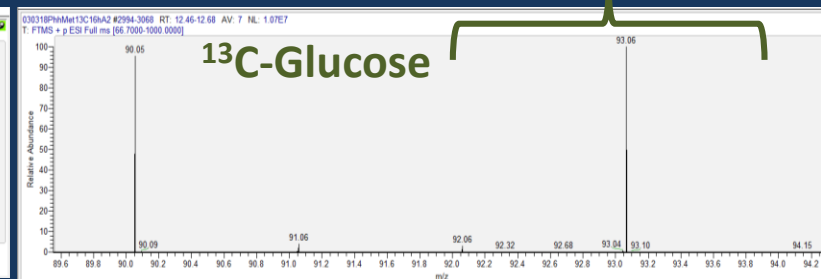
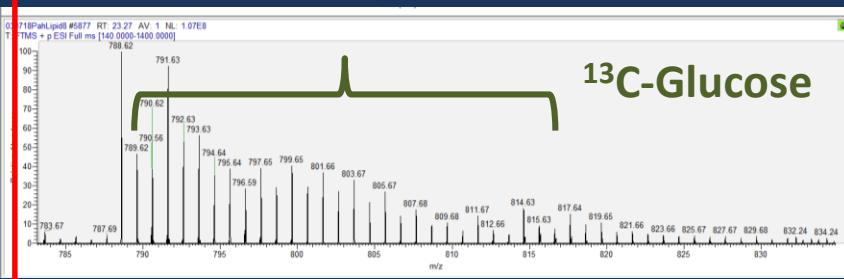
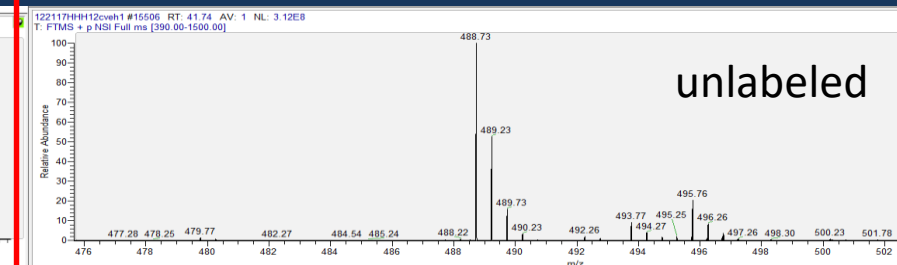
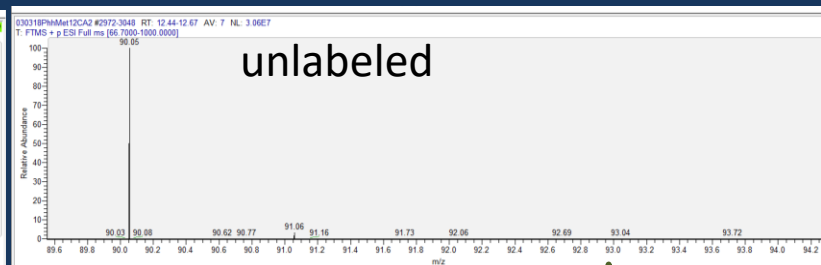
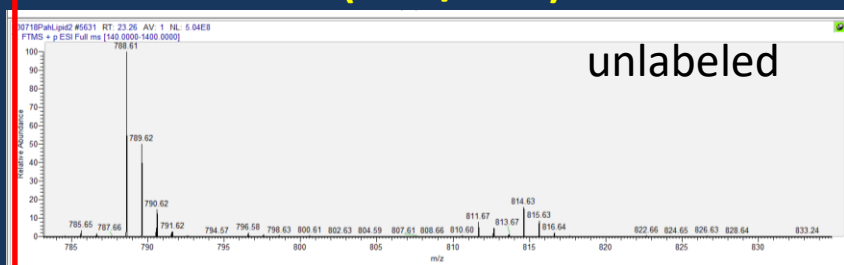
IsoSearch

In-house software

Peptidomics
AGFAGDDAPR, 2+

Lipidomics
PC(18:0/18:1)

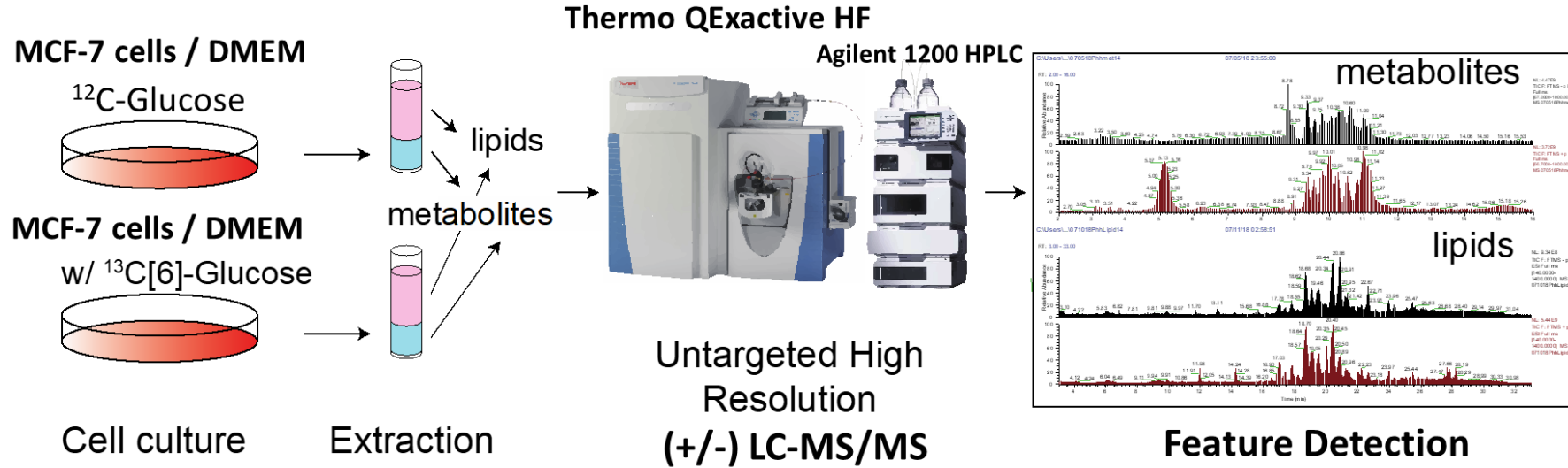
Metabolomics
Alanine



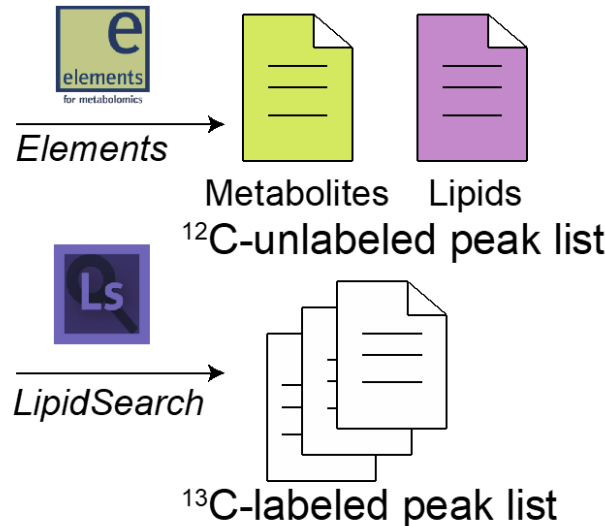
Workflow for IsoSearch Untargeted Isotopomer Analysis



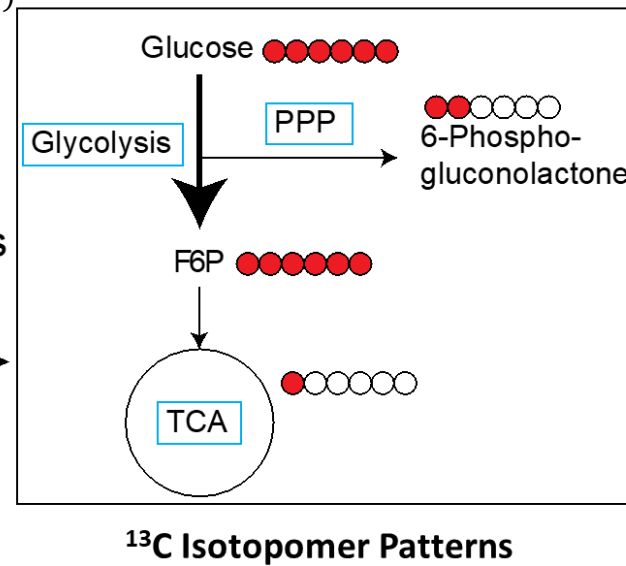
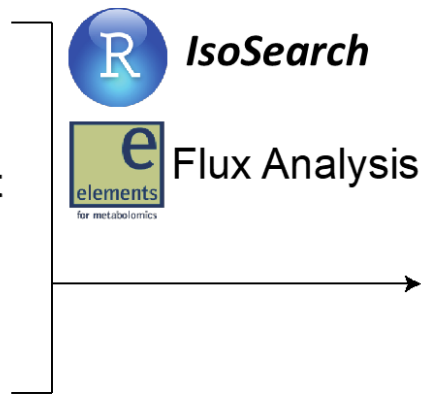
He Huang



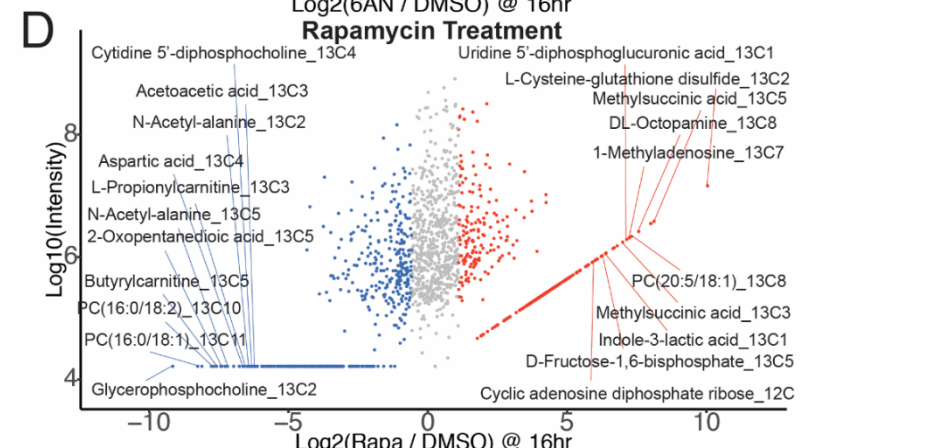
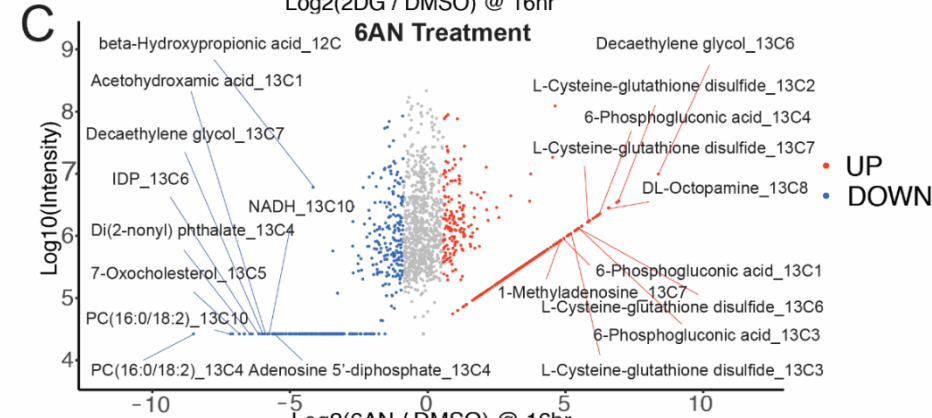
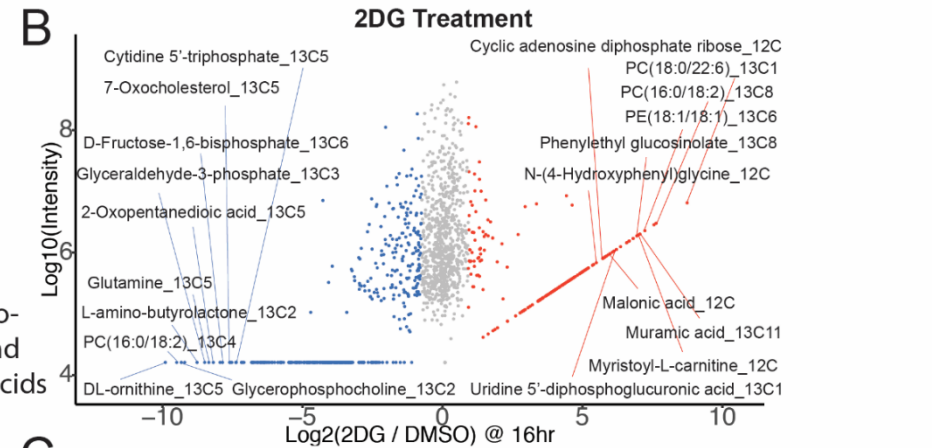
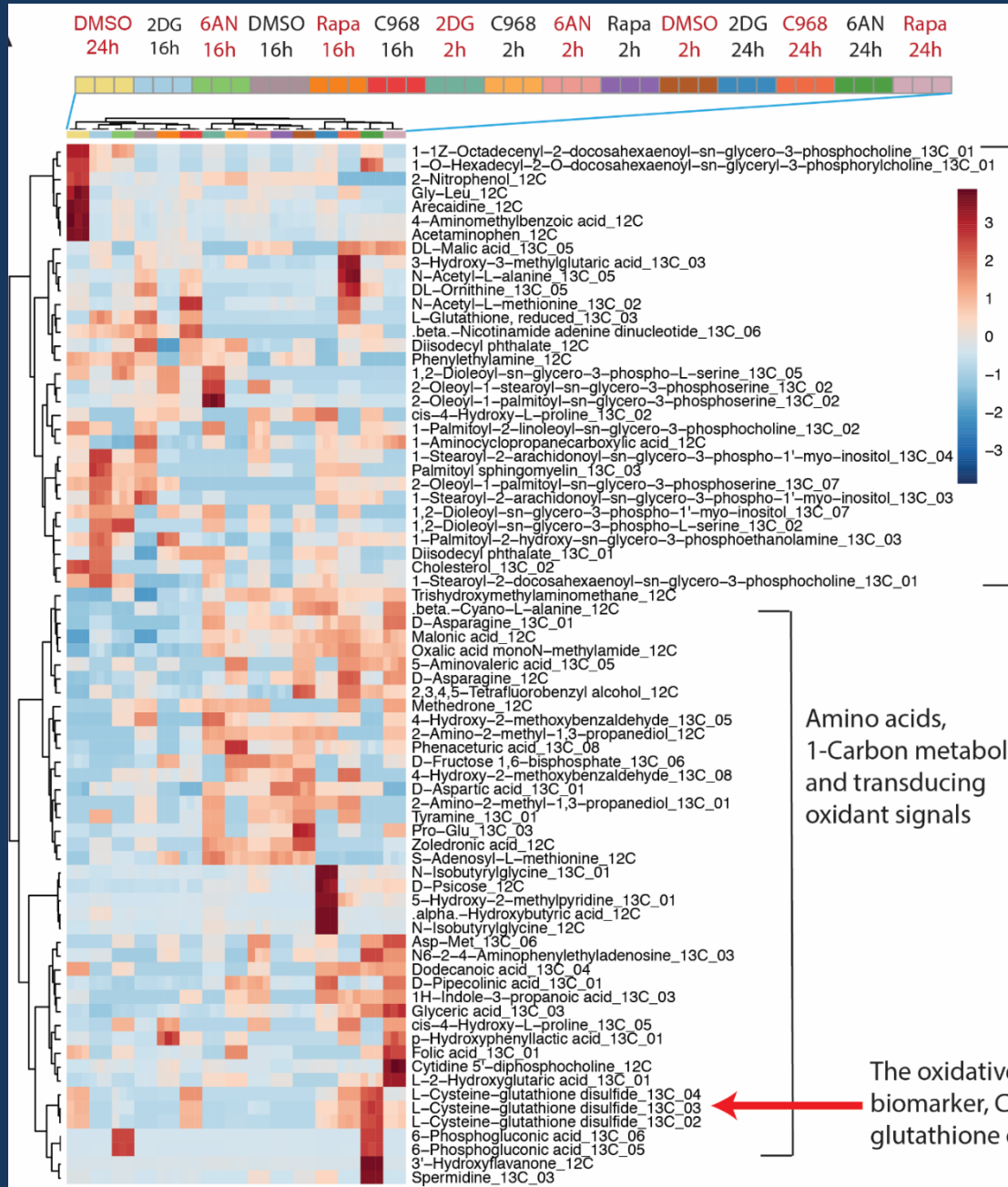
Identification



$$Score = 1 - \sqrt{(\Delta m/z \times \beta)^2 + (\Delta RT)^2}$$

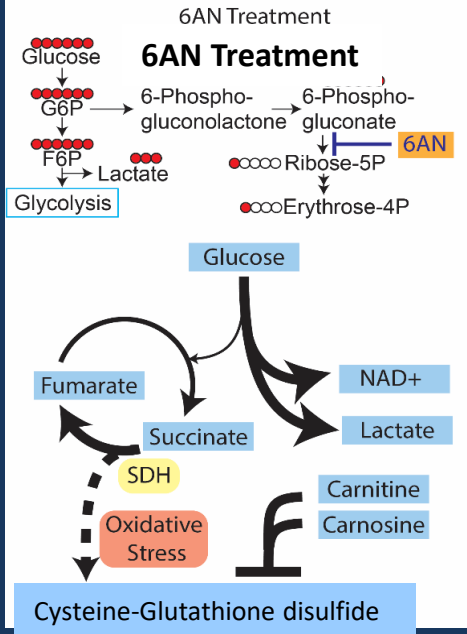
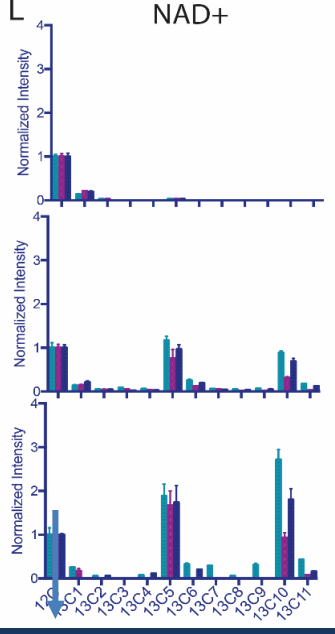
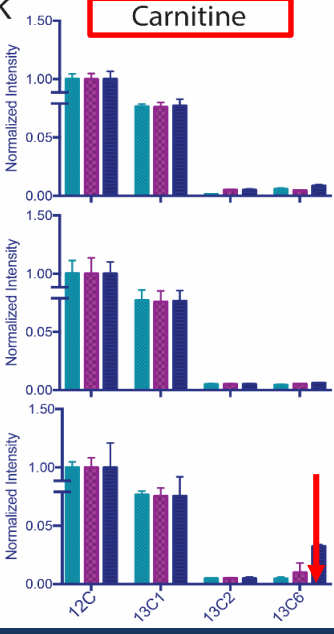
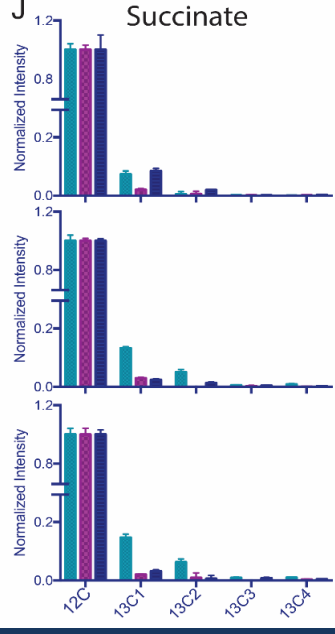
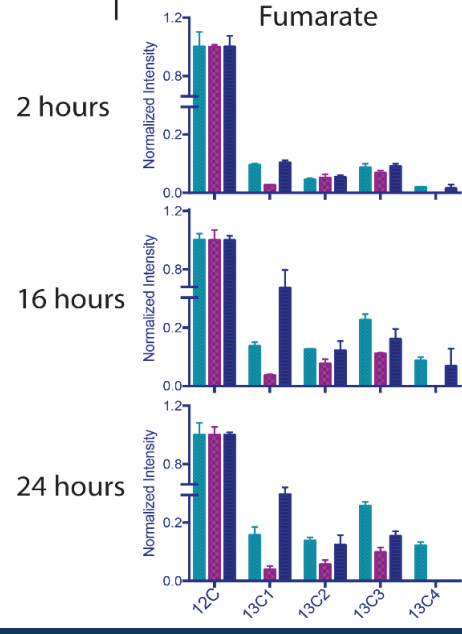
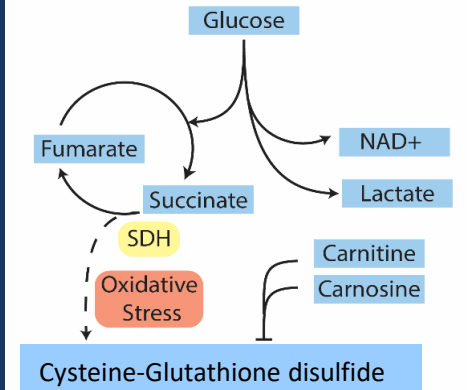
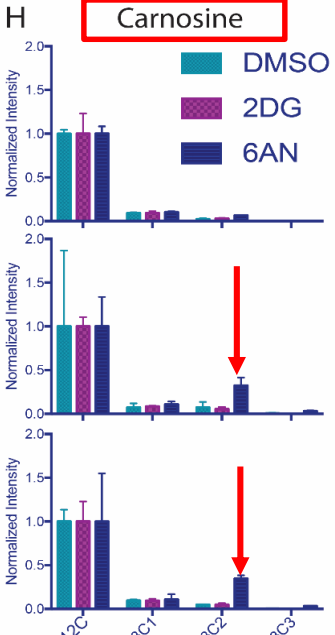
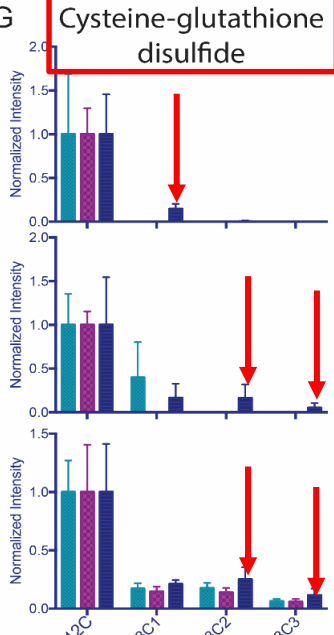
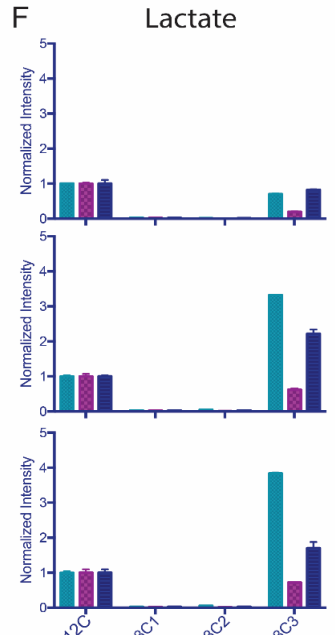
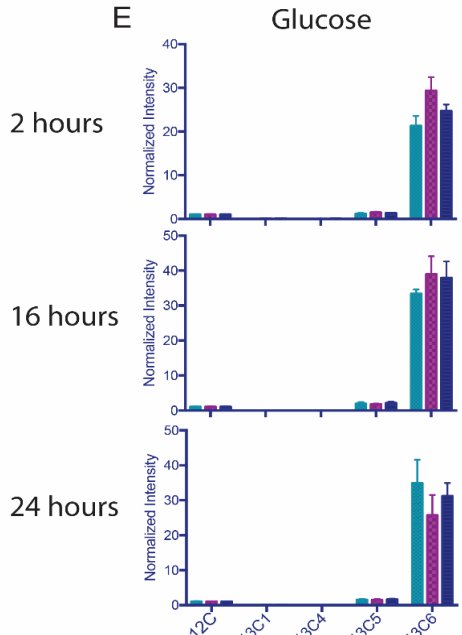
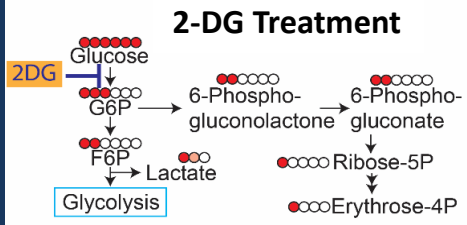


MCF-7 Breast Cancer Cells with Metabolic Inhibitors and Rapamycin – 2 hr, 16 hr, 24 hr treatments

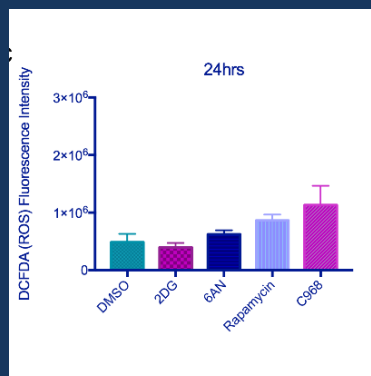
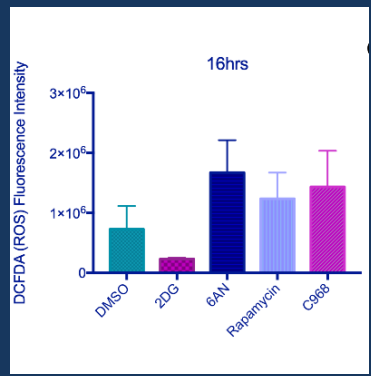
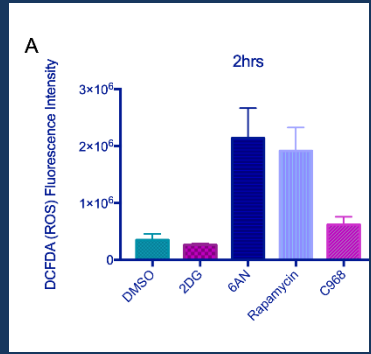


Many metabolites unique to untargeted metabolomics platform

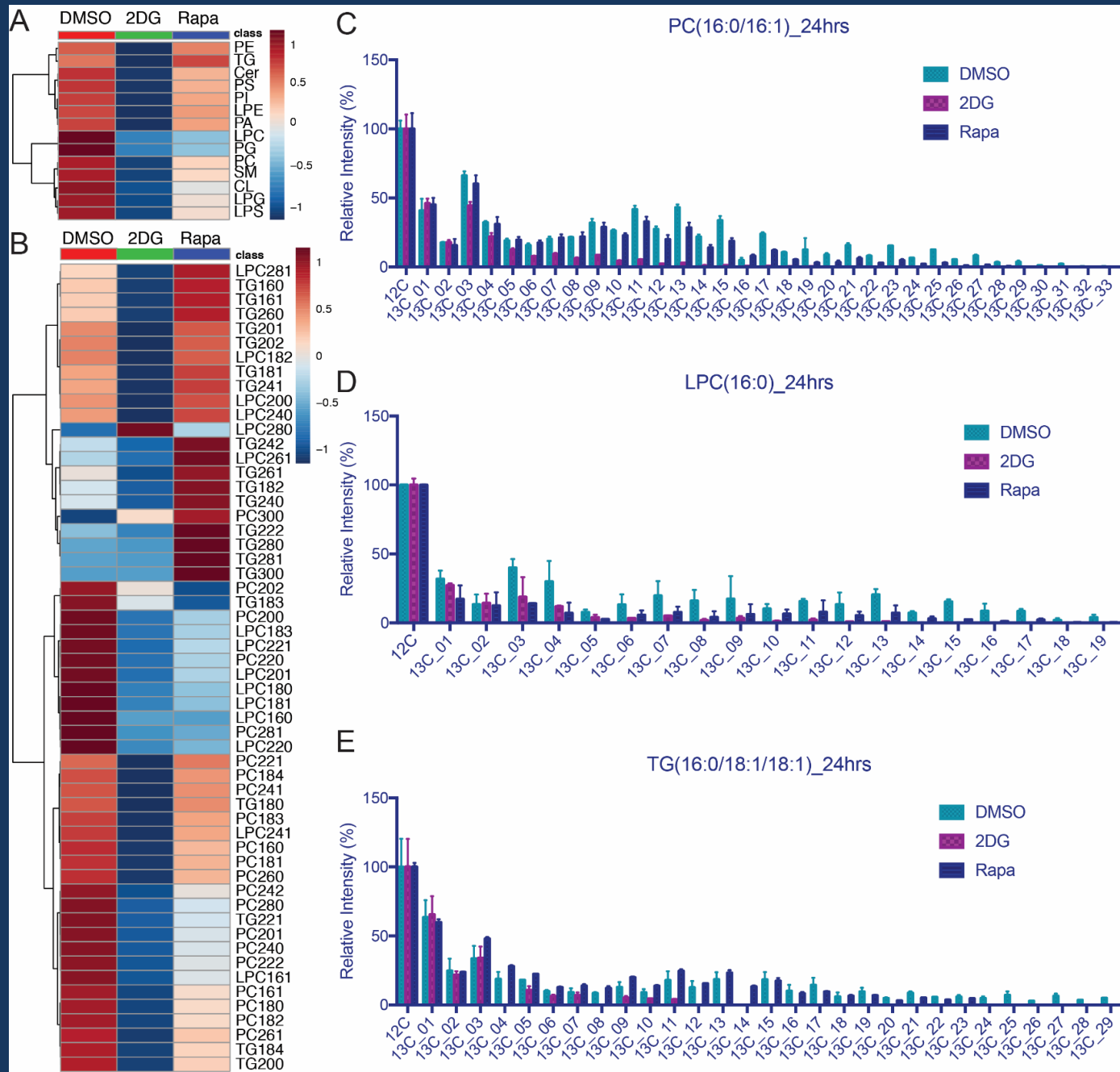
Discovery of Alternative Metabolites using Untargeted fluxomics in MCF-7 Breast Cancer Cells



ROS Assay

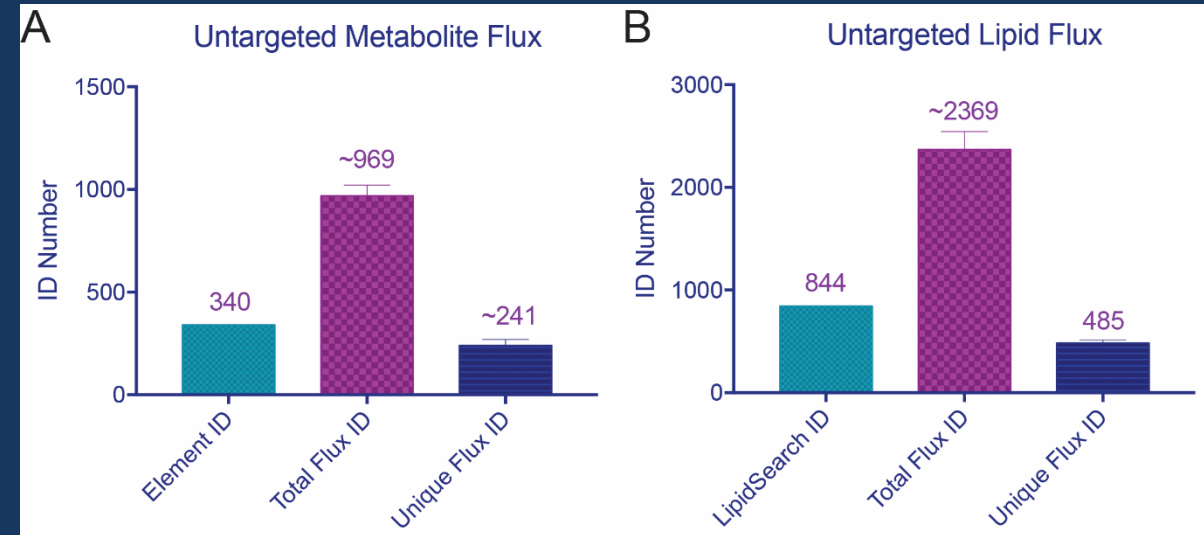
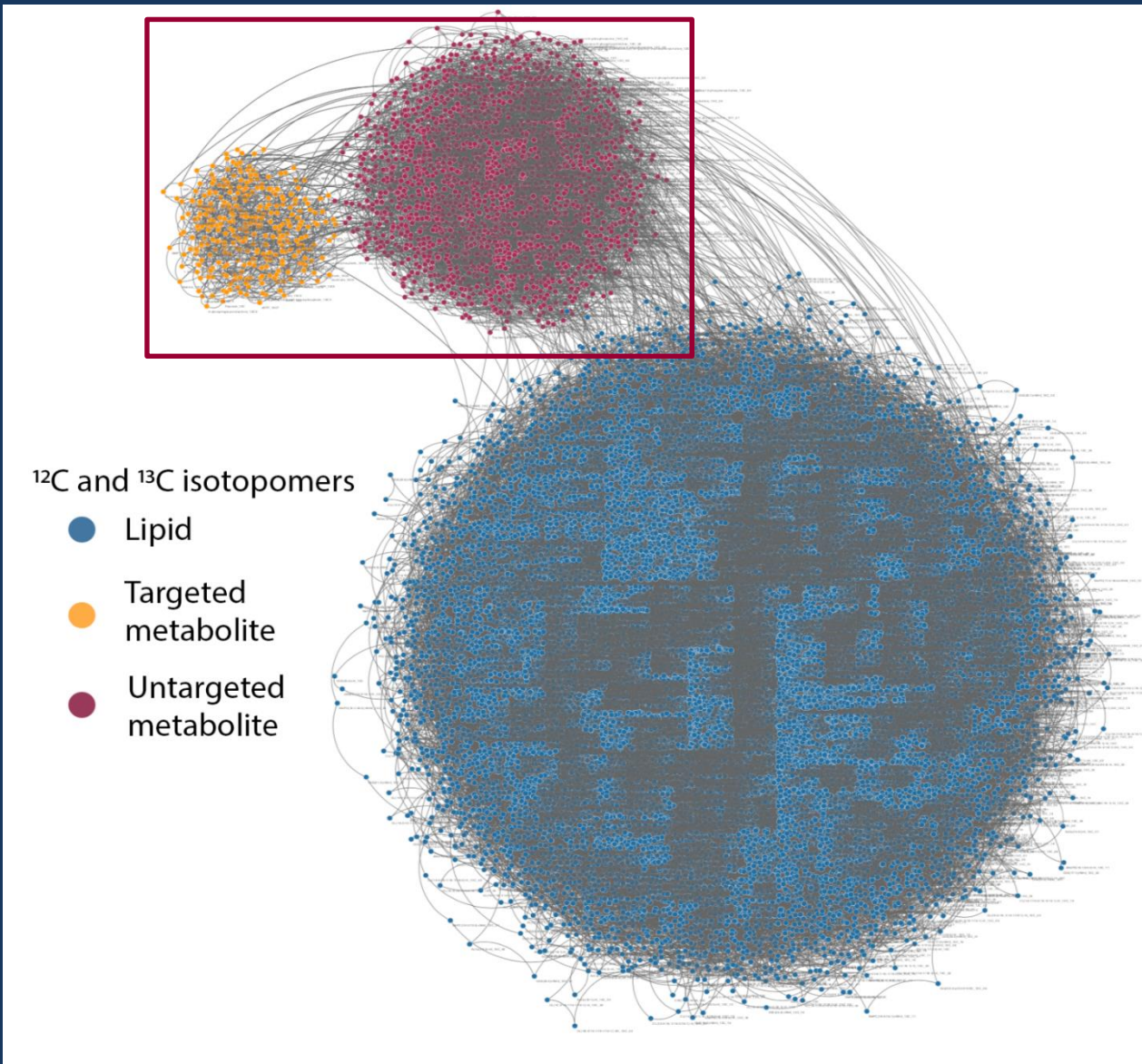


Example of Lipid Isotopomer Profile of MCF-7 Cells treated with 3 drugs for 24 hrs

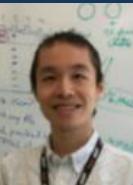


2-DG inhibits higher order Lipid Flux
Rapamycin is less inhibitory

Untargeted Fluxomics increases the number of isotopomers significantly



Huang et al, 2020, Anal Chem., in review



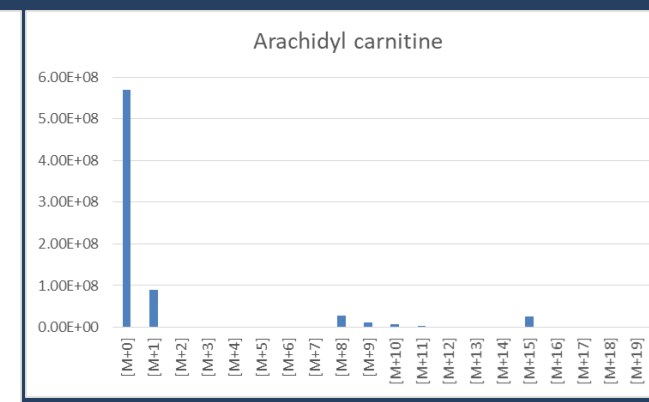
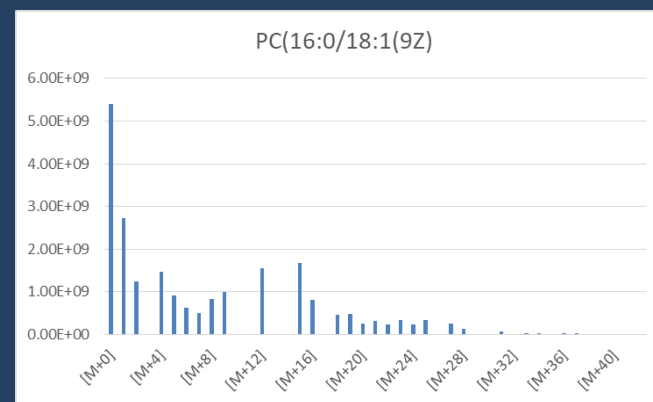
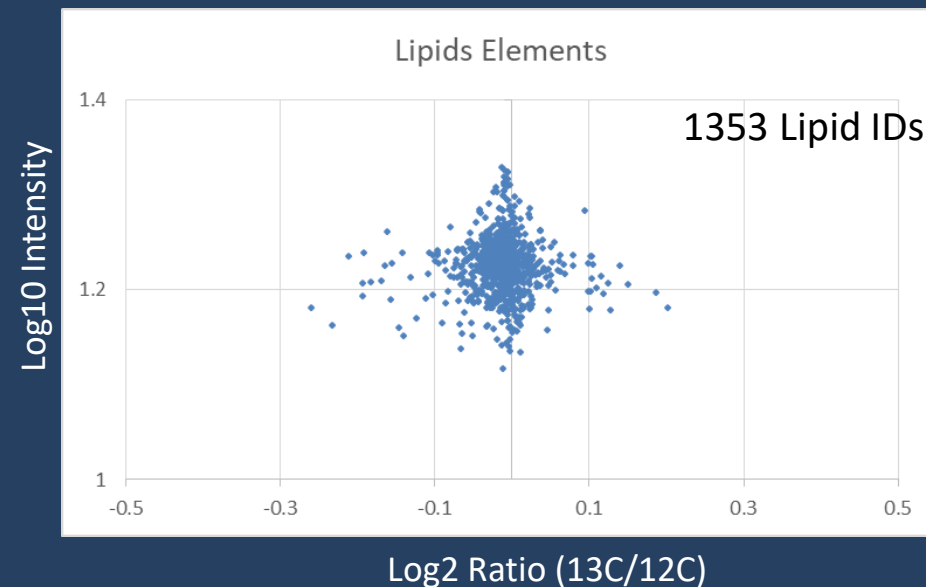
Lipid Isotopomer Profiles using Scaffold Elements (Truly Untargeted Profiling)

LipidSearch 4.1.3 Classes

Group	classKey	Lipid Name	Group	classKey	Lipid Name
P-Choline	LPC	lysophosphatidylcholine	Glycosphingolipids	Cer	Ceramides
PAF	platelet-activating factor		CerP	Ceramides phosphate	
PC	phosphatidylcholine		GM3	Gangliosides	
MePC	Methyl phosphatidylcholine		GM2	Gangliosides	
P-Ethanol Amine	LPE	lysophosphatidylethanolamine	GM1	Gangliosides	
LdMePE	lysodimethylphosphatidylethanolamine		GD1a	Gangliosides	
PE	phosphatidylethanolamine		GD1b	Gangliosides	
BisMePE	Bis-methyl phosphatidylethanolamine		GD2	Gangliosides	
dMePE	dimethyl phosphatidylethanolamine		GD3	Gangliosides	
P-Serine	LPS	lysophosphatidylserine	GT1a	Gangliosides	
PS	phosphatidylserine		GT1b	Gangliosides	
BisMePS	Bis-methyl phosphatidylserine		GT1c	Gangliosides	
P-Glycerol	LPG	lysophosphatidylglycerol	GT2	Gangliosides	
PG	phosphatidylglycerol		GT3	Gangliosides	
BisMePG	Bis-methyl phosphatidylglycerol		GQ1c	Gangliosides	
P-Inositol	LPI	lysophosphatidylinositol	GQ1b	Gangliosides	
PI	phosphatidylinositol		Steroid	ChE	Cholesterol Ester
PIP	phosphatidylinositol		ZyE	zymosterol	
PIP2	phosphatidylinositol		StE	Stigmasterol ester	
PIP3	phosphatidylinositol		SIE	Sitosterol ester	
P-Ethanol	LPET	lysophosphatidylethanol	AGlcSIE	AcylGlcSitosterol ester	
PEt	phosphatidylethanol		D7ChE	Deuterated Cholesterol Ester	
P-Acid	LPA	lysophosphatidic acid	Coenzyme	Co	Coenzyme (O-acyl)-1-hydroxy fatty acid
BisMeLPA	Bis-methyl lysophosphatidic acid		Fatty esters	OAHFA	
PA	phosphatidic acid		WE	wax esters	
BisMePA	Bis-methyl phosphatidic acid		AcCa	Acyl Carnitine	
cPA	cyclic phosphatidic acid		Glycoglycerolipid	MGMG	Monogalactosylmonoacylglycerol
P-Methanol	LPMe	lysophosphatidylmethanol	MGDG	Monogalactosyldiacylglycerol	
PMe	phosphatidylmethanol		DGMG	Digalactosylmonoacylglycerol	
Sphingolipids	SM	sphingomyelin	DGDG	Digalactosyldiacylglycerol	
LSM	lysosphingomyelin		SQMG	Sulfoquinovosylmonoacylglycerol	
phSM	sphingomyelin(phytosphingosine)		SQDG	Sulfoquinovosyldiacylglycerol	
Neutral glycerolipid	MG	monoglyceride	Neutral glycerolipid (deuterated) D5DG		Deuterated diglyceride
DG	diglyceride		D5TG		Deuterated triglyceride
TG	triglyceride				
Fatty Acid	FA	fatty acid			
Cardiolipin	CL	Cardiolipin			
Sphingoid base	So	Sphingosine			
SoP	Sphingosine phosphate				
Neutral Glycosphingolipids SoG1		Glucosylsphingosine			
CerG1	Simple Glc series				
CerG2	Simple Glc series				
CerG3	Simple Glc series				
CerG2GNAc1	Simple Glc series				
CerG3GNAc1	Simple Glc series				
CerG3GNAc2	Simple Glc series				
ST	Sulfatide				

Scaffold Elements 2.2

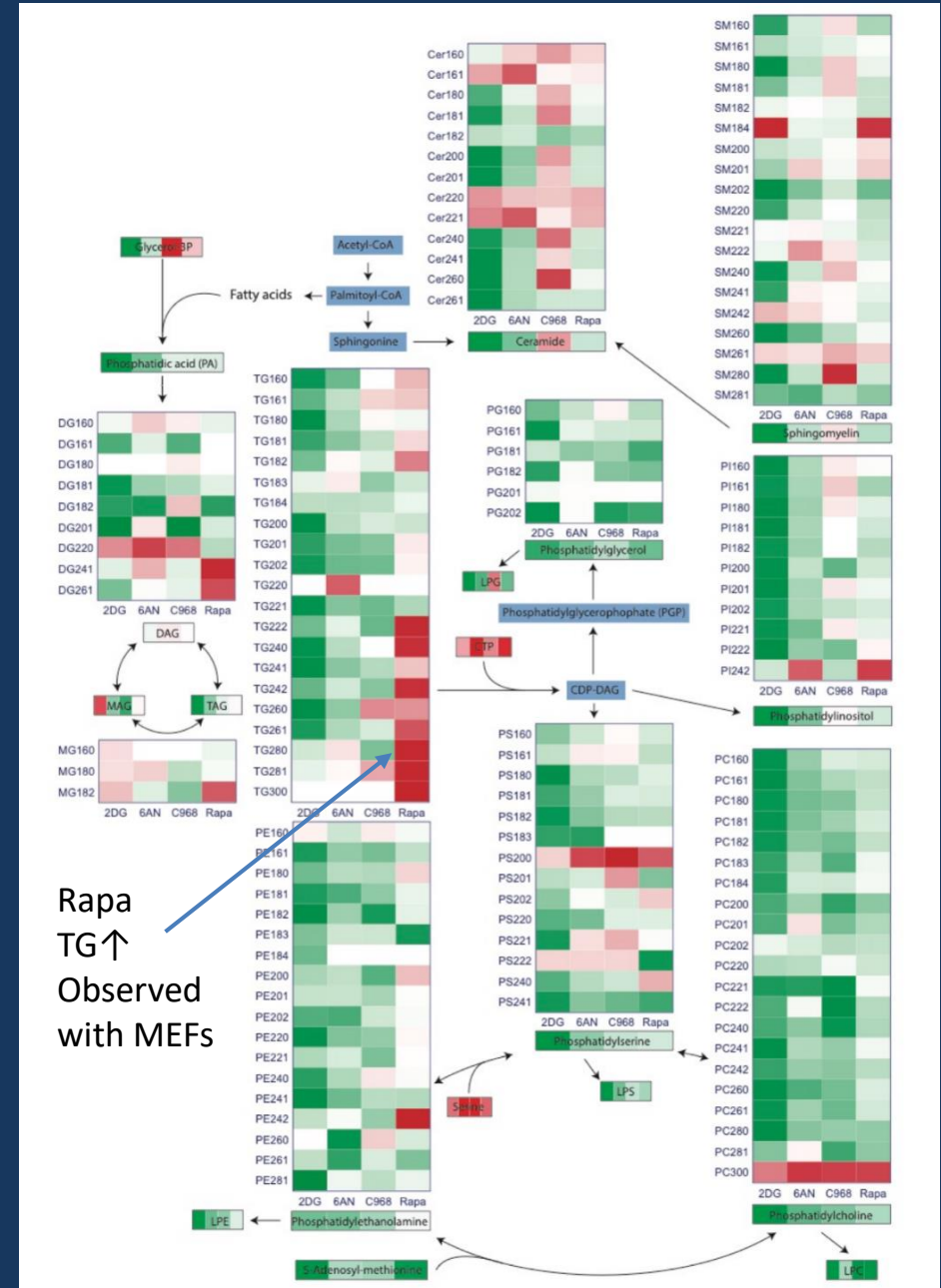
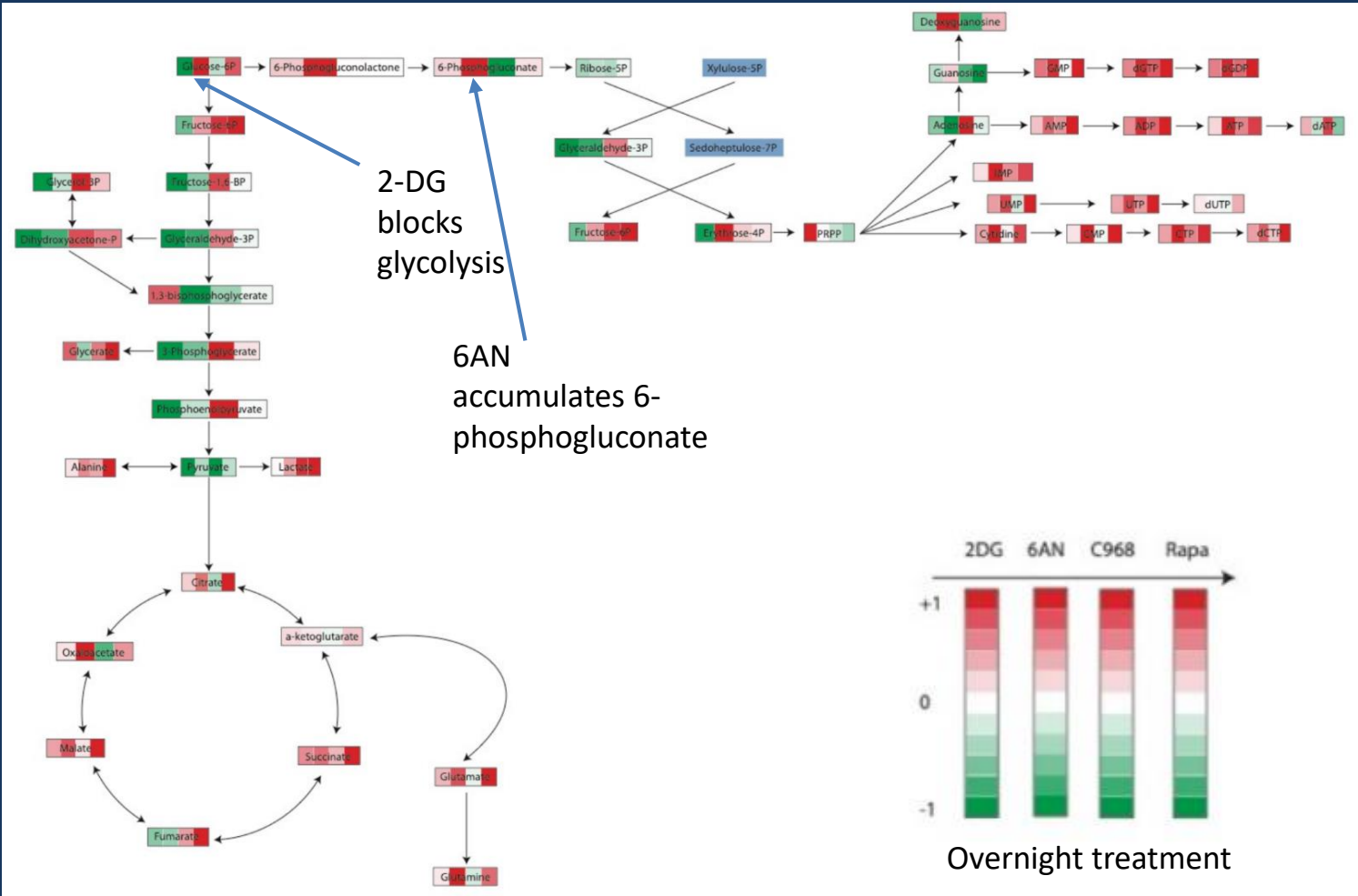
NIST 2017: 652,475 MS/MS spectra
LipidMaps: 22,185 structures



Visualization of IsoSearch Isotope Patterns in MCF-7 cells

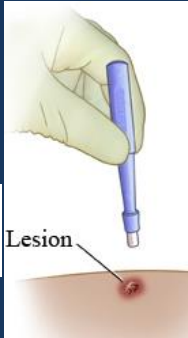
¹³C Lipidomics

¹³C Metabolomics



Our Goal is To Perform *Serial-Omics* from Tumor Biopsies, DBS, Urine, etc.

Needle Biopsy



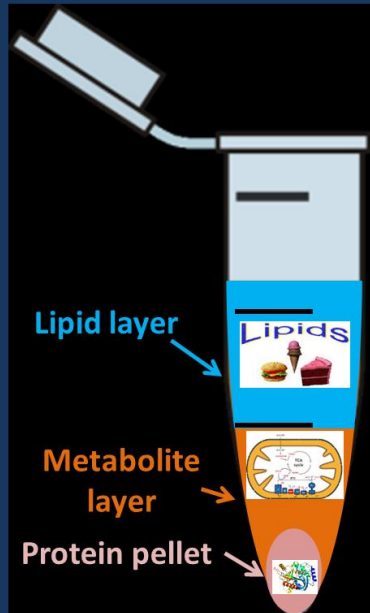
< 5-10 mg

Dried Blood Strips



~ 5 µL

Methyl tert-butyl ether (MTBE)



LC-MS/MS

Untargeted lipidomics

Targeted lipidomics

Untargeted Metabolomics/peptidomics

Targeted metabolomics

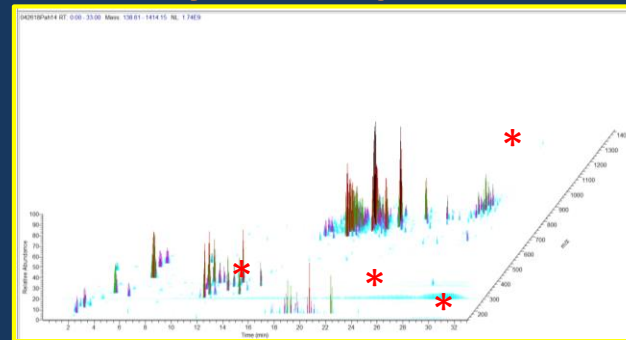
Untargeted Proteomics

TMT labeling (126-131)

TiO₂ phosphoproteomics



Assay Development

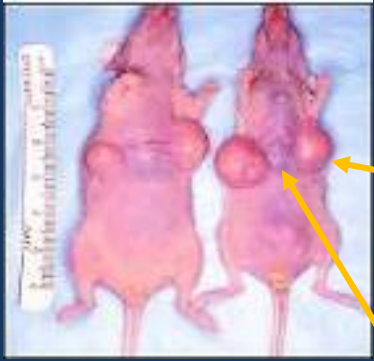


SRM

- Merge –omics data
- Save money/reagents
- Reduce chemical waste
- **Improve reproducibility (one prep)**
- *Biomarker discovery*
- *Robust assay development*

Mouse Breast Tumor vs. Mammary Gland

Brca1-/-, P53-/-

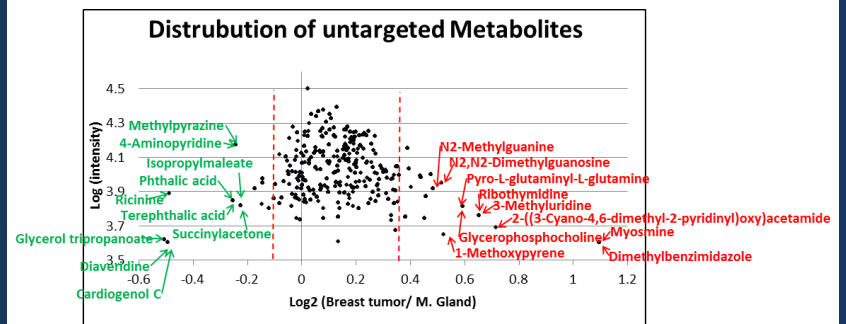
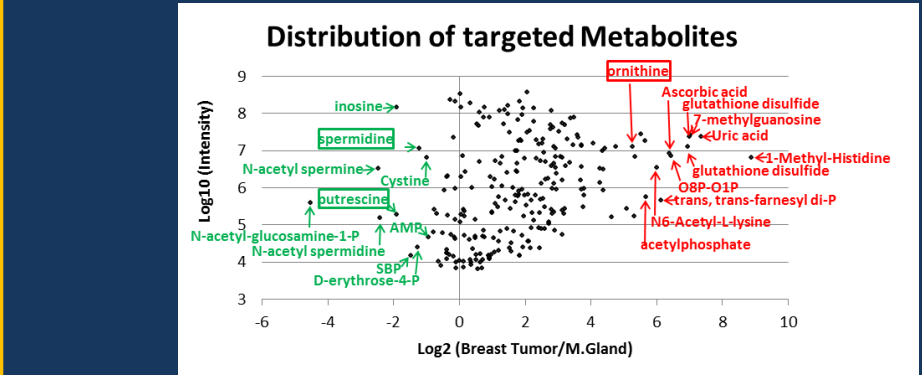
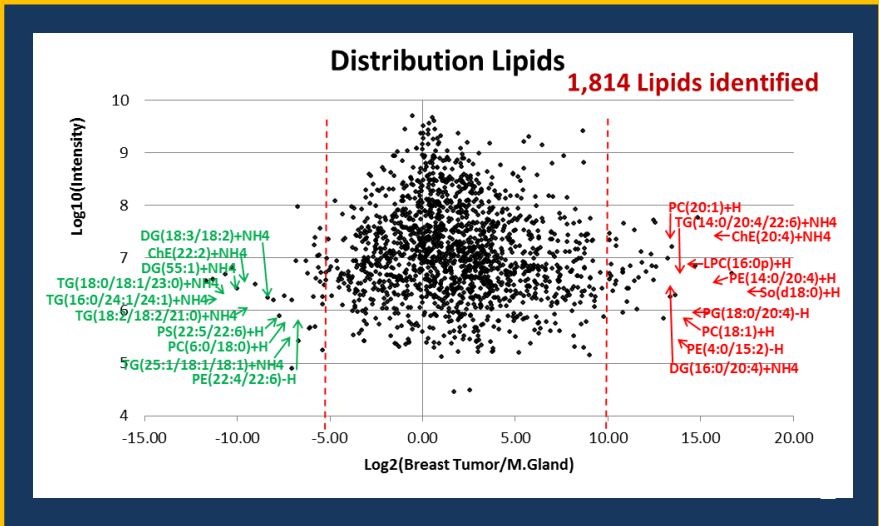
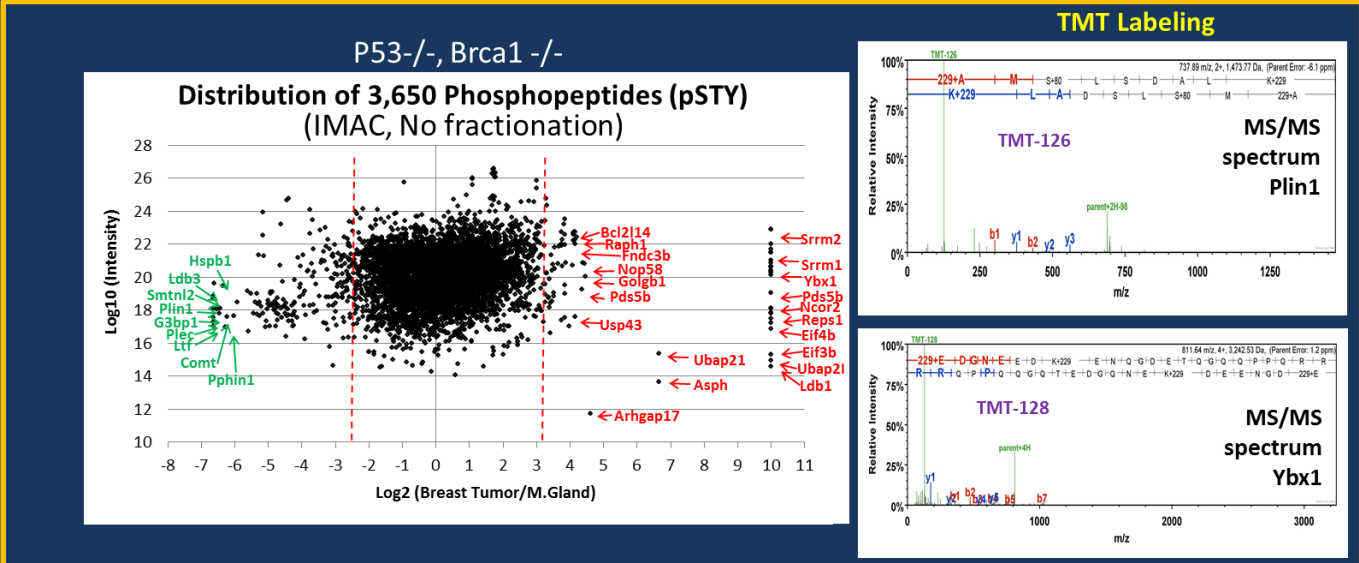


tumor tissue
(cancer)

mammary gland tissue
(normal)

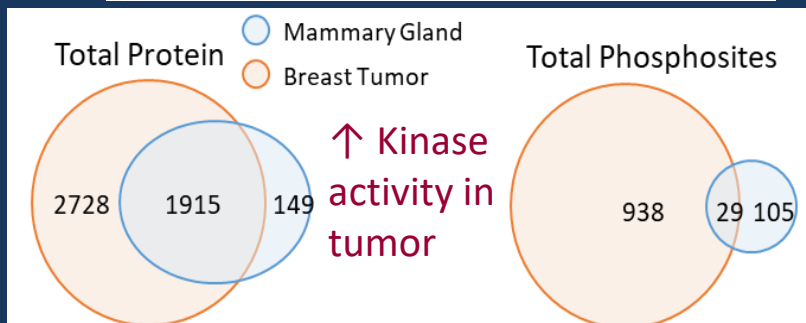
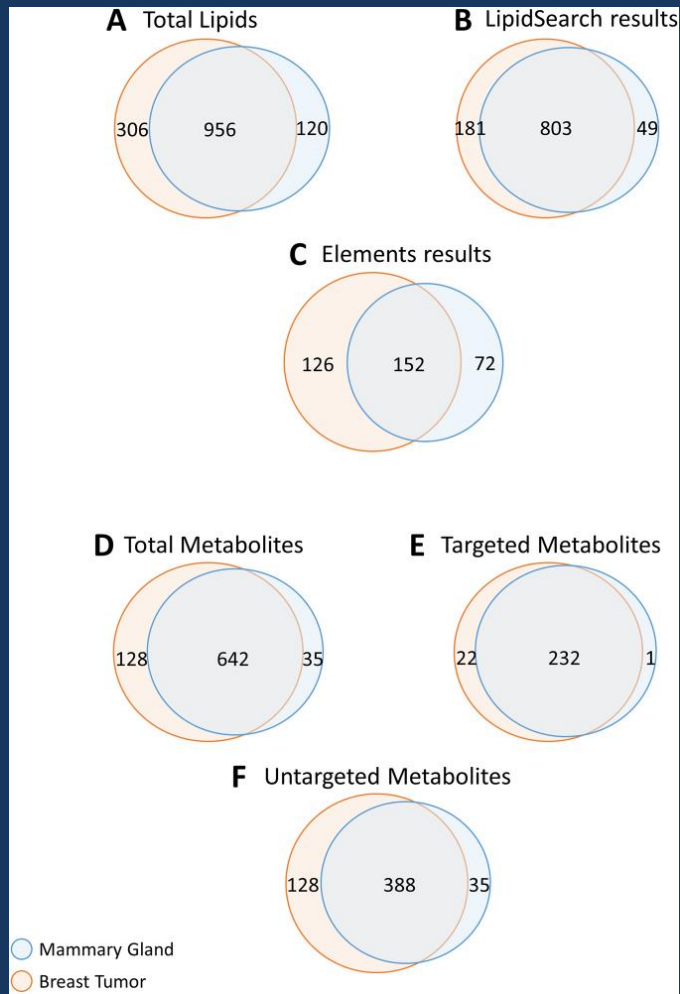


Gerburg Wulf

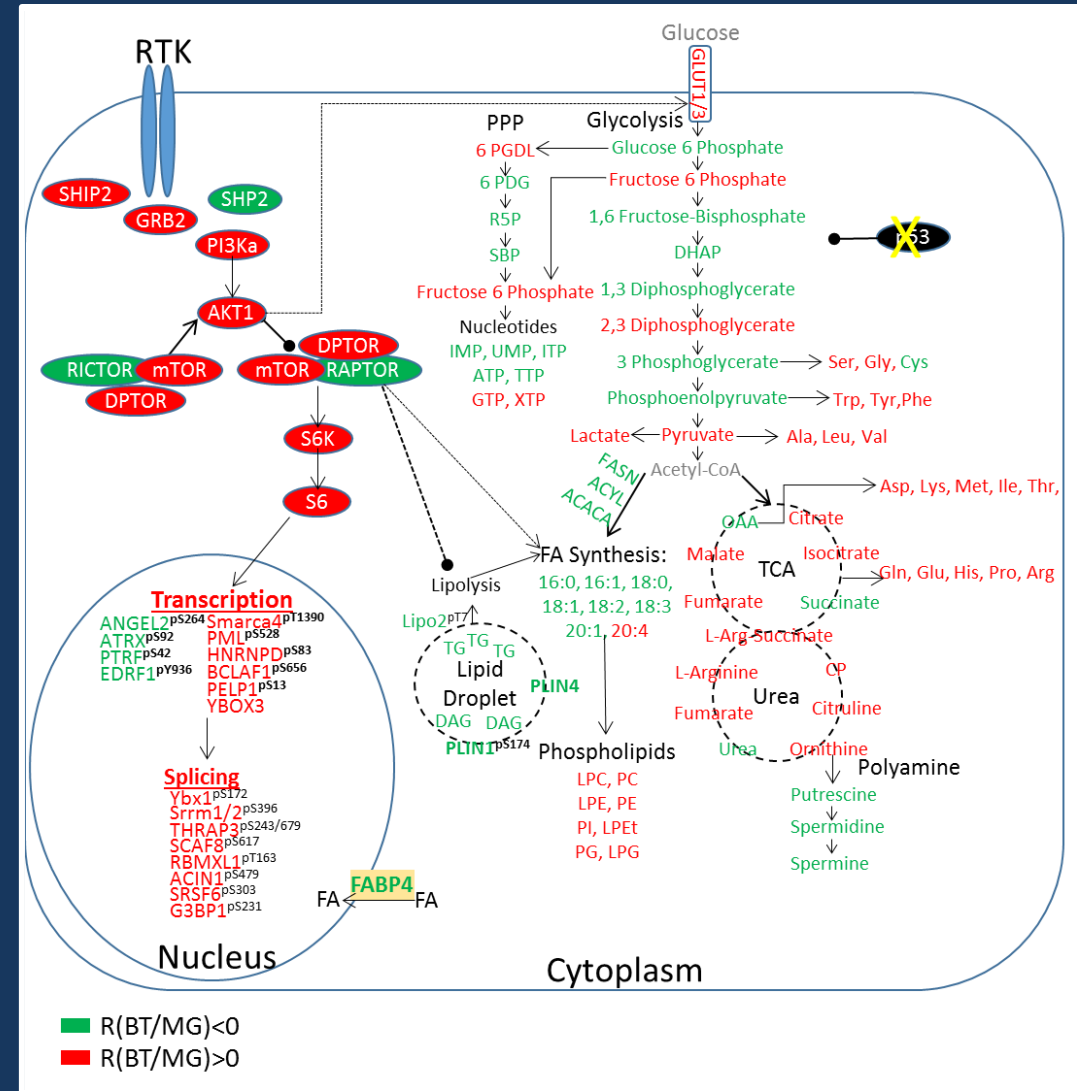
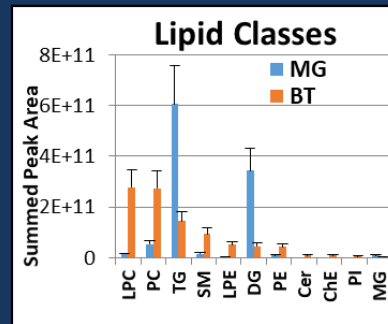
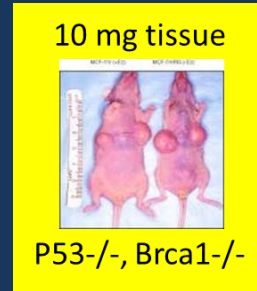


Merging the Phosphoproteome-Metabolome-Lipidome.....

Serial-Omics Data and Pathway Model (Lipidomics, Metabolomics and Phosphoproteomics)



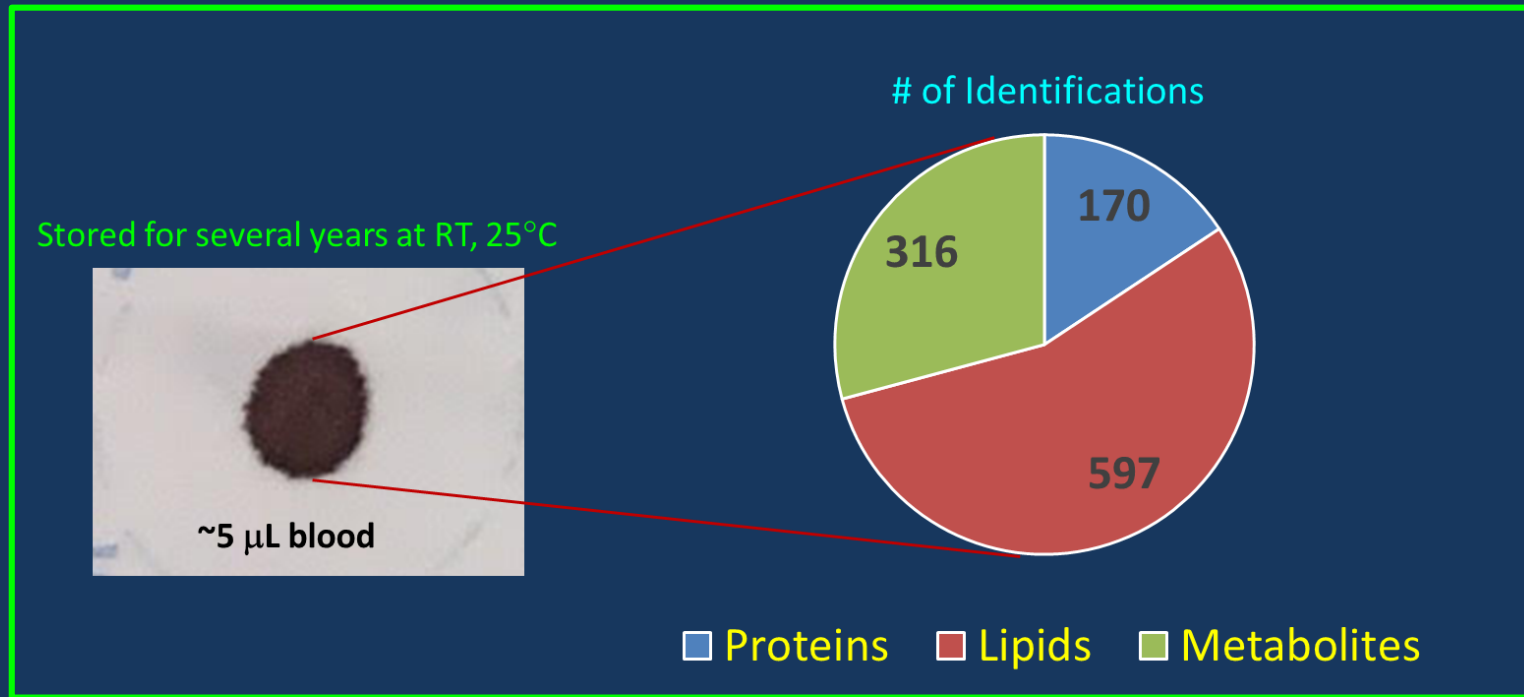
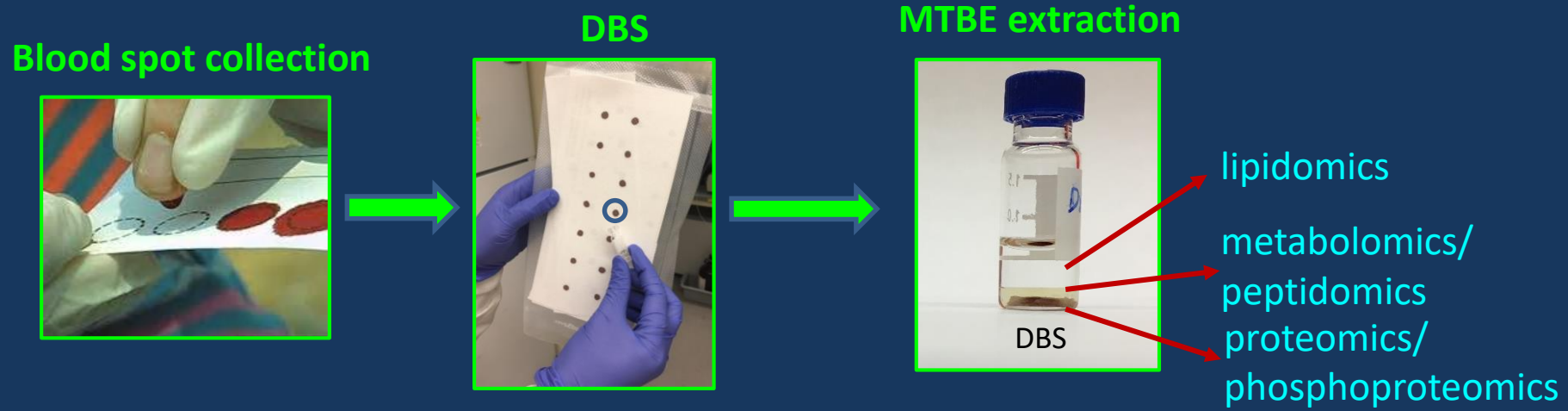
Mouse Breast Tumor



- Tumor concentrated in kinase activity (PO4)
- Mammary gland high TG content
- Tumor high TCA cycling and AA content



Serial-Omics of Dried Blood Spots – A step towards tumor biopsies



Analysis of the MTBE protein precipitate and Dried Blood Spot paper

DBS, post tryptic digestion



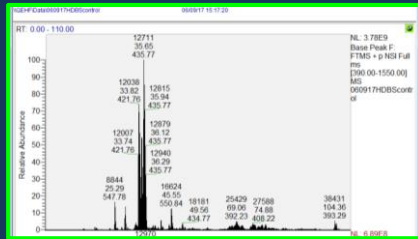
C₁₈ Zip tip

TiO₂ tip

LC-MS/MS

DBS

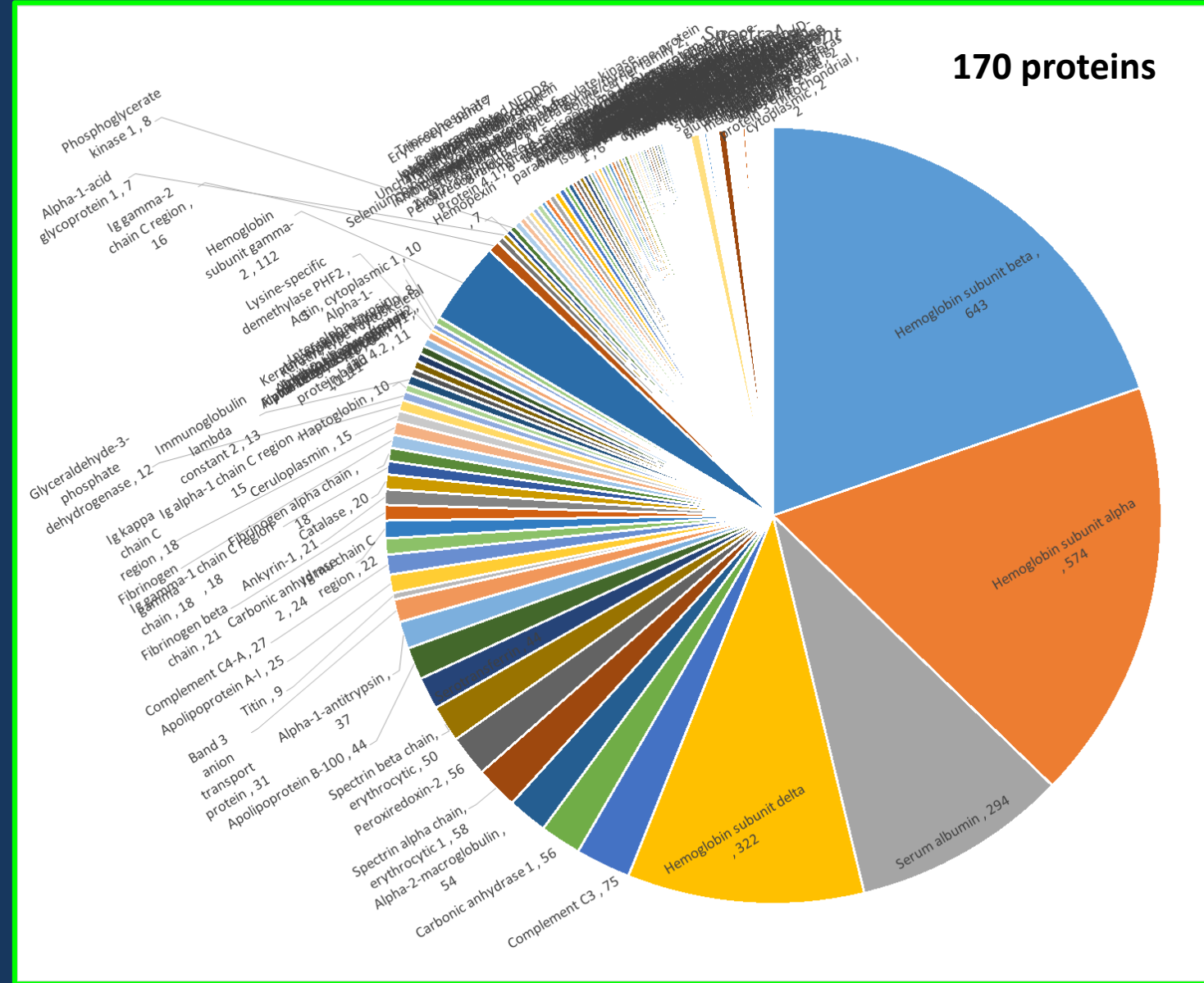
Control paper



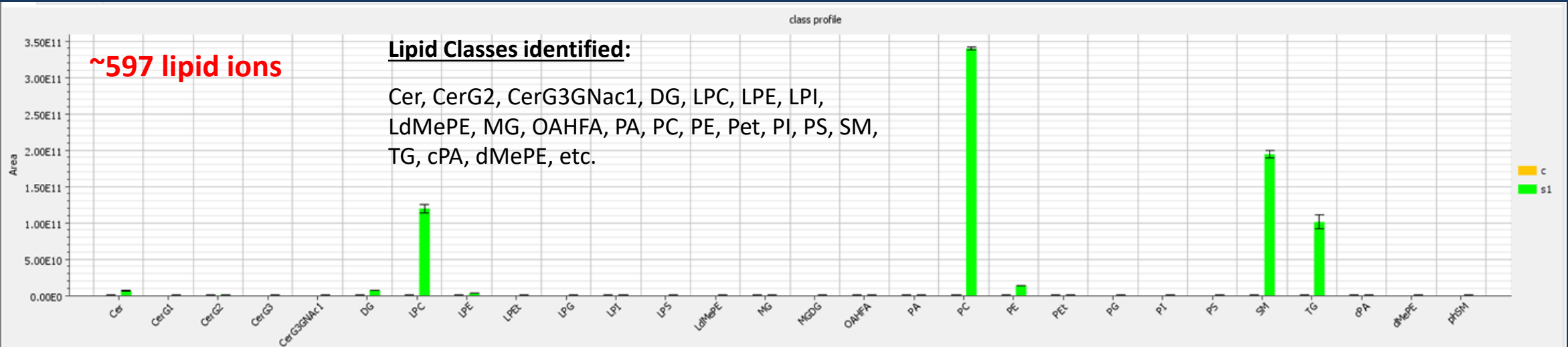
170 DBS proteins identified:

- Hemoglobin (α,β,δ) 52% & Albumin 10% of total signal
- No significant PO₄ info

Proteins identified from 3 year old dried blood spots



Dried Blood Spot Lipidomics & Metabolomics by LC-MS/MS



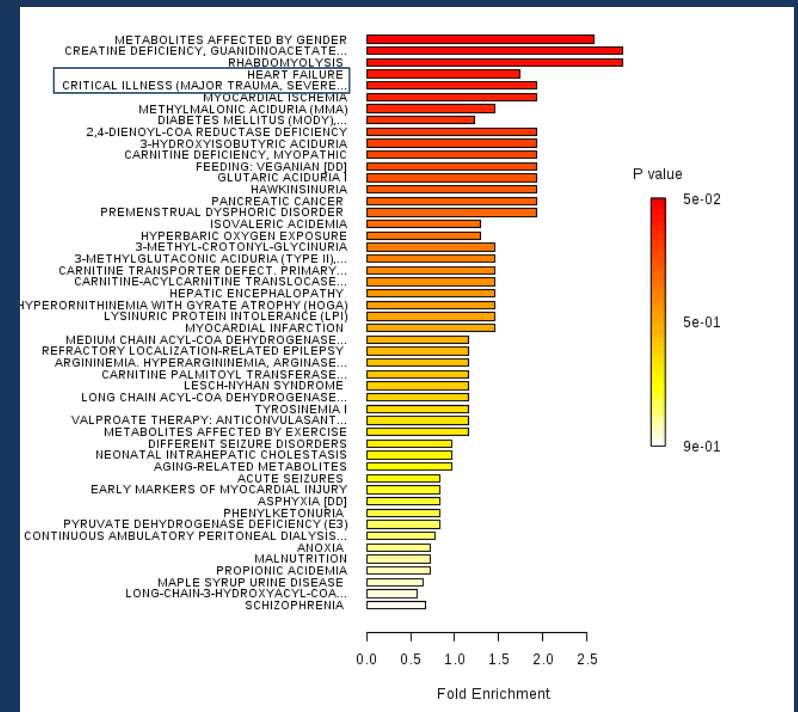
ID Score	MS2 Score	Metabolite Name	Molecular Formula	Dried Blood Spot (Log2 Peak Area)
0.716	0.88	Creatinine	C 4H 7N 3O	10.5
0.915	0.94	Hypoxanthine	C 5H 4N 4O	10.3
0.81	0.68	Edetic Acid	C 10H 16N 2O 8	10.3
0.978	0.98	Niacinamide	C 6H 6N 2O	10.2
0.972	0.98	Phosphoric acid	H 3O 4P	10.2
0.919	0.93	Alpha-D-Glucose 1,6-bisphosphate	C 6H 14O 12P 2	10.2
0.655	0.86	Cytosine	C 4H 5N 3O	10.2
0.941	0.91	2,3-Diphosphoglyceric acid	C 3H 8O 10P 2	10.1
0.973	1	L-Isoleucine	C 6H 13NO 2	10
0.885	0.85	Acetic acid, (aminoxy)-	C 2H 5NO 3	9.98
0.948	1	3-Phosphoglyceric acid	C 3H 7O 7P	9.86
0.971	0.99	L-Carnitine	C 7H 15NO 3	9.77
0.987	1	DL-Phenylalanine	C 9H 11NO 2	9.74
0.955	0.97	N-Methyl-a-aminoisobutyric acid	C 5H 11NO 2	9.72
0.929	0.98	Phosphoenolpyruvic acid	C 3H 5O 6P	9.7
0.67	0.95	Diethanolamine	C 4H 11NO 2	9.6
0.971	0.99	2-Imidazolecarboxaldehyde	C 4H 4N 2O	9.54
0.98	1	Creatine	C 4H 9N 3O 2	9.49
0.679	0.87	Senecioid acid	C 5H 8O 2	9.49
0.956	0.93	4-Pyridoxic acid	C 8H 9NO 4	9.46
0.986	0.99	Hexanoylcarnitine	C 13H 25NO 4	9.42
0.908	0.97	Deoxygalactonojirimycin	C 6H 13NO 4	9.42
0.954	0.91	Cadaverine	C 5H 14N 2	9.42
0.976	0.98	Citric acid	C 6H 8O 7	9.4
0.633	0.29	Glucaric acid	C 6H 10O 8	9.32
0.958	1	L-Glutamic acid	C 5H 9NO 4	9.3
0.899	0.87	3-Hydroxydodecanoic acid	C 12H 24O 3	9.25
0.699	0.79	Myristoleic acid	C 14H 26O 2	9.25
0.661	0.61	Gamma-Aminobutyric acid	C 4H 9NO 2	9.25
0.938	0.99	Succinic acid	C 4H 6O 4	9.24
0.991	1	L-Tyrosine	C 9H 11NO 3	9.21
0.941	1	Adenosine monophosphate	C 10H 14N 5O 7P	9.19
0.722	1	L-Pipecolic acid	C 6H 11NO 2	9.18
0.763	0.94	Adenine	C 5H 5N 5	9.18
0.889	0.87	Serricornin	C 11H 22O 2	9.16

Top 35 metabolites by abundance

116 untargeted + 200 targeted metabolites

Untargeted metabolomics helpful for non-cell applications

MetaboAnalyst Pathway Enrichment





John M. Asara, Ph.D. – Director, Associate Professor of Medicine
Min Yuan, B.S. – Sr. Research Associate, Lab Manager
He Huang, Ph.D. – Research Fellow, Research Associate

Contact and Info

- Contact John Asara, jasara@bidmc.harvard.edu for project discussion
- Per unique sample for BIDMC & DF/HCC affiliates;
- Prices and more info available at www.bidmcmassspec.org

Metabolomics

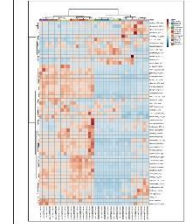
- Targeted Metabolic Profiling (~300 targets) by SRM
 - Central carbon metabolism
 - Glycolysis
 - TCA cycle
 - Pentose phosphate pathway
 - Amino acids
 - Nucleotides
 - Reactive oxygen species
 - Methionine metabolism, etc.
- ¹³C/¹⁵N Metabolic Flux Tracing (~150 targets)
- ¹³C/¹⁵N Isotopomer tracing of central carbon metabolites (~70 targets)
- Untargeted Metabolic Profiling by High Resolution LC-MS/MS (>450 molecules)
- Absolute quantitation with calibration curve or labeled internal standard

Metabolomics Instrumentation

Thermo QExactive Orbitrap HF coupled to Agilent 1200 HPLC



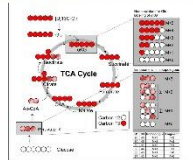
Metabolomics flux Heat Map



AB/SCIEX QTRAP 6500 triple quadrupole coupled to Shimadzu UF-HPLC



Example of ¹³C isotopomer Flux

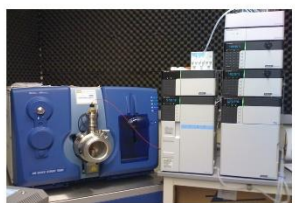


Lipidomics Instrumentation

Thermo QExactive Orbitrap Plus coupled to Agilent 1200 HPLC



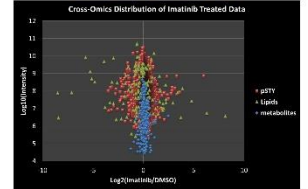
AB/SCIEX QTRAP 5500 triple quadrupole coupled to Shimadzu UF-HPLC



Lipidomics

- Untargeted Lipidomic Profiling (>1,200 molecules) by High Resolution LC-MS/MS
 - 20 main classes, >90 sub-class of lipids
 - Phospholipids, triglycerides, ceramides, etc.
 - Individual lipid molecule ID and quantification
 - Lipid class and fatty acid profiles
- ¹³C/¹⁵N Isotopomer tracing
 - All identified lipid ions
- Targeted Lipid Profiling
 - Coming soon

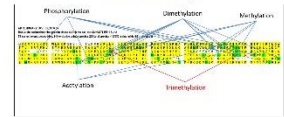
Multi-Omics Overlay Plot



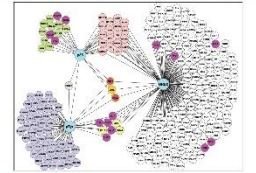
Proteomics (PTM-omics)

- Protein Identification from Gels and Solution
 - SDS-PAGE gel slices or small regions
 - On-bead digestion
 - Solution based or protein pellets
- Phosphoproteomics
 - pTyr enrichment
 - IMAC/TIO2 global Phospho enrichment
 - Phospho-specific Ab
- Post-Translation Modification Mapping of Proteins
 - Ubiquitination, acetylation, methylation, phosphorylation, etc.
- Quantitation with SILAC and TMT labeling
 - Immunoprecipitations
 - Label-free Quantitation
- Protein Complex analysis

PTM Site Map



Protein-Protein Interaction Network



Proteomics Instrumentation

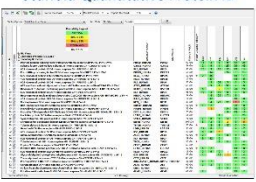
Thermo QExactive Orbitrap HF coupled to Proxeon EASY-nLCII nano-HPLC



Thermo hybrid Orbitrap Elite coupled to Proxeon EASY-nLCII nano-HPLC



Scaffold Quantitative Protein ID



Phosphorylation Site Maps

